Invited Speaker Abstracts and Bios

TUESDAY, April 16, 2013
11:00 – 12:00
Plenary Session IV: Keynote Lecture
Chairs: Clara Camaschella, MD; Paul A. Sharp, BSc, PhD
Oxygen and Iron Homeostasis
Invited Speaker: Christopher J. Schofield, MD, Oxford University, UK

On the basis of their roles in plant and microbial secondary metabolism, where they catalyse an astonishing array of oxidative reactions, ferrous iron and 2-oxoglutarate (2OG) dependent oxygenases have been proposed as amongst the most catalytically flexible of all enzyme families. In animals, pioneering work defined roles for them in collagen and epidermal growth factor like domain modification. Subsequently, we and others, defined roles for them in hypoxic sensing, chromatin modification, fatty acid metabolism, and, very recently, translational regulation.

The lecture will discuss studies attempting to link the kinetics of oxygenases and their cofactor, including their use of iron, requirements to their biological roles, focussing on hypoxia sensing. Evidence will be discussed suggesting that the catalytic promiscuity of some, but not all, human 2OG oxygenases may mirror that of their microbial homologues.

Christopher J. Schofield *studied for a first degree in chemistry at the University of Manchester from 1979 – 1982. In 1982, he moved to Oxford to study for a DPhil with Professor Jack Baldwin. In 1985, he became a departmental demonstrator in the Dyson PErrins Laboratory, Oxford University followed by his appointment as lecturer in chemistry and fellow of Hertford College in 1990. In 1998, he became professor of chemistry, and in 2011 was appointed head of organic chemistry. Dr. Schofield is an international-leader in functional, structural and mechanistic studies on enzymes employing oxygen and 2-oxoglutarate co-substrates. His work has opened up new fields in antibiotic research, oxygen sensing and gene regulation. After groundbreaking work on plant and microbial oxygenases, he pioneered structurally informed functional assignments for uncharacterised human oxygenases. His research has identified unanticipated roles for oxygenases in regulating gene expression, importantly in the cellular hypoxic response, and has revealed new post-translational modifications to chromatin and RNA splicing proteins. The work has identified new opportunities for medicinal intervention that are being pursued by numerous academic and commercial laboratories. Dr. Schofield has published over 370 papers in leading international journals.*

Invited Speaker Abstracts and Bios

Wednesday, April 17, 2013
11:00 – 12:00
Plenary Session VI: Special Keynote Lecture
Chairs: Kaila S. Srai, BSc, MSc, PhD; Greg Anderson, BSc, MSc, PhD
Iron, Energy and Origin of Life
Invited Speaker: Nick Lane, MD; Department of Genetics, Evolution and Environment, University College London, UK

Living is a continuous chemical reaction that passes back unbroken to the origin of life. To power growth, prokaryotes turnover about 10 – 100 times their own mass in ATP per cell division. Life is, in effect, a side-reaction of a main energy harvesting reaction. At the origin of life, before the evolution of enzymes or ribozymes that channelled energy and carbon metabolism efficiently, the first 'living' systems must have required orders of magnitude more energy and carbon, which in turn must have been channelled by purely geochemical processes. An iron-sulfur world has long been posited as a possible setting for the origin of life, but has so far failed to produce high rates of organic flux from gases such as H_2 and CO_2. Today, FeS proteins are critical for chemiosmotic coupling, which is central to both carbon and energy metabolism, but how and when chemiosmotic coupling arose is obscure. In this lecture, I will argue that the pH-dependent reduction potential of semi-conducting Fe(Ni)S minerals holds the key to the origins of biochemistry in the specific setting of deep ocean alkaline hydrothermal vents (1).

Alkaline hydrothermal vents are sustained far-from-equilibrium systems – microporous labyrinths suffused with geochemically sustained pH and redox gradients. These conditions give rise to thin-walled inorganic compartments that are remarkably similar in their energetic topology to chemoautotrophic prokaryotes, notably methanogens and acetogens that still live in such systems today. Both these groups synthesise acetyl CoA from H_2 and CO_2, and both are strictly dependent on electrochemical ion gradients over membranes. Recent work shows that these cells generate Na^+ or H^+ gradients from CO_2 and H_2 via flavin-based electron bifurcation, a process that depends on Fe(Ni)S proteins to reduce ferredoxin. Ferredoxin is central to both energy and carbon metabolism in different branches of the acetyl CoA pathway. Methanogens draw on the proton gradient to reduce ferredoxin via the energy-converting hydrogenase (Ech) for carbon assimilation. Acetogens reverse this process (using Ech or Rnf) to generate ion gradients by ferredoxin oxidation, utilising NADH and ATP to fix carbon. I shall outline how carbon and energy metabolism may have arisen from inorganic beginnings, giving rise to the emergence of free-living cells that depend on FeS proteins and the ATP synthase for the first simple chemiosmotic circuit, the basis for more sophisticated respiratory chains incorporating quinones and cytochromes.

References

(1) Lane N, Martin WF. The origin of membrane bioenergetics. Cell 151, 1406-1416 (2012)
(2) Sousa FL, et al. Early bioenergetic evolution. Phil Trans Roy Soc B. In press (2013).

Dr. Nick Lane *is a biochemist and writer in the department of genetics, evolution and environment at University College London. His research is on evolutionary biochemistry and bioenergetics, focusing on the origin of life and the evolution of eukaryotes. Nick Lane was awarded the inaugural UCL Provost's Venture Research Prize in 2009, and the BMC Research Award for Genetics, Genomics, Bioinformatics and Evolution in 2011. He leads the UCL Research Frontiers Origins of Life programme and is a founding member of the UCL Consortium for Mitochondrial Research. Nick has published some 60 papers, including research articles in Nature, Cell and Science, and three critically acclaimed books, the latest of which, Life Ascending, won the 2010 Royal Society Prize for Science Books. Nick's books have been translated into 20 languages, and he was described by the Independent as "one of the most exciting science writers of our time."*

Podium Abstracts

Podium #1

HOST IRON STATUS AND IRON SUPPLEMENTATION INFLUENCE HOST SUSCEPTIBILITY TO ERYTHROCYTIC STAGE OF PLASMODIUM FALCIPARUM BY ALTERING THE STRUCTURE OF THE HOST RBC POPULATION

Martha Clark, BS[2], Raj Kasthuri, MD[2], Anthony Fulford, PhD[3], Andrew Prentice, PhD[3], Steve Taylor, MD[4] and Carla Cerami Hand, MD, PhD[1]

[1]University of North Carolina-School of Public Health; [2]University of North Carolina; [3]London School of Hygiene and Tropical Medicine; [4]Duke University Medical Center

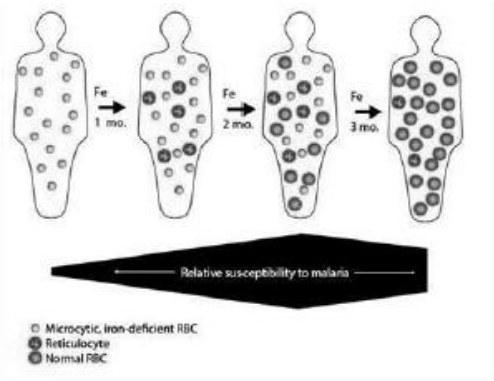
(Presented By: Carla Cerami Hand)

Introduction: Iron deficiency is prevalent in pregnant women and children in developing countries, and as a result the World Health Organization recommends routine oral iron supplementation. In malaria-endemic regions this recommendation is being reassessed in light of evidence that iron deficiency may protect against malaria and that supplementing iron-replete children with moderate doses of oral iron or iron-deficient children with high-dose iron may increase susceptibility to malaria. The underlying mechanisms behind these observations remain unclear.

Methods and Materials: We employed the in vitro model of Plasmodium falciparum infection to study the invasion and intraerythrocytic growth of the parasite in red blood cells (RBCs) collected from individuals with well-defined, physiologic iron states.

Results: We have observed (1) impaired P. falciparum invasion and growth in RBCs from individuals with Iron Deficiency Anemia (IDA), (2) no significant impact of iron supplementation upon parasite growth in RBCs from iron replete individuals, and (3) increased parasite growth in RBCs from individuals with IDA who were given therapeutic doses of iron. This increase was principally caused by increased parasite invasion and growth in newly-formed RBCs. In order to quantify the relative contributions of RBC states upon parasite growth, we mathematically modeled these effects: The model indicates that the host susceptibility to erythrocyte-stage falciparum infection is increased byhe replacement of microcytic RBCs with iron-replete, normocytic RBCs as well as the addition of younger RBCs to the RBC population.

Conclusion: These findings support well-described clinical patterns of differential susceptibility to malaria.



Podium #2

METABOLIC ADAPTATION TO TISSUE IRON OVERLOAD CONFERS TOLERANCE TO MALARIA

Raffaella Gozzelino, PhD[1], Bruno Bezerril Andrade[2], Rasmus Larsen[3], Nivea F. Luz[4], Liviu Vanoaica[5], Antonio Coutinho[3], Silvia Cardoso[3], Sofia Rebelo[3], Maura Poli[6], Manoel Barral-Netto[7], Deepak Darshan[5], Lukas Kühn[5] and Miguel P. Soares[3]

[1]Instituto Gulbenkian de Ciencia; [2]Immunobiology Section, Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA; [3]Inflammation Laboratory, Instituto Gulbenkian de Ciência, Oeiras, Portugal; [4]Centro de Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz (FIOCRUZ), Salvador, Bahia, Brazil; [5]Ecole Polytechnique Fédérale de Lausanne (EPFL), Swiss Institute for Experimental Cancer Research (ISREC), Switzerland; [6]Dipartimento Materno Infantile e Tecnologie Biomediche, University of Brescia, Italy; [7]Faculdade de Medicina, Universidade Federal da Bahia, Salvador, Bahia, Brazil

(Presented By: Raffaella Gozzelino)

Introduction: Disease tolerance is a host defense strategy that limits the fitness costs associated with infection, irrespectively of pathogen burden. As demonstrated hereby, tolerance to malaria, the disease caused by Plasmodium infection, is operational in mice as well as in humans.

Methods and Results: In mice, this defense strategy relies on the expression of the iron (Fe) sequestering protein ferritin H chain (FtH), while in humans, ferritin expression is associated with reduced tissue damage, irrespectively of pathogen burden. The protective effect of FtH relies on its ferroxidase activity, which prevents labile Fe from sustaining c Jun N terminal kinase (JNK) activation. FtH expression is inhibited by JNK activation, which promotes tissue Fe overload, tissue damage and malaria severity, irrespectively of pathogen burden. Pharmacologic approaches mimicking the anti oxidant effect of FtH or inhibiting JNK activation act therapeutically to confer tolerance to malaria in mice.

Conclusion: In conclusion, FtH provides metabolic adaptation to tissue Fe overload, conferring tolerance to malaria.

Podium Abstracts

Podium #3
HEPCIDIN REGULATION IN MALARIA INFECTION
Natasha Spottiswoode, BSc, MSc[1], Andrew Williams[2], Andrew Armitage[3], Lucy Eddowes[3], Chris Newbold[4], Bob Pinches[4], Simon Draper[2], Alain Townsend[3], Patrick Duffy[5] and Hal Drakesmith[3]
[1]Oxford University and the National Institutes of Health; [2]The Jenner Institute, Old Road Campus Research Building, Roosevelt Drive, Oxford UK; [3]Molecular Immunology Group, Weatherall Institute of Molecular Medicine, Oxford UK; [4]Molecular Parasitology Group, Weatherall Institute of Molecular Medicine, Oxford, UK; [5]Laboratory of Malaria Immunology & Vaccinology, NIAID, NIH, Bethesda, MD
(Presented By: Natasha Spottiswoode)

Objective: Malaria and anaemia are interrelated problems. Malaria may cause life-threatening anaemia; conversely, host iron restriction reduces the likelihood of malaria infection. To better understand these interactions, we examine the regulation of the iron control hormone hepcidin in malarial infection.
Materials and Methods: For a murine model of malaria infection, BALB/c mice were infected with 103 *Plasmodium berghei* ANKA sporozoites. Uninfected and infected mice were sacrificed at days 2, 4, 6, and 8 post-infection, and parasitemia determined by microscopy. Tissue mRNA levels were assayed using quantitative real-time PCR (qRT-PCR). Western blots were performed on liver lysate and quantitatively analyzed using a LiCor system. For in vitro experiments, hepatoma HepG2 cells were plated and treated with proteins of interest for 4 hours, and gene expression analyzed by qRT-PCR. Human peripheral blood mononuclear cells (PBMCs) were freshly harvested and co-cultured with uninfected red blood cells (uRBCs) or *P. falciparum* schizont-infected red blood cells, and after 3 hours, gene expression in PBMCs was assayed by qRT-PCR.
Results: In mice infected with *P. berghei* sporozoites, liver hepcidin (*Hamp1*) was increased on day 8 post-infection, concurrently with the development of ~4% blood-stage parasitemia. To identify the intrahepatic regulators responsible, we examined hepatic expression of genes controlled by the two major hepcidin control pathways: the inflammatory (Stat3-mediated) pathway and the BMP (Smad-mediated) pathway. *Fga* and *Saa-1,* inflammatory pathway responsive genes, were upregulated on day 2 post-infection and did not correlate with hepcidin expression. Similarly, Stat3 phosphorylation, indicating activation of the inflammatory pathway, correlated with *Fga* and *Saa-1* expression but not with hepcidin. Conversely, BMP-pathway responsive gene *Id1* was upregulated on day 8 and was significantly associated with hepcidin. Hypothesizing, therefore, that the BMP pathway might be responsible for hepcidin upregulation in malaria, we measured mRNA of several *Bmp* genes in spleen, bone marrow, and liver tissue, but found no increase in expression in infected mice. However, Activin B, a member of the TGFb superfamily, has recently been shown to upregulate hepcidin through the BMP pathway and is upregulated by LPS stimulation in mice. We therefore measured Activin B mRNA in murine hepatic tissue and found that it was upregulated significantly throughout malaria infection, and further increased on day 8 post-infection, thus presenting a possible mechanism for hepcidin stimulation in malaria. We also showed that Activin A, a close cousin to Activin B that is increased in white blood cells in response to inflammation and infection, is also capable of inducing hepcidin (*HAMP*) and *ID1* upregulation in vitro. We therefore tested human PBMCs exposed to P. falciparum schizonts, and found that schizonts, but not uRBCs, induced Activin A expression in PBMCs, providing a second potential link between malaria, activins, and hepcidin.
Conclusions: These data indicate that hepcidin is increased in murine blood-stage malaria and is likely controlled by the BMP pathway. Activin B, which may stimulate hepcidin through the BMP pathway, is increased in murine liver tissue during infection. Furthermore, the closely related protein Activin A is similarly capable of hepcidin upregulation, and Activin A mRNA is increased in human PBMCs exposed to schizonts. We propose that activins may link malaria infection and hepcidin upregulation. This study has implications for understanding host-parasite interactions, and may inform strategies for addressing the twin problems of anaemia and malaria.

Podium #4
IRON MODULATES BMP6 IN HEPATOCYTES AND LIVER SINUSOIDAL ENDOTHELIAL CELLS
Laura Silvestri, PhD[1], Antonella Nai, PhD student[2], Marco Rausa, PhD student[2], Alessia Pagani, PhD[2] and Clara Camaschella, MD[2]
[1]Vita-Salute University and San Raffaele Scientific Institute; [2]Vita-Salute University and San Raffaele Scientific Institute, Milan, Italy
(Presented By: Laura Silvestri)

Introduction: Hepcidin, the main regulator of iron homeostasis, is controlled by two pathways sensing circulating (Holo-Tf) and hepatic iron. Although the signals that modulate hepcidin according to Holo-Tf are still unknown, hepatic iron upregulates hepcidin through transcriptional activation of BMP6. The liver tissue is constituted by several heterogeneous cell types with different functions: 70% hepatocytes (HC), the remaining nonparenchimal cells, including Kupffer cells (KC), liver sinusoidal endothelial cells (LSEC), hepatic stellate cells and others. Which cell types modulate BMP6 in response to liver iron changes has not been explored yet.
Materilas and Methods: To investigate the potential contribution of the different cell types to the regulation of BMP6 and hepcidin in conditions of dietary-induced iron overload or iron deficiency, we separated HC, KC and LSEC by a combination of collagenase-based density gradient centrifugation and magnetic activated cell sorting (Liu et al., Proteomics 2011) and analyzed BMP6 expression by qRT-PCR. We investigated also the role of spleen-derived macrophages, F4/80 positive,

CD11b positive and F/4/80-CD11b negative cells, since the spleen, together with the liver, represents the main organ that release or store iron according to the body iron requests. Under basal conditions, HC are the major source of BMP6.
Results: We observed that in iron overload, BMP6 is strongly induced in HC and accordingly, the BMP-SMAD target genes Hamp and Id1 are increased. In addition, LSEC and, to a lesser extent, KC upregulate BMP6 in conditions of high iron suggesting that also these cell types, in addition to HC, participate in hepcidin regulation. In condition of low iron, BMP6 is only slightly downregulated in HC cells and no significant changes were observed in KC and LSEC. Both Hamp1 and Id1 are strongly reduced in HC, suggesting that in iron deficiency other signal/s, as hypoxia-dependent Tmprss6 modulation, are activated to negatively modulate the BMP-SMAD pathway. Neither low nor high iron caused transcriptional changes in BMP6 levels in the different spleen-derived cell types, suggesting that the liver is the main organ that regulates BMP6 in response to increased iron accumulation.
Conclusion: Additional studies on genetic mice models of iron overload (*HJV* and *TfR2* KO mice) or iron deficiency (*Tmprss6* KO mice) will allow to characterize more in detail the BMP6-mediated signaling in the different cell types under pathological conditions.

Podium #5
MICRORNA-130A DOWNREGULATES HEPCIDIN EXPRESSION DURING IRON DEFICIENCY BY TARGETING ALK2
Kimberly Zumbrennen-Bullough, PhD, Qifang Wu, PhD, Wenjie Chen, PhD and Jodie Babitt, MD
Division of Nephrology, Program in Membrane Biology, and Center for Systems Biology, Massachusetts General Hospital, Harvard Medical School
(Presented By: Kimberly Zumbrennen-Bullough)

Introduction: Systemic iron homeostasis is primarily controlled by the liver through the secretion of the peptide-hormone hepcidin, which regulates intestinal iron absorption and hepatocyte and macrophage iron sequestration. Hepcidin expression is upregulated via a canonical BMP-SMAD signaling pathway during iron-replete conditions, and results in the downregulation of intestinal iron uptake and cellular iron export. Here, we show that microRNA-130a (miR-130a) specifically targets members of the BMP-SMAD pathway to downregulate hepcidin expression during iron deficiency.
Methods and Materials: Using miRNA microarray and qRT-PCR analysis, we found miR-130a upregulated in iron-deficient and anemic mouse livers. Web-based miRNA target-prediction programs identified potential miR-130a targets within the BMP-SMAD pathway including the BMP type I receptor *ALK2* and the intracellular signaling molecule *SMAD5*. Using luciferase reporter constructs containing the 3' untranslated region (3' UTR) of the putative target transcripts, we found reduced luciferase activity in the presence of a miR-130a mimic for both *ALK2* and *SMAD5* 3' UTRs.
Results: Mutation of the putative miR-130a target sites in the *ALK2* and *SMAD5* 3' UTRs restored luciferase activity. Hep3b cells treated with miR-130a mimic showed reduced levels of endogenous *ALK2* mRNA while endogenous *SMAD5* mRNA and protein levels were unchanged. Using actinomycin D to inhibit cellular transcription, we found the half-life of *ALK2* mRNA was significantly decreased in miR-130a mimic treated cells. miR-130a mimic-transfected cells also showed reduced levels of phosphorylated SMAD1/5/8 protein (a marker of BMP-SMAD signaling) and hepcidin (*Hamp*) mRNA in response to BMP stimulation.
Conclusion: These data indicate that miR-130a is upregulated in iron deficient conditions and targets *ALK2*, and potentially *SMAD5*, to downregulate hepcidin expression. We hypothesize that by reducing the signaling capacity of the BMP-SMAD pathway, miR-130a functions to blunt hepcidin induction by iron thereby promoting iron uptake and facilitating resolution of the iron deficiency.

Podium #6
TESTOSTERONE PERTURBS SYSTEMIC IRON BALANCE THROUGH ACTIVATION OF EGFR SIGNALING AND REPRESSION OF HEPCIDIN
Chloe Latour[1], Leon Kautz, PhD[2], Celine Besson-Fournier[1], Marie-Laure Island, PhD[1], Tomas Ganz, MD, PhD[2], Olivier Loreal, MD, PhD[1], Helene Coppin, PhD[1] and Marie-Paule Roth, MD[1]
[1]Inserm; [2]UCLA
(Presented By: Marie-Paule Roth)

Introduction: Men and women exhibit significant differences in the progression of chronic liver diseases such as hereditary hemochromatosis, chronic hepatitis C, or alcoholic liver disease, all of which have been associated with altered hepcidin expression. These differences have been attributed at least in part to gender-related variations in iron metabolism.
Methods and Materials: To better understand the mechanisms underlying this sexual dimorphism, we took advantage of the very significant differences in hepcidin expression and tissue iron loading between Bmp6-deficient males and females.
Results: Prepubertal gonadectomies in both sexes and short term treatment of ovariectomized females with testosterone showed that testosterone is a hepcidin suppressor that robustly blocks hepcidin transcriptional regulation through upregulation of EGFR signaling. In contrast to hepcidin mRNA, hepatic Egfr expression and activation were higher in Bmp6-/- males than in females, and were reduced by castration but stimulated by testosterone. Furthermore, selective Egfr inhibition by gefitinib in males markedly increased hepcidin gene transcription. Enhanced Egfr signaling in males thus amplifies the previously described inhibitory effect of EGF on hepcidin transcription, possibly via regulation of Smad activity by phosphorylation and dephosphorylation of the linker and C-terminal sites. In males where the effects of testosterone and Bmp6-deficiency on

hepcidin regulation are combined, hepcidin is so strongly repressed that iron accumulates massively, not only in the liver like in females, but also in the pancreas, heart and
kidneys.
Conclusion: Testosterone-induced repression of hepcidin expression may become functionally important during homeostatic stress from various disorders that result in iron loading and/or reduced capacity for hepcidin synthesis. Novel therapeutic strategies targeting the testosterone/EGF/EGFR axis may thus be useful for slowing progression of these diseases.
This work was supported in part by a grant from the French National Research Agency (ANR, programme Genopat, project ANR-09-GENO-016).

Podium #7
IN VIVO DISRUPTION OF THE HEPCIDIN-FERROPORTIN REGULATORY CIRCUITRY CAUSES FATAL SYSTEMIC AND EXOCRINE PANCREATIC IRON OVERLOAD
Sandro Altamura[1], Hermann-Josef Groene[2], Regina Kessler[1], Bruno Galy[3],[4], Matthias Hentze[1],[3],[4] and Martina Muckenthaler[1],[4]
[1]MMPU – Molecular Medicine Partnership Unit, Heidelberg; [2]DKFZ – Deutsches Krebsforschungszentrum, Heidelberg; [3]EMBL – European Molecular Biology Laboratory, Heidelberg; [4]Equal contribution
(Presented By: Sandro Altamura)

Introduction: Systemic iron levels are tightly controlled by the hepatic hormone hepcidin in response to iron availability, inflammation, hypoxia or the iron demand for erythropoiesis. Hepcidin binds to the iron export protein ferroportin (FPN1) to regulate iron release from exporting cells. A mutation of cysteine 326 (C326S) of FPN1 was reported in a patient with non-classical ferroportin disease (Sham et al, 2005) and shown to abrogate hepcidin binding in vitro (Fernandes et al, 2009).
Methods and Materials: To study consequences of the disruption of the hepcidin-ferroportin interaction in vivo, we generated the first knock-in mouse model of C326S non-classical ferroportin disease.
Results: Mice with either heterozygous or homozygous C326S FPN alleles are viable and fertile. At 8-weeks of age both heterozygous and homozygous mice show profoundly increased transferrin saturation and serum ferritin levels as well as hepatic iron overload. Histological analysis by Perl's Prussian blue staining revealed that hepatic iron accumulation is restricted to hepatocytes and that Kupffer cells are spared of iron. In addition, splenic macrophages and duodenal enterocytes are iron-depleted.
Conclusion: Macroscopically, C326S homozygous mice show progressive, brown discoloration of the pancreas that correlates with profound iron deposition. Histological analysis reveals that iron localizes exclusively to the exocrine pancreas sparing the islets of Langerhans. Consistently, C326S homozygous mice do not show any signs of diabetes. Pancreatic iron accumulation is closely associated with increased reactive oxygen species (ROS), degeneration of exocrine pancreatic cells, increased plasma lipase and exocrine pancreatic failure. Starting at the age of 33 weeks, pancreatic failure is accompanied by progressive wasting and death. We believe that C326S FPN mice represent the first example of fatal iron overload in an animal model, opening avenues to investigate the underlying molecular mechanisms.

Sham R, Phatak PD, West C, et al. Autosomal dominant hereditary hemochromatosis associated with a novel ferroportin mutation and unique clinical features. Blood Cells Mol. Dis. 2005; 34:157-61.
Fernandes A, Preza GC, Phung Y, et al. The molecular basis of hepcidin-resistant hereditary hemochromatosis. Blood. 2009;114:437-443.

Podium #8
ZIP14 KNOCKOUT MICE EXHIBIT ALTERED IRON METABOLISM AND MARKEDLY IMPAIRED HEPATIC AND PANCREATIC UPTAKE OF INTRAVENOUSLY ADMINISTERED NON-TRANSFERRIN-BOUND IRON
Supak Jenkitkasemwong, MS[1], Chia-Yu Wang, PhD[1], Shintaro Hojyo, PhD[2], Toshiyuki Fukada, PhD[2] and Mitchell Knutson, PhD[1]
[1]University of Florida; [2]RIKEN Research Center for Allergy and Immunology, Yokohama, Japan
(Presented By: Supak Jenkitkasemwong)

Introduction: ZIP14 (ZRT/IRT-like protein 14) is a transmembrane metal-ion transporter that is abundantly expressed in the liver, pancreas, and heart. We previously found that ZIP14 mediates the uptake of non-transferrin-bound iron (NTBI) by AML12 mouse hepatocytes (Liuzzi et al., *Proc Natl Acad Sci USA*, 2006), HepG2 human hepatoma cells (Gao et al., *J Biol Chem,* 2008) and in the *Xenopus* oocyte heterologous expression system (Pinilla-Tenas et al., *Am J Physiol*, 2011). We also found that ZIP14 in HepG2 cells promotes the assimilation of iron from transferrin (Zhao et al, *J Biol Chem*, 2010). Collectively, these observations suggest that ZIP14 may function *in vivo* not only in the uptake of plasma NTBI, which appears during iron overload, but also in normal cellular iron acquisition from transferrin. Therefore, the aim of the present study was to characterize the iron status of ZIP14 knockout (*Zip14-/-*) mice (Hojyo et al., *PLoS ONE*, 2011) and to measure plasma NTBI uptake in these animals.
Methods and Materials: Iron status was assessed by measuring tissue non-heme iron concentrations, plasma iron, and hemoglobin levels in wild-type controls and *Zip14-/-*mice at 4 and 16 weeks of age. To assess NTBI uptake, 6-wk-old wild-type and *Zip14-/-*mice were intravenously injected with radiolabeled 59Fe-ferric citrate (after saturating plasma transferrin) and 59Fe uptake by the liver, pancreas, and heart was measured 2 hours later.

Results: At 4 weeks of age, hepatic non-heme iron concentrations were lower by 44% ($P < 0.0001$) in *Zip14-/-* mice (n=13) compared with controls (n=11). Diminished hepatic iron levels in *Zip14-/-* mice were associated with higher levels of transferrin receptor 1 ($P < 0.001$) and lower levels of ferritin ($P < 0.01$), as measured by immunoblotting. At 16 weeks of age, however, hepatic iron concentrations did not differ between *Zip14-/-* mice and controls. Interestingly, splenic non-heme iron concentrations did not differ between groups at 4 weeks of age, but were 42% lower ($P < 0.01$) in *Zip14-/-*mice at 16 weeks of age (n=10). The 16-week-old *Zip14-/-* mice also displayed 30% higher ($P < 0.01$) plasma iron levels than controls (n=10). Hemoglobin levels did not differ between groups at either age. Intravenous administration of radiolabeled 59Fe-NTBI revealed a 70% ($P < 0.0001$) and 75% ($P < 0.0001$) reduction in NTBI uptake by the liver and pancreas, respectively, in *Zip14-/-* mice (n=10) compared with controls (n=5). NTBI uptake in the heart was unaffected in *Zip14-/-* mice.
Conclusion: In conclusion, our data indicate that loss of *Zip14* alters iron homeostasis in mice in a tissue- and age-specific manner and that ZIP14 appears to be the primary NTBI uptake pathway in the liver and pancreas. Supported by NIH grant R01 DK080706.

Podium #9

HEPCIDIN ASSAY AND IV IRON THERAPY ARE SUPERIOR TO FERRITIN AND ORAL IRON THERAPY IN IDENTIFYING PATIENTS WITH IRON DEFICIENCY ANEMIA (IDA)

Lawrence Goodnough, MD, David Morris, Todd Koch, Andy He and David Bregman
Stanford University
(Presented By: Lawrence Goodnough)

Introduction: Oral iron therapy may not increase hemoglobin levels in IDA due to poor compliance and/or suboptimal gastrointestinal absorption due to hepcidin, which regulates iron homeostasis. This study evaluated whether hepcidin levels and IV iron therapy can better identify patients with IDA compared to use of traditional biomarkers (ferrin, TSAT) and empiric oral iron therapy.
Methods: Hepcidin levels were assessed in a subset of subjects enrolled in a randomized trial comparing oral iron (ferrous sulfate) to intravenous iron (Injectafer®[ferric carboxymaltose, FCM]) in subjects with IDA (Hemoglobin [Hb] ≤ 11 g/dL; and ferritin ≤ 100 ng/mL, or ≤ 300 ng/mL when transferrin saturation (TSAT) was ≤ 30%) (Szczech et al Amer Soc Nephrol 2011; 22:405A). Subjects who met the inclusion criteria underwent a 14-day (run-in) course of ferrous sulfate 325 mg, three times per day. Subjects with an increase in Hb ≥ 1 g/dL were considered to be "responders" and not studied further. "Non-responders" were randomized to FCM (2 injections of 750 mg given on Day 0 [day of randomization] and Day 7) or oral iron for 14 more days. Hb levels and markers of iron status were assessed at screening (day-15), day-1 and day 35. Hepcidin levels were analyzed at screening (Day -15) in an initial Cohort (I) of 44 patients. A hepcidin value of >20 ng/mL was identified for further analysis for predictive values for non-responsiveness to 14 day oral iron run-in in 240 patients (Cohort II). Hepcidin levels were also analyzed at Day -1 and Day 35 in a Cohort (III) of patients who were then randomized to FCM vs. oral iron therapy.
Results: Hepcidin screening levels in Cohort I were significantly higher in the non-responders vs. responders (33.2 vs. 8.7 ng/mL, $p < 0.004$). Only 1/22 responders had hepcidin values > 20 ng/mL. Utilizing a hepcidin criterion of > 20 ng/mL, we found a sensitivity of 41.3% specificity of 84.4% and a positive predictive value (PPV) of 81.6% for non-responsiveness to oral iron. While ferritin < 30ng/mL or TSAT <15% had greater sensitivity (77.3% and 64.7%, respectively), their PPVs (59.2% and 55%) were inferior to PPVs for hepcidin. Patients subsequently randomized to FCM vs. oral iron responded with Hgb increases of ≥1 g/dL for 65.3% vs. 20.8% ($p <0.0001$)and mean Hgb increases of 1.7 ± 1.3 vs. 0.6 ± 0.9 g/dL ($p = 0.0025$), respectively. Hepcidin levels increased significantly in the IV iron cohort (39.2 to 160.5 ng/mL) but not in the oral iron cohort (32.0 to 29.0 ng/mL), indicating a lack of effect for oral iron in iron store repletion, compared to IV iron.
Conclusion: Our analysis provides evidence that non-responsiveness to oral iron in patients with iron deficiency anemia can be predicted from patients' baseline hepcidin levels, which have superior positive predictive values compared to transferrin saturation or ferritin levels. Furthermore, non-response to oral iron therapy does not rule out iron deficiency, since two thirds of these non-responders to oral iron, responded to IV iron.

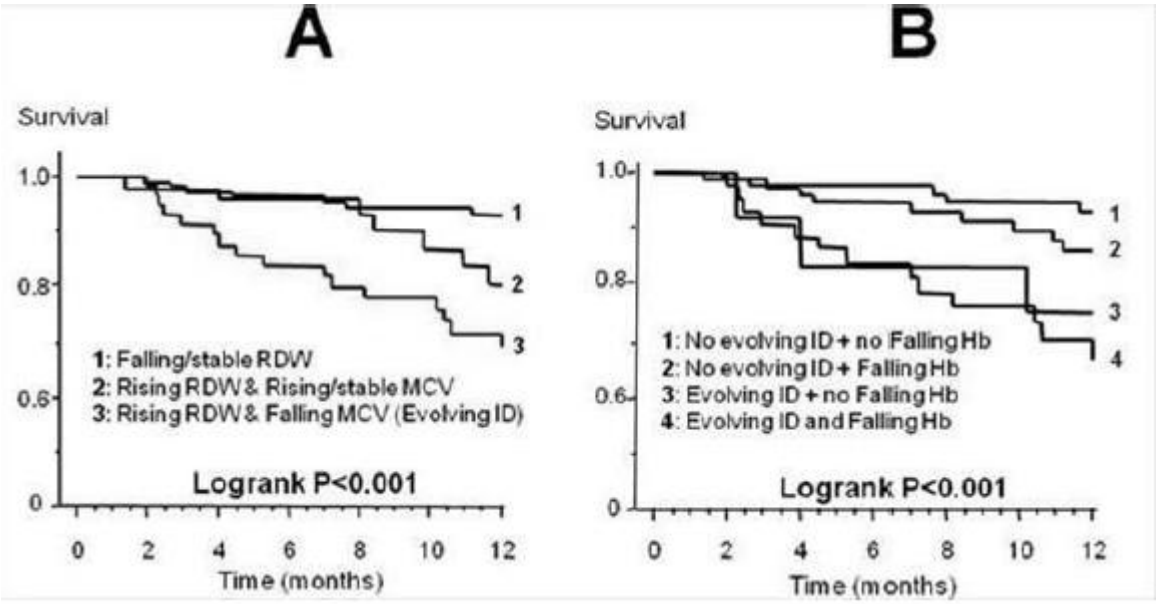
Podium #10

EVOLUTION OF SIMPLE ERYTHROCYTE INDICES TOWARDS AN IRON DEFICIENT PICTURE PREDICTS WORSENING MYOCARDIAL SYSTOLIC FUNCTION AND PROGNOSIS IN CHRONIC HEART FAILURE

Hazel Turner, MBBS, Klio Konstantinou, MBBS, Karl Norrington, MBBS, Mihir Kelshikir, BSc, Mohamad Barakat, MBBS, Richard Jabbour, MBBS, Nelson Amaral, MD, Hua Zen Ling, MBBS, Suneil Aggarwal, MBBS, Julia Flint, MBBS, Nay Aung, MBBS, Simon Woldman, MBBS, Surjit Kalia Srai, PhD and Darlington Okonko, PhD
Imperial College London
(Presented By: Hazel Turner)

Introduction: Whilst a progressive rise in the red cell distribution width (RDW) can reflect many aberrations that exacerbate chronic heart failure (CHF), the combination of a rising RDW and falling mean cell volume (MCV) signifies evolving iron deficiency (ID). Because iron is critical for multiple physiological processes and ID in animal models subtly impairs myocyte contractility, we hypothesised that evolving ID might be primarily responsible for the adverse prognosis conferred by a rising RDW, and might attenuate sensitive indices of myocardial systolic performance.

Methods and Materials: We analysed the 1 year survival implications of evolving ID in a derivation cohort of 211 CHF outpatients (age 68±12 years, Left ventricular ejection fraction 29±9%, New York Heart Association class 2±1) seen at University College Hospital, London and validated the results in another 174 CHF patients seen at St Marys Hospital, London. Repeat tissue doppler mitral annular systolic velocities (S'), a sensitive measure of myocardial contractility, were available in 34 patients.
Results: In the derivation cohort, 56 (27%) patients had evolving ID and 31 (15%) died. Patients with evolving ID had greater reductions in S' over time than 5 of 38 those who did not (-0.5 cm/sec vs. 1.0 cm/sec, P<0.05). In Cox survival analyses, evolving ID predicted increased mortality (Hazard ratio 3.43, 95% confidence interval 1.68 – 7.04, P<0.001) independently of all covariates and enhanced $\chi 2$ values when added to baseline models implying incremental prognostic utility. A ΔRDW > 1.1% and a ΔMCV ≤ -1 fL optimally forecasted death with their combination identifying those at a 6-fold escalated risk
(Hazard ratio 5.84, 95% confidence interval 2.73 – 12.51, P<0.0001). Patients with evolving ID had a worse outcome than those with a rising RDW alone (Fig A), and those with evolving ID without falling haemoglobin levels had a poorer prognosis than those with a falling haemoglobin without evolving ID (Fig B). In the validation cohort, 30 (17%) patients died. Again, evolving ID was more ominous than a rising RDW or a falling haemoglobin alone.
Conclusion: Evolution of simple erythrocyte indices towards an iron deficient picture can identify CHF patients at a 6-fold increased risk for mortality. Evolving ID is associated with worsening subtle measures of left ventricular contractility and should be targeted.



Podium #11
TRANSFERRIN RECEPTOR 1 (TFR1) IS REQUIRED FOR MAINTENANCE OF THE INTESTINAL EPITHELIUM
Alan Chen[1], Renee M. Ned, PhD[2], Adriana Donovan, PhD[3] and Nancy C. Andrews, MD, PhD[1]
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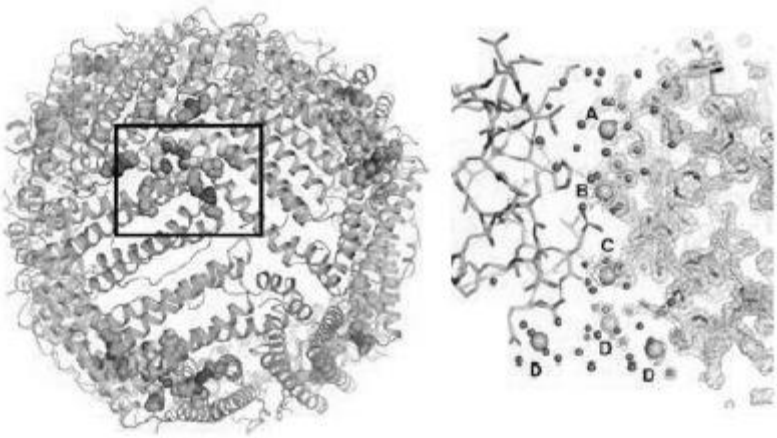
(Presented By: Alan Chen)

Introduction: Small intestinal epithelial proliferation is driven by stem cells and transit amplifying cells residing in crypts. Tfr1 is enriched along the basolateral surface of proliferating intestinal epithelial cells. We previously showed that Tfr1 is required to assimilate transferrin-bound iron from the serum into erythroid precursors. However, its role in the intestinal epithelium was unknown.
Methods and Materials: We generated and characterized a conditional knockout mouse model in which Tfr1 is deleted throughout the intestinal epithelium, using a floxed Tfr1 allele and Cre recombinase expressed under the control of the villin promoter. Tfr1 intestinal epithelium-specific knockout mice died by postnatal day 2 with major disruption of epithelial integrity, blunted villi, edema, and neutrophilic infiltration. Loss of Tfr1 severely abrogated proliferation in the crypts as assessed through staining for Ki67. This phenotype was replicated in adult animals by Tamoxifen-inducible deletion of Tfr1 using an estrogen-responsive Cre recombinase.
Results: We were unable to rescue the mutant mice by administration of large doses of iron dextran, even though increased iron was observed in the epithelium. However, the phenotype was corrected by transgenic expression of a mutant allele of Tfr1, Tfr1RR654A, which is unable to bind transferrin. In the rescued animals, the intestinal epithelial morphology and proliferation were indistinguishable from wild type mice. Taken together, these experiments suggest that Tfr1 has a novel,

iron-independent role in the intestinal epithelium. Characterization of the mutant epithelium revealed that Sox9 expression was decreased in the transit amplifying cells. Furthermore, the percentage of Lgr5+ intestinal epithelial stem cells was significantly reduced in the Tfr1 conditional knockout mice.
Conclusion: We conclude that Tfr1 is essential for early steps in intestinal epithelial maintenance and/or differentiation and that its role is independent of iron assimilation.

Podium #12
HOW FERRITIN PROTEIN CAGES LINK ION CHANNEL FE2+ TRANSPORT TO THE ACTIVE SITES: MORE SOPHISTICATION IN FERRITIN FUNCTION
Elizabeth Theil, PhD and Rabindra Behera, PhD
CHORI
(Presented By: Elizabeth Theil)

Introduction: Ferritins are a diverse group of protein cages, which reversibly synthesize caged iron oxide bionanominerals, by a combination of protein-based (enzymatic) reactions of Fe2+ and O2 or H2O2 substrates and protein cage -controlled mineral growth. The Fe2O3•H2O biomineral is a metabolic iron concentrate and antioxidant used by humans, other animals, plants, bacteria and archaea; in humans and other animals, the protein cages mix enzymatically active (H) and inactive (L) subunits; tissue-specific H:L ratios coincide with tissue – specific ferritin biomineral order (crystallinity) and different iron physiology, e.g., in heart and liver. Fe2+ reaches ferritin active sites through ion channels that cross the cage and connect the cytoplasm to the ferritin interior, much as ion channels cross cell membranes. Even though ferritin active sites vary in cage location, amino acids and catalytic mechanisms, all ferritins use ion channels, which are constructed from helical sections of three protein subunits assembled symmetrically around the three-fold cage axes (Figure 1). While many studies examine effects of ion channel and active site amino acids on overall mineralization, none have identified molecular links between the ferritin Fe2+ ion channel transport and delivery to the active sites buried in the middle of each H subunit.
Methods and Results: By substitution of conserved amino acids, which have flexible positions in ferritin protein crystal structures, we observed: 1.Conserved carboxylates act as a “bucket brigade” moving Fe2+ from: Channel constriction E130 to Channel flare D127 to E136 to E57 to Active site E58. (E136 flips between D127 and E57; E57 flips between E136 and E58). In E136A and E57A, Kcat is inhibited, Km and active site saturation increase, and turnover slows (longer T1/2 diferric peroxo). 2. An animal specific –SH, C126, pulls Fe2+ into the ion channels: (Cu2+, inhibitor of Fe2+/O2 catalysis, and Cys126, form a Cys-Cu complex in E130D; absorbance: 373 and 455 nm). Moving parts in stable (6 M urea or 80oC) ferritin protein cage scaffolds. e.g., gated pores (see Tosha et al., JBC ¬287:13017, 2012) and “flipping”, internal transit residues, E136 and E57, show Nature’s evolutionary tweaking of old protein coats. (Part support: NIH DK20251, CHORI Partners).
Figure 1. Multiple metal ions in ferritin ion channels, which distribute Fe2+ to three active sites, are aligned from top (cytoplasm) to bottom (inside the ferritin protein cage), and cluster in the flared part of the channel near the internal exits, in eukaryotic ferritins. Left: External view of the protein cage; black box: one of the eight ion channels. Right: Side view of an ion channel; note line of metal ions (green) and exit cluster of metal ions. Modified from: Tosha et al., JBC 287: 2012, 13017; PDB file 3KA3.



Podium Abstracts

Podium #13

REGULATION OF PLANT IRON RESPONSE AND ACCUMULATION BY IRON-BINDING REGULATORS

Takanori Kobayashi, PhD[1], Seiji Nagasaka, PhD[2], Takeshi Senoura, PhD[3], Reiko Nakanishi Itai[2], Hiromi Nakanishi, PhD[2] and Naoko K. Nishizawa, PhD[2]
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(Presented By: Takanori Kobayashi)

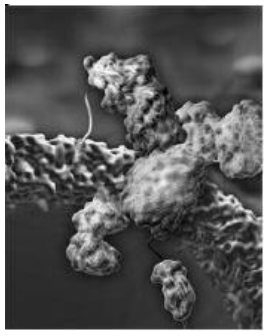
Introduction: Most living organisms, including all the animals and plants, absolutely require iron for their survival and proliferation. Since humans rely on plants as the primary source of both energy and iron, clarification of iron homeostasis in plants is of particular importance. Although abundant in mineral soils, iron is sparingly soluble in the physiological pH range under aerobic conditions. To avoid a deficit of iron, higher plants transcriptionally induce various genes involved in iron uptake and translocation in response to low iron availability.
Methods and Materials: We have previously identified and characterized the rice transcription factor IDEF1 (Iron Deficiency-responsive Element-binding Factor 1), which plays a central role in iron deficiency response and tolerance in rice. IDEF1 expression was constitutively observed in various organs including roots, leaves, flowers and seeds throughout rice life, and was not affected by iron nutritional status.
Results: IDEF1 positively regulated the majority of known iron uptake and/or translocation-related genes during iron sufficiency and the early stages of iron deficiency. In subsequent stages of iron deficency, however, IDEF1 partially changed the species of its downstream genes. IDEF1 possessed characteristic histidine–asparagine repeat and proline-rich regions, which bound to various divalent metals, including iron and zinc. Deletion of these metal-binding regions impaired the IDEF1-mediated gene activation primarily at an early stage but not at subsequent stages of iron deficiency. Recently, we identified another kind of metal-binding regulators in rice, which we designated OsHRZ1 and OsHRZ2. Expression of OsHRZ1 and OsHRZ2 was transcriptionally up-regulated under iron deficiency and was positively regulated by IDEF1. OsHRZ1 and OsHRZ2 bound to iron and zinc, at higher metal/protein ratios compared with IDEF1. HRZ knockdown plants exhibited tolerance to iron deficiency both in hydroponic culture and calcareous soil, and accumulated more iron in shoots and grains irrespective of soil iron conditions. Expression of iron deficiency-inducible genes involved in iron uptake and translocation was markedly enhanced in HRZ knockdown plants, mostly under conditions of iron sufficiency. These results suggest that OsHRZ1 and OsHRZ2 are novel iron-binding sensors that negatively regulate iron acquisition under conditions of iron sufficiency.
Conclusion: Our results suggest that plants possess both positive and negative iron regulators that bind to iron and other metals, including zinc, thus possibly sensing cellular metal ion balance caused by changes in iron availability rather than absolute iron concentration. Our results also pave the way for the production of iron-efficient and iron-fortified plants for the production of enhanced food and biomass in problem soils.

Podium #14

STRUCTURAL BASIS FOR IRON PIRACY BY PATHOGENIC NEISSERIA

Susan Buchanan[1], Nicholas Noinaj, PhD[1], Nicole Easley, BS[1], Muse Oke, PhD[1], Naoko Mizuno, PhD[2], James Gumbart, PhD[3], Evzen Boura, PhD[1], Ashley Steele, PhD[4], Olga Zak, PhD[5], Philip Aisen, PhD[5], Emad Tajkhorshid, PhD[3], Robert Evans, PhD[6], Andrew Gorringe, PhD[7], Anne Mason, PhD[4] and Alasdair Steven, PhD[2]
[1]NIDDK / NIH; [2]NIS / NIH; [3]U. IL Urbana; [4]U. Vermont; [5]Albert Einstein College of Medicine; [6]Brunel U.; [7]Health Protection Agency, Porton Down
(Presented By: Susan Buchanan)

Introduction: Neisseria are obligate human pathogens causing bacterial meningitis, septicemia, and gonorrhea. Neisseria require iron for survival and can extract it directly from human transferrin for transport across the outer membrane. The transport system consists of TbpA, an integral outer membrane protein, and TbpB, a co-receptor attached to the cell surface; both proteins are potentially important vaccine and therapeutic targets. Two key questions driving Neisseria research are: 1) how human transferrin is specifically targeted, and 2) how the bacteria liberate iron from transferrin at neutral pH.
Methods and Materials: To address them, we solved crystal structures of the TbpA-transferrin complex and of the corresponding co-receptor TbpB. We characterized the TbpB-transferrin complex by small angle X-ray scattering and the TbpA-TbpB-transferrin complex by electron microscopy.
Conclusion: Collectively, our studies provide a rational basis for the specificity of TbpA for human transferrin, show how TbpA promotes iron release from transferrin, and elucidate how TbpB facilitates this process.



Podium Abstracts

Podium #15

IRP2 REGULATES TUMOR GROWTH IN BREAST CANCER

Wei Wang, PhD[2], Zhiyong Deng, PhD[1], Heather Hatcher, PhD[3], Lance Miller, PhD[3], Xiumin Di, MS[3], Lia Tesfay, MS[1], Ralph B. D'Agostino Jr., PhD[4], Suzy Torti, PhD[1] and Frank Torti, MD[5]

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(Presented By: Suzy Torti)

Introduction: Iron regulatory proteins (IRP1 and IRP2) regulate cellular iron by controlling the expression of many genes involved in iron transport, storage and utilization. Iron metabolism has been shown to be important in some cancers. However, the role of IRPs in breast cancer is not yet understood.

Methods: 1. Cell culture: breast cells included the breast cancer cell lines R5, MCF7, and MDA-MB-231-LUC; the non-tumorigenic breast cancer cell line MCF10A, and primary mammary epithelial cells HME.

2. shRNA gene silencing: Two individual lentiviral shRNA vectors were created for IRP2. Gene knockdown was conducted by lentiviral infection.

3. Cell growth assay: Cell proliferation was measured using a WST-1 assay kit following the manufacture's instruction (Roche Applied Science). Cell survival was analyzed by a clonogenic assay.

4. Gene expression analysis: Real-time RT-PCR was performed on the ABI Prism 7000 to detect mRNA expression. Western blotting was used to detect protein levels.

5. Xenograft animal experiments: All animal protocols were approved by the Wake Forest Health Sciences Animal Care and Use Committee. MDA-MB-231-LUC cells (containing scrambled shRNA or IRP2 knockdown shRNA) were inoculated into the fourth inguinal mammary fat pad of female nude mice and tumor growth was monitored over time.

Results: We observed that several breast cancer cell lines exhibit high expression of IRP1 and IRP2 relative to non-malignant breast cells. Gene silencing experiments demonstrate that IRP2 plays a major role in mediating cellular iron homeostasis in the MDA-MB-231 breast cancer cell line, because IRP2 rather than IRP1 knockdown reduced TfR1 and increased ferritin H expression. The critical function of IRP2 in iron metabolism in breast cancer cells was further indicated by a significant decrease in the liable iron pool (LIP) following silencing of IRP2. Levels of IRP2 affect the proliferation of breast cancer cells: proliferation of MDA-MB-231 cells was significantly decreased following IRP2 knockdown. In an orthotopic xenograft model, MDA-MB-231-Luc cells infected with IRP2 shRNA lentivirus or control shRNA were injected into the mammary fat pad of nude mice. IRP2 knockdown significantly suppressed tumor growth in vivo. Microarray analysis of tumors of breast cancer patients demonstrates that increased IRP2 expression is associated with high grade cancer and molecular subtypes with poor prognosis.

Discussion: Although both IRP1 and IRP2 are highly expressed in breast cancer cells, IRP2 plays the predominant role in regulation of iron metabolism in these cells. Our study suggests that dysregulation of IRP2-mediated iron metabolism plays an important role in breast cancer development and may contribute to poor outcome of breast cancer patients.

Conclusions: 1. Breast cancer cells have a high level of IRP2.

2. IRP2 plays a key role in maintenance of iron homeostasis in breast cancer cells.

3. Knockdown of IRP2 decreases breast cancer cell growth.

Podium #16

EXPRESSION OF THE NEET PROTEIN FILY MEMBERS MITONEET AND NAF-1 IS ESSENTIAL FOR CELL PROLIFERATION AND PLAYS A ROLE IN CANCER CELLS BY AFFECTING IRON METABOLIC PROPERTIES

Sagiv Tamir, MSc[2], Yung-Sung Sohn, PhD[2], Yael Harir, MSc[2], Luma Song, PhD[3], Ron Mittler, PhD[3], Patrica Jennings, PhD[4], Ioav Cabantchik, MD PhD[2] and Rachel Nechushtai, PhD[1]

[1]Hebrew University of Jerusalem; [2]The Alexander Silberman Institute of Life Sciences, Hebrew University of Jerusalem, Israel; [3]Department of Biological Sciences, University of North Texas, TX, USA; [4]Departments of Chemistry, Biochemistry and Physics, UCSD, La Jolla, CA, USA

(Presented By: Rachel Nechushtai)

Introduction: MitoNEET (mNT:CISD1) and Miner1 (Naf-1:CISD2) are the major members of a novel class of 2Fe-2S ISC proteins that share a NEET fold characterized by a ISC that is comprised of a labile iron coordinated to 1 His and 3 Cys residues. NEET proteins are localized in mitochondria and ER and their demonstrable interactions with resident mitochondrial VDAC and Bcl2, implicate them in vital cell functions. Recent observations indicated that that mitoNEET overexpression is highly correlated with cell proliferation and especially with cancer, where mitoNEET expression levels are among the 10 top ones of the expressed human genome.

Methods and Materials: To assess the biological role of NEET proteins in normal and cancerous cells we opted for selectively repressing (by shRNA transfection) mitoNEET and NAF-1 in HEK 293 lines and mammary cancer MDA-231 and MCF-7 cells.

Results: The selective repression of either NEET protein led to a demonstrable retardation in cell growth and to morphological changes that were manifested by EM as distorted mitochondria cristae, increased number of mitochondrial particles and the appearance of autophagic vesicles. These changes were accompanied by a. reduction in respiration rates (both basal and under uncoupling conditions), b. reduction in MMP (measured with the fluorescent TMRE and JC-1 by microscopy and by flow cytometry) and c. increased ROS formation (measured with H2CDCF) that was markedly abrogated by treating cells with the permeant chelator deferiprone. The results of NEET repression in cancer cells that can be reverted or prevented by some iron chelators, implicate mitochondrial iron accumulation in ROS formation and ensuing damage. Moreover, they place the mitochondrial accumulated metal at the crossroads of disease as well as a potential target for cancer therapy.
Conclusion: Ongoing translational studies with nude mice subjected to the MDA-231 malignant cell genetically manipulated to express different levels of NEET provide direct support for the NEET cancer hypothesis
Supported by ISF (863/09

Podium #17
EXPLOITING IRON TOXICITY IN MULTIPLE MYELOMA: A STRATEGY TO INCREASE THE EFFICACY OF PROTEASOME INHIBITION THERAPIES
Alessandro Campanella, PhD[1], Sabrina Bertilaccio, PhD[1], Paolo Santambrogio, PhD[1], Magda Marcatti, MD[1], Roberto Sitia, MD, PhD[2], Giovanni Tonon, MD, PhD[1] and Clara Camaschella, MD[2]
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(Presented By: Alessandro Campanella)

Introduction: Multiple Myeloma (MM) is a malignant still incurable plasma cell disorder. Pharmacological treatment based on the proteasome inhibitor bortezomib has improved patient outcome. However, bortezomib−resistance remains a major clinical problem.
Methods and Materials: Since bortezomib promotes oxidative stress, we explored whether cellular iron metabolism may contribute to the resistant phenotype. We analyzed iron and oxidative status together with cell viability in 7 human MM cell lines (MMc) characterized by a range of responsiveness to bortezomib and in plasma cells from 5 patients. Cells were treated with increasing bortezomib concentrations with or without iron supplementation.
We found that bortezomib resistance directly correlates with ferritin levels and that iron supplementation upon bortezomib increases sensitivity.
Results: Bortezomib blocked the translation of new ferritin molecules in response to iron addition, likely through the induction of the unfolded protein response, without affecting iron export. In this way, MMc with low basal ferritin developed ROS induced damage after iron supplementation in the presence of bortezomib, while iron toxicity was less evident in bortezomib−resistant cells with high basal ferritin. Down−regulation of basal ferritin levels by DFO pre−treatment or by shRNA sensitized MMc to bortezomib and to iron toxicity. We confirmed that bortezomib prevents ferritin increase and that DFO pre−treatment followed by bortezomib and iron overcomes resistance also in patients CD138 positive cells (plasma cells). To confirm whether iron supplementation increases bortezomib sensitivity in vivo, we injected the human bortezomib−resistant U266 MMc in rag2−/−,gamma−/− mice. In this xenograft model malignant cells localization is quite exclusively restricted to the bone marrow. Animals injected with U266 and treated with bortezomib and iron showed a statistically significant increase in median survival with respect to untreated animals, at variance with animals treated with bortezomib alone. Bone marrow samples from animals treated with bortezomib plus iron showed a statistically significant reduction of U266 (CD138 and CD45R positive) cells with respect to samples from untreated or bortezomib treated animals.
Conclusion: In conclusion, basal ferritin limits oxidative damage and correlates with bortezomib resistance. Moreover, bortezomib blocks ferritin up−regulation sensitizing cells to the additional oxidative damage induced by iron supplementation. Ferritin down−regulation and iron supplementation are strategies worth to be explored to improve the efficacy of proteasome inhibition therapies.

Podium #18
EXTRACELLULAR FERRITIN IS A TUMORIGENIC FACTOR AND IS SECRETED BY TUMOR-ASSOCIATED MACROPHAGES: A FUNCTIONAL LINK BETWEEN INFLMATION AND TUMORIGENESIS
Ahmed Alkhateeb, BS[2], B. Han, MD, PhD, Kim Leitzel, MS, A. Ali, MD, Allan Lipton, MD and James Connor, PhD[1]
[1]Pennsylvania State University College of Medicine; [2]Pennsylvania State University
(Presented By: James Connor)

Introduction: Tumor-associated macrophages play a critical role in breast tumor development and progression; however, it is still unclear what effector molecular mechanisms they employ to impact tumorigenesis. Ferritin is the primary intracellular iron storage protein and is also abundant in circulation. In breast cancer patients, ferritin is detected at higher levels in both serum and tumor lysates, and its increase correlates with poor clinical outcome.
Methods and Materials: In this study, we have performed a comprehensive examination of the distribution of ferritin in normal and malignant breast tissue at different stages in tumor development. We demonstrated decreased ferritin expression in cancer cells but increased infiltration of ferritin-rich CD68-positive macrophages with increased histological grade. Interestingly, ferritin stained within the stroma surrounding tumor tissue suggesting local release within the breast. In cell

culture, primary macrophages, but not breast cancer cells, were capable of ferritin secretion and this secretion was further increased in response to the inflammatory cytokines TNFα and IL-1β. We next examined the possible functional significance of extracellular ferritin in a breast cancer cell culture model. Ferritin stimulated the proliferation of the human breast cancer cell lines MCF7 and T47D in an iron-independent manner suggesting a direct and novel function for this protein in tumorigenesis. To provide further support to this concept, we measured the inflammatory biomarker serum ferritin in advanced breast cancer patients receiving combinational therapeutics (n=66). Serum ferritin was elevated in approximately 50% of the patients and this elevation was predictive of progression-free survival (P=0.004, median 8.30 vs. 23.90 months) and overall survival (P<0.0001, median 12.73 vs. 69.57 months).

Results: When patients were stratified based on their levels for serum ferritin and the hepatic inflammatory biomarker C-reactive protein (CRP), elevation in serum ferritin alone was predictive of shorter survival while elevation in CRP alone was not. Moreover, serum ferritin and CRP showed independent prognostic value in a multivariate model. Overall, the independent prognostic value of serum ferritin indicates that its increase is not caused exclusively by inflammation-induced secretion by the liver and that it may have direct functional significance on breast tumors.

Conclusion: In conclusion, the identification of tumor-associated macrophages as the major site of ferritin expression and release within breast tumors as well as the tumorigenic effects of extracellular ferritin introduces novel therapeutic targets and molecular diagnostic tools for breast cancer. The targeting of ferritin's functionality or secretion within the tumor microenvironment may have clinical efficacy regardless of tumor site or molecular subtype.

Podium #19

THE IRON CHELATOR DP44MT IS HIJACKING P-GLYCOPROTEIN IN THE LYSOSOMES TO OVERCOME DRUG RESISTANCE

Patric Jansson, MSc, PhD, Tetsuo Yamagishi, Danae Sharp and Des Richardson
Department of Pathology and Bosch Institute, University of Sydney
(Presented By: Patric Jansson)

Introduction: P-glycoprotein (P-gp) is a drug export pump known to cause drug resistance. We have demonstrated that novel thiosemicarbazones developed in our laboratory possess potent and selective anti-cancer activity. We have also shown that P-gp localized to lysosomes sequesters substrates to confer drug resistance. Intriguingly, a leading compound, di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone (Dp44mT) showed potentiated cytotoxicity to cells over-expressing P-gp. Dp44mT is known to accumulate within lysosomes and induce lysosomal damage causing cell death. This study aimed to determine: **(a)** if the increased cytotoxicity is P-gp-dependent, **(b)** if Dp44mT is a P-gp substrate and **(c)** the link between potentiated cytotoxicity of Dp44mT in Pgp expressing cells and lysosomal damage.

Methods and Materials: MTT assay examined the effect of Dp44mT on cellular proliferation using the specific P-gp inhibitors, valspodar (Val) and elacridar (Ela). Knockdown of P-gp was achieved using siRNA. The 14C-Dp44mT efflux studies determined if Dp44mT is a substrate of P-gp. Lysosomal damage was studied using Lysotracker dye.

Results: Increased cytotoxicity of Dp44mT was observed in only P-gp expressing cells, but not in cells without P-gp. This was abrogated in the presence of Val and Ela. Knockdown of P-gp using siRNA also reduced the cytotoxicity of Dp44mT, demonstrating the importance of P-gp for mediating increased cytotoxicity. Efflux of 14C-Dp44mT was significantly less (p<0.001) in P-gp expressing cells compared to cells without P-gp. P-gp inhibitors significantly decreased 14C-Dp44mT efflux, suggesting that Dp44mT is a substrate of P-gp, leading to accumulation of Dp44mT in the lysosme. Lysosomal damage caused by Dp44mT was increased in P-gp cells and was reduced in the presence of Val and Ela, demonstrating the hijacking ability of Dp44mT to cause increased cytotoxicity in P-gp expressing cells via lysosomal damage.

Conclusion: The ability of Dp44mT to hijack lysosomes to exert potentiated cytotoxicity is a novel mechanism to combat multi-drug resistance.

Podium #20

NOVEL THIOSEMICARBAZONE IRON CHELATORS INHIBIT EPITHELIAL TO MESENCHYMAL TRANSITION AND MIGRATION OF CANCER CELLS VIA THE IRON REGULATED METASTASIS SUPPRESSOR NDRG1

Zaklina Kovacevic, Zhiqiang Chen, MD, PhD, Jing Sun, MD, Daohai Zhang, PhD and Des R. Richardson, BSc, MSc, PhD, DSc (UWA), FFSc (RCPA)
University of Sydney
(Presented By: Zaklina Kovacevic)

Introduction: Cancer mortality is largely attributed to metastasis of the primary tumour, migration and invasion to other sites in the body. One novel strategy for the treatment of cancer is to target proteins that have the ability to inhibit the metastatic and invasive properties of cancer cells. A promising new target molecule is the iron regulated metastasis suppressor, N-myc down-stream regulated gene 1 (NDRG1). Indeed, NDRG1 is often down-regulated in advanced cancers and metastases of prostate, lung, colon, pancreatic and breast cancers, with its expression being correlated with increased survival of cancer patients.

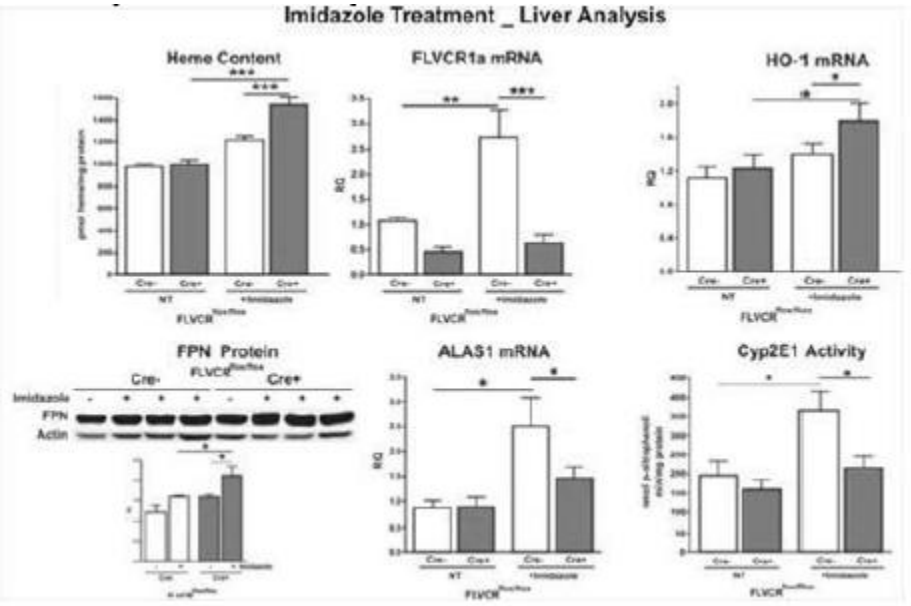
Novel thiosemicarbazone iron chelators Dp44mT and DpC are currently being developed as anti-cancer agents and were found to be highly effective at inhibiting the progression of numerous cancers both in vitro and in vivo (Richardson D.R. et al. 2004 BLOOD 104:1450). Apart from their ability to generate cytotoxic reactive oxygen species, these agents are also able to

markedly up-regulate NDRG1 expression in cancer cells via HIF-1α dependent and independent mechanisms (Le N.T. and Richardson D.R. 2004 BLOOD 104:2967).
Methods: We examined the molecular mechanisms behind the anti-metastatic effects of NDRG1 using prostate, colon and pancreatic cancer cells. NDRG1 was over-expressed or silenced in these cells to determine its down-stream effects. Novel iron chelators Dp44mT and DpC were also utilized to determine their effects on cancer cell migration and invasion.
Results and Discussion: We found that NDRG1 was able to inhibit the key step in metastasis, namely the epithelial to mesenchymal transition (EMT) leading to reduced cell migration and invasion. NDRG1 also promoted the membrane expression of the adherens junction proteins E-cadherin and β-catenin, while inhibiting the ROCK1/pMLC2 pathway that promotes stress fiber formation and cell motility.
Considering the ability of Dp44mT and DpC to up-regulate NDRG1 expression in cancer cells, we further examined the effect of these agents on the NDRG1 down-stream targets. Indeed, we found that both Dp44mT and DpC induced similar effects to NDRG1 over-expression, resulting in inhibition of EMT and cancer cell motility, migration and invasion. Using siRNA to silence NDRG1 expression, we found that the effects of the iron chelators on EMT and cell motility were largely mediated by NDRG1.
Conclusions: We have identified a promising new strategy for the treatment of cancer, one that involves novel thiosemicarbazone iron chelators that target the iron regulated metastasis suppressor, NDRG1.

Podium #21
FLVCR1A MAINTAINS HEPATIC HEME AND IRON HOMEOSTASIS AND CONTROLS CYTOCHROME ACTIVITY BY REGULATING THE FREE HEME POOL
Francesca Vinchi, PhD, Giada Ingoglia, PhD Student, Deborah Chiabrando, PhD, Emilia Turco, PhD, Lorenzo Silengo, MD, Fiorella Altruda, PhD and Emanuela Tolosano, PhD
Molecular Biotechnology Center, University of Turin
(Presented By: Francesca Vinchi)

Background: Recently, several heme exporters, such as FLVCR1a and ABCG2, have been described, suggesting the existence of an heme-iron export pathway that parallels the inorganic iron export one, mediated by FPN. We asked whether this mechanism actually acts in hepatocytes and when its function is required.
Methods and Results: To address these points, we generated a liver-specific FLVCR1a-null mouse and we analyzed its phenotype under steady state or stess conditions. 6 month-old FLVCR1a-null mice developed hepatic heme loading and showed an increased iron burden in this tissue, resulting in oxidative status alteration. Interestingly, they showed a higher hepatic HO activity, as well as a significant upregulation of HO-1, H-/L-Ferritin and FPN.
Results: The role of FLVCR1a was further evaluated upon hemolysis or enhanced heme biosynthesis. Upon PHZ-induced hemolysis, FLVCR1a, is strongly downregulated in the liver of wild-type mice, and FLVCR1a-null mice did not show any obvious difference compared to wild-type, suggesting that FLVCR1a is not essential in this condition. Upon enhanced heme biosynthesis after ALA treatment, FLVCR1a was strongly upregulated in the liver of wild-type mice, suggesting that its function is essential to detoxify heme-iron excess. Conversely, ALA-treated FLVCR1a-null mice induced HO-1, H-/L-Ferritin and FPN to an higher extent and developed hepatic heme-iron loading and tissue oxidation, indicating that the enhancement of the heme-degrading/iron-storage system compensates for the lack of FLVCR1a, thus leading to a misbalance in hepatic iron homeostasis. Since more than half of the hepatic heme production is used for the formation of microsomal cytochromes P450 (CYPs), that are involved in steroid and xenobiotic metabolism, we asked whether FLVCR1a function is required upon cytochrome induction, that in turn stimulates heme synthesis. To assess this aspect, we treated our mice with Imidazole or Benzo(a)Pyrene, two different CYP inducers. Upon these treatments, FLVCR1a-null mice accumulated an higher amount of heme in the liver compared to wild-type. Heme overload resulted in the upregulation of the genes of the heme degrading pathway and in the downregulation of those of the heme biosynthetic pathway, aimed at reducing intracellular heme as a compensation for the lack of heme export. The reduction in newly synthesized heme, despite the accumulation of an higher amount of “old and inactive” heme, led to a strong reduction in hepatic CYP activity in Imidazole- and Be(a)P-treated liver-specific FLVCR1a-null mice compared to wild-type.
Conclusions: These data demonstrate that FLVCR1 has a key role in the maintenance of physiological hepatic heme/iron homeostasis. Moreover, our results suggest that FLVCR1a has a regulatory function on CYP activity. In particular, FLVCR1a controls CYP activity by regulating heme synthesis and the composition of the free heme pool.

Podium Abstracts



Podium #22

A POTENTIAL ROLE OF THE HEME EXPORT PROTEIN FLVCR IN IRON HOMEOSTASIS

Raymond Doty, PhD, Elizabeth Broussard, MD, Christopher Savard and Janis Abkowitz, MD
University of Washington
(Presented By: Raymond Doty)

Introduction: FLVCR is a widely expressed heme export protein with highest levels present in tissues that have a high flux of heme, such as placenta, uterus, duodenum, liver, and macrophages. Mice that have FLVCR deleted systemically develop profound anemia and have severe iron loading in the liver, duodenum, and kidney while mice lacking FLVCR only in hematopoietic cells develop profound anemia, but have only slightly elevated liver and kidney iron levels. This suggests that loss of FLVCR in non-hematopoietic tissues results in the higher tissue iron loading and is consistent with FLVCR playing a role in trafficking iron (as heme-iron) independent of its role in erythropoiesis. Previous studies demonstrated heme was secreted into bile after hemolysis or exogenous heme administration, thus we suggest FLVCR may be able to export heme into bile as a way to regulate hepatic iron levels. We wanted to test whether hepatocyte FLVCR is capable of excreting heme-iron via the bile and whether FLVCR plays a role in iron homeostasis independent of erythropoiesis.

Methods and Materials: To perform these studies, we bred our *Flvcr* floxed mice with *Albumin-cre* mice to generate mice lacking FLVCR in hepatocytes. We analyzed the heme content of bile collected from control mice and mice lacking FLVCR in hepatocytes under basal and hemolytic stress conditions to determine if FLVCR is able to secrete heme into bile. Control mice secrete 13.8 ± 10.8 pg/min of heme into bile while mice lacking hepatocyte FLVCR do not secrete any detectible heme into bile. After phenylhydrazine induced hemolysis, control mice secrete 34.5 ± 17.5 pg/min of heme into bile while mice lacking hepatocyte FLVCR secrete less than 25% of that: 7.4 ± 3.3 pg/min. This demonstrates that under basal conditions FLVCR is likely the only pathway to secrete heme into bile, however other heme exporters may be capable of secreting heme into bile as demonstrated by the secretion of heme into bile under hemolytic stress conditions in mice lacking hepatocyte FLVCR.

Results: These rates of heme secretion into bile would result in the excretion of 1.8-4.5 ng iron per day, however, which is unlikely to directly impact body iron levels since mice absorb about 5 µg of iron daily. We then tested if FLVCR-mediated heme excretion from hepatocytes may impact liver iron levels and play a regulatory role in fine tuning iron homeostasis, and characterized iron parameters and regulatory genes in mice lacking hepatocyte FLVCR. We found no changes in serum iron, transferrin saturation, or hemopexin levels. Interestingly, we found a small but significant reduction in splenic *Flvcr* expression that is not caused by off target deletion of *Flvcr*. We also find a mild increase in splenic and duodenum iron levels with no changes in hepatic iron levels. Concomitant with these small increases in tissue iron levels, we found a reduction in liver *Hmox2*, *Hamp*, and *Fpn1* expression. These results are consistent with reduced heme delivery to the liver caused by the reduction of splenic macrophage expression of FLVCR.

Conclusion: These studies indicate hepatocyte FLVCR at most plays a minor role in iron homeostasis and suggests that either macrophages, including Kupffer cells, or enterocyte FLVCR may be major contributors of the severe iron loading in mice lacking FLVCR systemically.

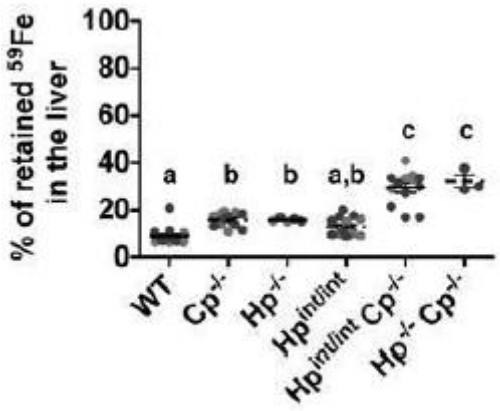
Podium Abstracts

Podium #23
EACH MEMBER OF THE PCBP FILY EXHIBITS IRON CHAPERONE ACTIVITY TOWARD FERRITIN
Sebastien Leidgens, PhD[1], Kimberley Bullough, PhD[1], Haifeng Shi, PhD[1], Toshiki Yabe, PhD[1], Minoo Shakoury-Elizeh, BA[1], Poorna Subramanian, BA[2], Emory Hsu, BS[1], Navin Natarayan, BS[1], Anjali Nandal, PhD[1], Timothy Stemmler, PhD[2] and Caroline Philpott[1]
[1]NIDDK, NIH; [2]Wayne State University
(Presented By: Caroline Philpott)

Introduction: The mechanisms through which iron-dependent enzymes receive their metal cofactors are largely unknown. Poly r(C) Binding Protein 1 (PCBP1) is an iron chaperone for ferritin and both PCBP1 and its paralog PCBP2 are required for iron delivery to the prolyl hydroxylase that regulates HIF1.
Methods and Materials: Here we show that PCBP2 is also an iron chaperone for ferritin. Expression of PCBP2 in yeast cells expressing human H and L ferritin activated the cell's iron deficiency response and led to increased iron deposition into ferritin. Depletion of PCBP2 in Huh7 cells led to diminished iron incorporation into ferritin. Both PCBP1 and PCBP2 were co-immunoprecipitated with ferritin in HEK293 cells and both PCBPs exhibited micromolar binding affinity for Fe(II) and for ferritin by isothermal titration calorimetry. Mammalian genomes contain four PCBP paralogs.
Results: While PCBPs 1 and 2 are ubiquitously expressed, PCBP3 and PCBP4 exhibit only limited expression in some tissues. Expression of PCBPs 3 and 4 in yeast cells activated the iron deficiency response, but only PCBP3 exhibited genetic interaction with ferritin and increased iron loading into ferritin. Expression of PCBP1 and ferritin in a *ccc1* mutant yeast strain, which is sensitive to iron toxicity, intensified the toxic effects of iron. In contrast, expression of PCBP4 in the *ccc1* strain protected the cells from the toxic effects of iron. Both PCBPs 3 and 4 co-immunoprecipitated with ferritin in HEK293 cells, but PCBP4 required high levels of overexpression before interaction could be detected.
Conclusion: Thus, iron chaperone activity is shared by all PCBP family members, although PCBP4 appears to exhibit reduced interaction with ferritin.

Podium #24
SEVERE DEFECTS IN IRON METABOLISM IN MICE WITH DOUBLE KNOCKOUT OF THE MULTICOPPER FERROXIDASES HEPHAESTIN AND CERULOPLASMIN
Brie Fuqua, Deepak Darshan, David Frazer, Sarah Wilkins, Chris Vulpe and Gregory Anderson
University of California, Berkeley, and the Queensland Institute of Medical Research, Brisbane
(Presented By: Brie Fuqua)

Introduction: Multicopper ferroxidases (MCFs) are important for intestinal iron absorption and iron recycling. MCFs are hypothesized to oxidize iron from the only known iron exporter ferroportin 1 for delivery to the ferric iron carrier transferrin in the blood. Hephaestin (Hp), the only MCF known to be expressed in intestinal enterocytes, is important for the transfer of dietary iron to the blood. Mice with ablation of Hp in the whole body (Hp-/-) or intestine alone (Hpint/int) exhibit defects in iron absorption and a microcytic anaemia when young. However, the anaemia improves with age and the mice survive and grow. Circulating ceruloplasmin (Cp) is the only other known MCF likely to interact with intestinal enterocytes, and has been shown to be important for iron absorption during severe iron need (S. Cherukuri et al., Cell Metab., 2005). The aim of this study was to assess the effects of combined deletion of Hp and Cp on iron homeostasis in mice.
Methods: Mice lacking both Hp and Cp (Hp-/- Cp-/- mice) and littermate controls were generated by crossing Hp-/- and Cp-/- strains. In order to differentiate between phenotypes due to ablation in the intestine versus other tissues, mice with whole body knockout of Cp but deletion of Hp only in the intestine (Hpint/int Cp-/- mice) were generated by crossing Hpint/int and Cp-/- mice. The general phenotype, hematology, tissue iron levels, and the absorption and distribution of radiolabeled iron given by gavage or via a ligated duodenal gut loop were then studied and compared for these mice.
Results: Hp-/- Cp-/- mice were pale and severely anaemic throughout life, and died of unknown causes between 23-30 weeks of age. Intestinal iron absorption was inappropriately low relative to the anaemia. Surprisingly, marked iron loading was observed not only in duodenal enterocytes of these mice but also in the liver and peripheral tissues, including the exocrine pancreas, kidney medulla, and heart. Hpint/int Cp-/- mice exhibited a moderate anaemia that was more severe than that of Hp-/- and Cp-/- single knockout mice. Iron loading, however, was seen only in the duodenum and liver, as also observed in Hpint/int and Cp-/- single knockout mice, respectively. Although both double knockout mice were able to absorb iron in radiolabeled iron absorption studies, the iron was inappropriately distributed, with relatively high levels in the liver (figure; groups with shared letters are not significantly different). Interestingly, increased liver iron retention was also observed, although to a significantly lesser extent, in Hp-/- and Cp-/- single knockout mice relative to WT controls.
Conclusions: Hp-/- Cp-/- and Hpint/int Cp-/- mice were both viable and able to absorb iron, demonstrating that Hp and Cp together, and likely MCFs in general, are not absolutely required for intestinal iron absorption. Both models, however, exhibited defects in iron absorption and distribution, and the phenotype of Hp-/- Cp-/- mice was severe. Together, these models suggest that an alternative mechanism for iron absorption, ferroxidase mediated or not, may exist. They also point to important extraintestinal roles for Hp in maintaining whole body iron homeostasis.



Podium #25

OVEREXPRESSED DROSOPHILA FERRITIN ALTERS IRON, ZINC AND MANGANESE HOMEOSTASIS

Fanis Missirlis, PhD[1], Lucía Gutiérrez, PhD[2], Kristina Zubow, PhD[3], Jon Nield, PhD[3] and Francisco J Lázaro, PhD[4]
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(Presented By: Fanis Missirlis)

Introduction: The primary function of ferritin is to store bioavailable iron in the form of ferrihydrite. In animals, ferritin is also used to traffic and recycle iron, and to modulate intestinal iron absorption, however, the effect on iron bioavailability when ferritin accumulation in cells is altered remains poorly understood. Putative *in vivo* interactions of ferritin with other metal ions have been proposed, but their physiological relevance remains unclear. These questions are highly pertinent given the role of labile metals in the propagation of oxidative stress and their vital function as prosthetic groups in enzymes.
Methods and Materials: Here we have subjected heterozygous mutant and overexpression ferritin strains of *Drosophila melanogaster* to dietary iron manipulations in order to study the partition of iron between ferritin and other proteins *in vivo*. Using quantitative magnetic analysis and single particle transmission electron microscopy imaging we have obtained evidence of a variable ferritin iron core size.
Results: Magnetic calculations suggested that total paramagnetic iron content, a likely correlate of bioavailable iron, is reduced in flies overexpressing ferritin. Further, we report that purified *Drosophila* ferritin also contained detectable zinc and manganese. Flies that overexpressed ferritin accumulated in their bodies half the amount of manganese compared to their respective controls.
Conclusion: Our results suggest that ferritin is involved in the homeostasis of other divalent metals, besides iron, and that overexpression of ferritin, sometimes employed to rescue neurodegenerative models of disease, functions to limit divalent metal bio-availability in cells.

Podium #26

TRANSCRIPTIONAL REGULATION OF HEME HOMEOSTASIS IN LACTOCOCCUS LACTIS

Shigetoshi Aono, PhD[1], Hitomi Sawai, PhD, Masaru Yamanaka, PhD, Hiroshi Sugimoto, PhD[2] and Yoshitsugu Shiro, PhD[2]
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(Presented By: Shigetoshi Aono)

Introduction: Numerous lactic acid bacteria including Lactococcus lactis acquires heme molecules as an exogenous source of heme to establish an aerobic respiratory chain. As free heme molecules are toxic for cells, cellular concentrations of heme should control strictly. L. lactis controls cellular heme concentrations by operating a heme efflux system. The expression of the heme efflux system is regulated by a heme-sensing transcriptional regulator HrtR (heme related transporter regulator). In this work, we have determined the crystal structures of apo-HrtR/DNA complex, apo-HrtR, and holo-HrtR at a resolution of 2.0, 2.8, and 1.9 Å, respectively, to elucidate the structure and function relationships of HrtR.
Methods and Materials: We have found that apo-HrtR can bind the target DNA, but holo-HrtR can not. The apo-HrtR/DNA complex structure reveals how the apo-HrtR dimer binds target DNA. The helix-turn-helix motif of each protomer contacts two consecutive DNA major grooves, and α2 and α3 helices in the helixturn-helix motifs interact with the target DNA. Arg46 (εNH) forms a hydrogen bond with a base (N7 of G11 (guanine 11)), which is the sole hydrogen bond involving the bases of DNA. The guanidium group of Arg46 also forms hydrogen bonds with the phosphate backbone of T10 (thymine10) and Tyr50. There are also hydrogen bonds between the imidazole group of His37 and the phosphate group of G9 (guanine 9), between the NH group of Ile35 and the phosphate group of T10, between the backbone NH group of Met36 and the phosphate group of T10, and between the OH group of Tyr50 and the phosphate group of G11. In addition to these hydrogen bonds, a CH-π interaction should be present between the methyl group of T12 (thymine 12) and Tyr50. The methyl group of T12 is oriented perpendicularly to the phenyl ring of Tyr50, and the distance is 3.8 Å between the carbon atom of the methyl group of T12 and

the Cg of Tyr50, which are reasonable conditions for a CH-π interaction.
Results: The crystal structure of holo-HrtR revealed that the heme is accommodated in a large cavity that is open to solvent, with His72 and His149 as the axial ligands to form a 6-coordinated heme. The α4, α5, α8, and α9 helices compose a hydrophobic heme pocket in the cavity, where the heme is surrounded by hydrophobic residues Ile71, Phe75, Phe76, Leu95, Leu99, Phe112, Val148, and Val152. These hydrophobic interactions along with the axial ligation of His72 and His149 will be responsible for a high heme-binding affinity of HrtR.
Conclusion: A comparison of the apo-HrtR/DNA complex and holo-HrtR structures revealed that heme-binding triggers a coil-to-helix transition at the α4a-α4b region. In apo-HrtR, a loop (residues 68-71) intervenes between α4a and α4b helices. Upon heme-binding, a coil-to-helix transition occurs in this intervening loop, which results in the formation of a long α4 helix in holo-HrtR. A change in the relative orientation of the DNA-binding domain is induced by the coil-to-helix transition of the α4 helix upon heme-binding, which results in the regulation of DNA-binding activity of HrtR.

Podium #27
DIFFERENCES IN HEPCIDIN REGULATION DISTINGUISH MILD AND SEVERE PHENOTYPES OF E-BETA THALASSAEMIA
Emma Jones[2], Angela Allen, Patricia Evans, Chris Fisher, Sant-Rayn Pasricha, Anuja Premawardhena, Nancy Olivieri, John Porter, Timothy St. Pierre, Hal Drakesmith[1] and David Weatherall
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(Presented By: Hal Drakesmith)

Introduction: Iron overload remains the primary cause of death in the β-thalassaemias. The suppression of hepcidin by erythropoietic drive provides an explanation for increased iron accumulation in β-thalassaemia Major and Intermedia. Co-inheritance of the haemoglobin E variant with β-thalassaemia gives rise to E-β thalassaemia, a condition that comprises around 50% of severe β thalassaemias globally and is the most common form in many parts of Asia. However many individuals with E-β-thalassaemia have very mild disease. The remarkably diverse phenotype of this condition is poorly understood, making clinical management challenging. To our knowledge, hepcidin has not been measured in this form of thalassaemia. We hypothesized that differences in phenotypic variability might be due in part to hepcidin regulation.
Methods: The interrelationships between iron parameters, erythropoiesis, inflammation and hepcidin in 70 E-β-thalassaemia patients were investigated. These patients attended a clinic in Kurenegala, Sri Lanka, and were categorised as having either mild or severe disease based on their clinical presentation including their transfusion requirements. Liver iron was assessed by MRI, and serum was analysed for ferritin, Hb, sTFR, EPO, GDF15, CRP, ALT, hepcidin and NTBI. Pairwise correlations evaluated associations between the clinical and serum parameters for the patient group as a whole and the mild and severe subgroups. T-tests compared parameters between the mild and severe patients.
Results: In the patient set as a whole, Hb was inversely correlated with both sTFR and GDF15; sTFR and GDF15 both positively correlated with EPO; sTFR and GDF15 were inversely correlated with hepcidin; hepcidin inversely correlated with NTBI, and NTBI was positively correlated with liver MRI iron and serum ferritin, which both correlated with ALT. Patients with severe phenotype were younger, had higher levels of liver MRI iron and were transfusion dependent. In this group, Hb no longer negatively correlated with indices of erythropoiesis (due to transfusion), but erythropoietic drive still inversely correlated with hepcidin, which in turn inversely correlated with NTBI. The total number of transfusions positively correlated with serum ferritin, which correlated with liver MRI iron; both ferritin and liver iron correlated with liver inflammation. In mild disease, Hb inversely correlated with GDF15 and EPO, but importantly, correlations between erythropoietic drive markers and hepcidin were no longer observed in this patient group, while the inverse correlation between hepcidin and NTBI, and the positive correlations between NTBI and ferritin and liver MRI remained.
Interpretation: Our data provide further support for the concept of an inhibitor of hepcidin that is produced by erythropoiesis. The findings suggest that severe E-β-thalassaemia is characterised by erythropoietic suppression of hepcidin despite transfusion, and the combination of low hepcidin and transfusion together exacerbate accumulation of NTBI and toxic liver iron. In patients with milder phenotype, erythropoietic suppression of hepcidin is less dominant and may be partly counterbalanced by hepcidin activating signals resulting from iron accumulation. Further investigation into hepcidin regulation in E-β-thalassaemia may assist prediction of disease course and help assess the appropriateness of chelation therapy.

Podium #28
HEPCIDIN IS ASSOCIATED WITH THE PRESENCE OF PLAQUE IN THE GENERAL POPULATION
Tessel Galesloot, Suzanne Holewijn, Jacqueline de Graaf, Sita Vermeulen and Dorine Swinkels
Radboud University Medical Centre
(Presented By: Tessel Galesloot)

Introduction: According to the “iron hypothesis”, iron deficiency plays a protective role against cardiovascular disease (CVD). Several underlying mechanisms have been proposed. Storage iron is able to catalyze the formation of reactive oxygen species (ROS), of which hydroxyl radicals are of major concern. They oxidize low-density-lipoprotein cholesterol, which has been shown to induce the formation of foam cells, and ultimately atherosclerosis. Until now, however, epidemiologic studies on associations between body iron stores and cardiovascular risk remain inconclusive. Furthermore, the peptide hormone hepcidin was hypothesized to play a role in CVD. As key regulator of body iron distribution, increased hepcidin concentrations

may enhance cardiovascular risk by reduction of iron mobilization from macrophages. This iron trapping in macrophages would activate them to become more atherogenic. Here, we assessed the associations between iron parameters and hepcidin with presence of plaque and CVD in a population-based sample.
Methods: We included 766 participants of the Nijmegen Biomedical Study (NBS) aged 46-67 years for whom measurements of hepcidin, iron parameters and non-invasive measurements of atherosclerosis were available. We excluded premenopausal women, participants with a history of CVD at baseline and persons who used antihypertensives, lipid lowering medication or medication for CVD at baseline (2003). Serum hepcidin (measured by an in house ELISA) and iron parameters were measured in 2003. Presence of plaque (assessed between 2005-2008) was defined as a focal thickening of the arterial wall of at least 1.5 times the mean intima-media thickness (IMT), an established measure of (subclinical) atherosclerosis. Information on development of CVD, defined as myocard infarct or cerebro-vascular accident, was obtained by use of questionnaires and validated by hospital records (end of follow-up at the end of 2010).
We performed multivariable logistic regression analyses using quartiles (Q) of hepcidin and the iron parameters. Analyses were stratified by gender and adjusted for age, time of blood sampling, body-mass index, C-reactive protein, glomerular filtration rate and available traditional risk factors of CVD based on the Framingham risk score: smoking, systolic blood pressure, total cholesterol and High-Density-Lipoprotein (HDL) cholesterol and diabetes.
Results: Hepcidin was significantly associated with the presence of plaque in males and females: odds ratio (OR) [95% confidence interval (95% CI)] Q2 vs. Q1 1.89 (1.00 - 3.57), Q3 vs. Q1 1.12 (0.59 - 2.14), Q4 vs. Q1 1.88 (0.97 - 3.64) for males and Q2 vs. Q1 2.65 (1.21 - 5.81), Q3 vs. Q1 1.72 (0.77 - 3.82), Q4 vs. Q1 3.45 (1.55 - 7.67) for females. Serum iron, total iron binding capacity, transferrin saturation and ferritin were not associated with presence of plaque. Associations for hepcidin with CVD in both males and females were inconclusive, due to a low frequency of events and therefore low power.
Conclusions: The results of our study are compatible with the hypothesis that increased concentrations of hepcidin increase atherosclerosis. However, the design of our study does not allow to draw conclusions about causality. In addition, even though we carefully selected potential confounding factors, residual confounding might be present in our results.
Our results suggest that hepcidin plays a role in the development of atherosclerosis, indicating that the body iron distribution is important for this process.

Podium #29

SPR BIOSENSORS AND "NATURAL HEPCIDIN RECEPTOR" IMMOBILIZATION FOR HEPCIDIN-25 DETERMINATION IN SERUM

Annalisa Castagna[1], Alessandra Maria Bossi, PhD[2], Michele Bovi, PhD[2], Oliviero Olivieri, Prof.[1] and Domenico Girelli, Prof.[1]
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(Presented By: Annalisa Castagna)

Introduction: Hepcidin-25, the main regulator of iron metabolism, has been proven difficult to measure in biological fluids, particularly through traditional immunochemical approaches. The available Mass Spectrometry-based assays developed to date were proven reliable but are limited by costs and the need of highly specialized personnel. Alternative approaches are being recently explored, including some based on the technology of Surface Plasmon Resonance (SPR). Here we propose the measurement of bioactive hepcidin by affinity capture through its natural receptor, i.e. the hepcidin binding domain (HBD) of ferroportin, immobilized onto SPR chips, using two different instruments for the detection.
Material and Methods: HBD peptide was immobilized covalently to CM5 chip. SensiQ Pioneer and Biacore3000 istruments were used for signal detection. Hepcidin 25 from different suppliers was used as standard for calibration curves implementation. Serum samples previously measured by SELDI-TOF MS were assayed. Cross reactivity experiments were also performed.
Results: The linearity range for Biacore measurements was 1.5-37 nmol/L while the linearity range for SensiQ Pioneer was 1-667 nmol/L. LOD was 1 nmol/L measured on two independent chips. The range of responses observed, especially regarding the SensiQ, fit well with the physiological concentration range reported. Our results indicate the HBD approach as a suitable way for detecting and measuring hepcidin 25 in clinical application. Synthetic hepcidin handling was strictly controlled to minimize plastic adhesion or temperature dependent unfolding, according to previous findings and guidelines. HBD-SPR was obtained after an improved binding protocol and showed good response and reproducibility either with synthetic hepcdin or with serum samples. Preliminary experiments comparing the HBD-SPR assay with a validated in-house MS assay in human serum samples indicated, in fact, an encouraging agreement. No response for the truncated forms of the hormone was detected, signifying the sensor measures the active form Hepcidin 25.
Conclusions: Biosensor technology might represent not only the first step towards the development of reliable alternative to the existing methods for the measurement of active Hepcidin 25 in body fluids but also give insights into the performance of available synthetic Hepcidin standards in terms of interaction with HBD.

Podium Abstracts

Podium #30
THE ERYTHROID FACTOR ERYTHROFERRONE AND ITS ROLE IN IRON HOMEOSTASIS
Léon Kautz, PhD, Elizabeta Nemeth, PhD and Tomas Ganz, PhD, MD
University of California Los Angeles, Center for Iron Disorders
(Presented By: Léon Kautz)

Introduction: In humans and other mammals, erythroid precursors in the bone marrow are the main consumers of iron. The availability of iron for erythropoiesis is controlled by hepcidin-induced endocytosis and degradation of ferroportin, the iron exporter which delivers iron to plasma from absorptive enterocytes and erythrocyte-recycling macrophages. In humans, within less than a day after hemorrhage or the administration of erythropoietin, duodenal iron absorption is increased, a mechanism which presumably evolved to provide for the iron requirement of increased erythropoiesis. Increased iron availability appears to be mediated by the suppression of the hormone hepcidin, thereby increasing ferroportin and delivering more iron to plasma. Increased erythropoietic activity is known to suppress hepcidin, but the molecular mechanism is not understood despite extensive investigation. We report that bleeding or administration of erythropoietin leads to the release of an erythroid factor made by erythroid precursors which acts on hepatocytes to suppress hepcidin.
Results: We examined the mouse hepcidin mRNA response to hemorrhage in wild-type mice or mice lacking hemojuvelin or TfR2, two of the critical mediators of hepcidin synthesis. Wild-type, hemojuvelin and iron-depleted TfR2 mutant mice all responded to hemorrhage by similar suppression of hepcidin mRNA within 9-15h, indicating that hemojuvelin and TfR2 are not essential for this response. We therefore initiated an unbiased search for potential suppressors of hepcidin by examining the time course of bone marrow response to hemorrhage (500 µl) using gene chip-based expression profiling. We identified less than a dozen erythroid-specific transcripts that change prior to the suppression of hepcidin mRNA. Searching for secreted proteins, we focused on a previously unidentified transcript that is highly induced prior to hepcidin suppression, and provisionally named it “erythroferrone” (ErFe). ErFe mRNA expression was greatly increased in the bone marrow and the spleen 4h after phlebotomy or EPO stimulation, preceding hepcidin suppression. ErFe-deficient mice did not suppress hepcidin mRNA after phlebotomy or EPO injection. ErFe-deficient mice also recovered more slowly from phlebotomy-induced anemia than their wild-type counterparts. We did not observe any significant defects in their baseline erythropoiesis or the composition or maturation of erythroid precursors suggesting that ERFE exerts its effect specifically on hepcidin regulation, either directly or indirectly by regulating production of another factor. Importantly, we also found that ErFe mRNA is greatly increased in the marrow and spleen of the mouse model of β-thalassemia Hbbth3/+ compared to wild-type controls.
Conclusion: Erythroferrone may be the long-sought erythroid factor repressing hepcidin during increased erythropoietic activity, and may contribute to the pathogenesis of iron-loading anemias like thalassemia.

Podium #31
AMP-ACTIVATED PROTEIN KINASE PLAYS A CRITICAL ROLE IN THE REGULATION OF HEPCIDIN EXPRESSION IN RESPONSE TO ERYTHROPOIETIC DEMAND
Hua Huang, PhD, Lara Krieg, BS and Xin Du, PhD
University of California, San Diego
(Presented By: Hua Huang)

Introducation: Systemic iron homeostasis is controlled by hepcidin-regulated iron absorption, recycling and mobilization. Increased body iron level, inflammation and endoplasmic reticulum stress upregulate hepcidin transcription, whereas erythropoietic demand and hypoxia suppress hepcidin transcription.
Methods and Materials: Although our knowledge of the iron homeostatic regulation has been enriched tremendously over the past decade, there is still a considerable gap in the molecular details. In this regard, we carried out a forward genetic screening for mutations that disturb the systemic iron balance in mice. The screen entails a simple colorimetric assay to measure serum iron concentration in the mutant mice that carry homozygous mutations induced by *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis. Here, we report a mutant phenotype, named *iron22*, displaying dysregulation of hepcidin expression in response to erythropoietic demand.
Results: Homozygous *iron22* mice show significantly reduced serum iron concentration. Further characterization reveals hemolytic anemia with dramatically increased erythropoiesis in the bone marrow as well as extramedullary tissues. In spite of increased erythropoietic activity and serum iron deficiency, hepcidin expression is significantly elevated in homozygous *iron22* mice compared to that in wild type control mice. The *iron22* phenotype was caused by a point mutation in gene *Prkag1* encoding AMP-activated protein kinase (AMPK) gamma1 subunit. The mutation creates a premature stop codon (Y38*) that triggers nonsense mediated decay of the mutant transcript. The molecular mechanism of AMPK*iron22* is currently under investigation.
Conclusion: AMPK signaling represents a critical mechanism in the regulation of iron homeostasis that is specifically responsive to erythroid signal.

Podium Abstracts

Podium #32
HEPCIDIN-INDEPENDENT IRON RECYCLING IN A MOUSE MODEL OF HAEMOLYTIC ANAEMIA
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(Presented By: David Frazer)

Introduction: The liver-derived peptide hepcidin plays a central role in body iron homeostasis by inhibiting cellular iron export. Stimulated erythropoiesis reduces hepcidin expression which in turn increases both macrophage iron release and intestinal iron absorption. This can lead to iron loading in chronic haemolytic anaemias such as beta-thalassaemia intermedia. However, in certain chronic haemolytic anaemias, iron absorption is not elevated despite a high erythropoietic rate, and no tissue loading occurs.
Methods: To investigate this in more detail, we made use of a novel mouse model created using ENU mutagenesis called MommeD7. The MommeD7 strain exhibits a mild chronic haemolytic anaemia without iron loading. We compared the MommeD7 mouse to *Hbbth3/+* mice, a model of the iron loading anaemia beta-thalassaemia intermedia. Liver, spleen, duodenum and blood were collected from four and eight week old MommeD7 and *Hbbth3/+* mice and from littermate controls, and were used to measure gene expression and tissue iron levels. Iron absorption was determined in whole animals using 59Fe. The half-life of plasma iron was analysed by following the disappearance of intravenously injected 59Fe.
Results: In both mouse strains, raised reticulocytes and an enlarged spleen suggested stimulated erythropoiesis, with less severe anaemia in MommeD7 mice (haemoglobin was 77% of littermates versus 52% for *Hbbth3/+* mice). Plasma iron half-life was decreased in both mouse strains (74% of littermates for Mommed7; 57% for *Hbbth3/+*). Despite increased iron turnover, the expression of hepatic *hepcidin* mRNA and intestinal ferroportin1 protein were unaltered in MommeD7 mice, and hepatic iron levels were normal at eight weeks. In contrast, *Hbbth3/+* mice exhibited reduced *hepcidin* expression (24% of littermates), increased intestinal ferroportin1 expression (226% of littermates), increased iron absorption (245% of littermates) and significant liver iron accumulation (241% of littermates).
Conclusions: These data suggest that in low-grade chronic haemolytic anaemia, such as that in MommeD7 mice, the increased erythroid iron requirements can be met through enhanced macrophage iron release without the need to increase iron absorption. Since this increased iron recycling can occur without changes in hepcidin, the data imply that hepcidin is not the sole regulator of macrophage iron release. The MommeD7 mouse strain provides a unique animal model with which to examine the molecular basis of hepcidin-independent macrophage iron recycling.

Podium #33
SMALL MOLECULE HIF-2α INHIBITORS SUPPRESS ERYTHROCYTOSIS AND PATHOLOGIC ANGIOGENESIS OF VHL -/- ZEBRAFISH
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(Presented By: Ana Metelo)

Introduction: Hypoxia Inducible Factor (HIF) target genes are involved in several cellular processes including angiogenesis, erythropoiesis, metabolism and stem cell proliferation. The von Hippel-Lindau (VHL) tumor suppressor protein targets HIF for degradation in conditions of normal oxygen tension. Loss-of-VHL leads to constitutive expression of HIF. We have discovered small molecules that repress HIF-2α translation by enhancing the binding of Iron-Regulatory Protein 1 (IRP1) to the Iron-Responsive Element (IRE) within the 5'-UTR of HIF-2αmRNA and showed that these small molecules specifically down-regulate HIF-2α expression in vitro.
Methods and Materials: To validate the HIF-2α inhibitors in vivo, we used two different zebrafish models; wild-type (wt) zebrafish embryos challenged with the hypoxia mimetic DMOG and a *vhl* zebrafish line. We also generated a Fli-GFP::*vhl*+/- line to image and quantify the blood vessels abnormalities of vhl embryos. Quantitative RTPCR was used to measure mRNA levels of different HIF target genes. Erythrocytosis and blood vessel sprouting were assayed using O-dianisidine staining. We employed a pixel-learning machine method for quantification. Confocal microscopy was used to image Fli-GFP::*vhl*+/- embryos. First we tested the efficacy of these compounds to inhibit acute hif induction in wt zebrafish embryos challenged with the hypoxia mimetic DMOG. Treatment of wt embryos with 100μM of DMOG up-regulated the expression of zebrafish HIF-target genes, such as vascular endothelial growth factor (vegf), erythropoietin (epo) and prolyl-hydroxylase 3 (phd3). Pre-treatment of embryos with the small molecule HIF-2α inhibitor 76 (10nM) significantly decreased the DMOG-induced up-regulation of HIF target genes and resulted in a decrease of DMOG-induced erythrocytosis and blood vessel sprouting.
Furthermore, we tested the efficacy of this HIF-2α inhibitor in a *vhl* zebrafish model (homozygougous mutants harbouring inactivating vhl mutations), which recapitulates aspects of human VHL disease (erythrocytosis, abnormal vascular proliferation in sites such as retina and brain, early lethality). Treatment with inhibitor 76 (10nM, 100nM and 1μM) down regulated the expression of the HIF-target genes phd3, epo and vegf in a dose response manner and rescued the erythrocytosis and sprouting phenotype of *vhl* embryos.
Results: Using a Fli-GFP::*vhl*+/- line we showed that compound 76 improves the abnormal connections between the intersegmental vessels of the Fli-GFP::*vhl* embryo tail, as well as their human hemangioblastoma-resembling brain vessel proliferation. Finally, we showed that treatment of *vhl* embryos with compound 76 rescues early lethality.

Conclusion: Our data demonstrate that HIF-2α inhibitor 76 is active in vivo at low concentrations and encourage further development and preclinical testing of this HIF-2α inhibitor for treatment of VHL-associated lesions, such as renal cell carcinoma, hemangioblastoma and neuroendocrine tumors.

Podium #34

IRON REGULATORY PROTEINS MEDIATE MACROPHAGE INNATE IMMUNITY AGAINST SALMONELLA

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(Presented By: Bruno Galy)

Introduction: Iron is a key determinant of host-pathogen interactions. Indeed, iron affects cellular effector pathways of both the innate and adaptive immune response; moreover, it is required by most pathogens to achieve full virulence, and iron sequestration during infection is a major defense strategy.
Methods and Materials: Iron metabolism in mammalian cells is regulated through iron-regulatory proteins (IRP)-1 and -2 that bind iron-responsive elements (IRE) in regulated messenger RNAs. We have generated mice lacking IRP1 and/or IRP2 in macrophages, key players at the interface between iron and immunity. While macrophages lacking either of the two IRPs display nearly normal expression of IRP-target genes, cells lacking both proteins exhibit marked changes in the expression of iron-handling molecules, reflecting their important molecular functions and indicating strong functional redundancy between them.
Results: Surprisingly, doubly deficient mice are asymptomatic under standard laboratory conditions. By contrast, they show higher mortality upon systemic infection with *Salmonella Typhimurium*, a pathogenic bacterium that multiplies within macrophages. Increased *S. Typhimurium* virulence in IRP-mutant mice is associated with high pathogen loads in spleen and liver, and ex vivo infection experiments show that IRP-null macrophages fail to limit the intracellular growth of *S. Typhimurium*. Interestingly, higher *Salmonella* proliferation in IRP-deficient macrophages correlates with increased bacterial uptake of radiolabelled iron. A mutant *Salmonella* strain impaired for siderophore biosynthesis no longer exhibits increased virulence in IRP-null versus control macrophages, while disruption of other known microbial iron uptake systems had little or no effect. IRP-deficient macrophages thus seem unable to restrict *S. Typhimurium* growth because they cannot limit siderophore-mediated Fe acquisition by the bacterium.
Conclusion: This work thus uncovers a novel role of the IRP/IRE system in macrophage-mediated innate immunity.

Podium #35

THE IRP1-HIF-2α AXIS: COORDINATING IRON AND OXYGEN SENSING WITH ERYTHROPOIESIS AND IRON ABSORPTION

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(Presented By: Rick Eisenstein)

Introduction: Red blood cell production is a finely tuned process that requires coordinated oxygen- and iron-dependent regulation of cell differentiation and iron metabolism.
Methods and Materials: Here we show that translational regulation of HIF-2α synthesis by IRP1 is critical for controlling erythrocyte number. IRP1 null mice (Irp1-/-) display a marked transient polycythemia with hematocrits as high as 75% between 4 and 6 weeks of age compared to ~50% in Irp1+/+ mice. Medullary and especially extramedullary erythropoiesis was substantially elevated in Irp1-/- mice at 5 weeks of age. At 5 weeks of age an altered splenic architecture, with a decreased white pulp and a substantial increase in red pulp consistent with the increased extramedullary erythropoiesis was noted. Splenomegaly was readily apparent with a 2-fold increase in mass at 4 and 5 weeks that declined thereafter. Also noted at 5 weeks of age was an increase in the primitive BFU-E (burst-forming unit-erythroid) and late-stage CFU-E (colony forming unit-erythroid) erythroid progenitors in bone marrow and spleen of Irp1-/- mice, while progenitors for other hematopoietic lineages were not altered. By 8 weeks age only about one-third of Irp1-/- mice exhibited an elevated hematocrit such that as a group Irp1-/- was not statistically different from Irp1+/+.
Results: HIF-2α mRNA is derepressed in kidney of Irp1-/- but not Irp2-/- mice leading to increased renal erythropoietin (Epo) mRNA and inappropriately elevated serum Epo levels indicating that Irp1-/- mice are not able to appropriately suppress Epo expression as they reach their adult weight and growth slows. Expression of the iron transport genes DCytb, DMT1 and ferroportin as well as other HIF-2α targets is enhanced in IRP1-/- duodenum suggesting that a state of HIF-2α hyperactivity exists in Irp1-/- mice. Analysis of mRNA translation state in liver revealed IRP1-dependent dysregulation of HIF-2α mRNA translation while IRP2 deficiency derepressed translation of all other 5' IRE-containing mRNAs expressed in liver.
Conclusion: These results uncover novel separable roles of each IRP and identify IRP1 as a therapeutic target for coordinately manipulating Epo production and iron absorption in hematologic disorders. Taken together, we propose that through it's regulation of HIF-2α, IRP1 has a critical role in influencing systemic iron metabolism and the adaptive responses to altered availability of iron and oxygen.

Podium Abstracts

Podium #36

IRP-1 CONTROLS IRON METABOLISM IN FRATAXIN-DEFICIENT TISSUES

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(Presented By: Alain Martelli)

Introduction: Friedreich ataxia (FA) is the most common recessive ataxia in the Caucasian population and is characterized by a spinocerebellar ataxia associating cardiomyopathy. FA is caused by severely reduced levels of frataxin, a mitochondrial protein involved in iron-sulfur (Fe-S) cluster biosynthesis.

Methods and Materials: We have previously generated conditional knockout (cdko) mouse models of FA, based on the Cre-LoxP strategy, which reproduce important pathophysiological and biochemical features of the human disease: progressive cardiac hypertrophy, progressive sensory and cerebellar ataxia, multiple Fe-S cluster enzyme deficit, time-dependent mitochondrial iron accumulation. Through biochemical characterizations, we showed that Fe-S cluster deficit precedes the mitochondrial iron accumulation in the cardiac mouse model (FxnMck), as well as in a mouse model in which frataxin was specifically deleted in liver (FxnAlb). By measuring IRP1 and IRP2 IRE-binding activities and expressions, we further showed that IRP1 was activated due to Fe-S cluster deficit in both FxnMck and FxnAlb, whereas no difference could be observed between control and deleted mice regarding IRP2 levels.

Results: As IRP1 activation was correlating with both transferrin receptor 1 and ferritin protein levels, we hypothesized that IRP1 activation was a key molecular event explaining the iron deregulation observed in FA. To validate this hypothesis, we crossed our frataxin cdko mouse models (FxnMck and FxnAlb) with Irp1 knockout mice to generate double Irp1ko;Fxncdko mice. Characterization of these mice revealed that activated IRP1 is indeed providing a pool of IRE-binding proteins that modifies iron distribution within the cells and that is essential to sustain the mitochondrial iron accumulation observed in the absence of frataxin. Furthermore, whereas no difference could be observed in FxnMck mice, the absence of IRP1 worsens the phenotype observed in the liver of FxnAlb mice since survival was reduced in the Irp1ko;FxnAlb compared to the FxnAlb mice. Further analysis showed that this reduced survival was a result of earlier liver failure associated with cellular iron depletion.

Conclusion: Altogether, our results show that IRP1 is essential to control iron metabolism and to adapt to the mitochondrial dysfunction associated with frataxin deficiency.

Podium #37

SNX3 REGULATES RECYCLING OF THE TRANSFERRIN RECEPTOR AND IRON ASSIMILATION

Caiyong Chen, PhD[1], Daniel Garcia-Santos, PhD[2], Yuichi Ishikawa, MD, PhD[1], Alexandra Seguin, PhD[3], Liangtao Li, MD[3], Gordon Hildick-Smith, BA[1], Matthew King, BS[1], Iman Schultz, PhD[1], Paul Kingsley, PhD[4], James Palis, MD[4], Shilpa Hattangadi, MD[5], Harvey Lodish, PhD[5], Takahiro Maeda, MD, PhD[1], Diane Ward, PhD[3], Jerry Kaplan, PhD[3], Prem Ponka, MD, PhD[2] and Barry Paw, MD, PhD[1]

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(Presented By: Barry Paw)

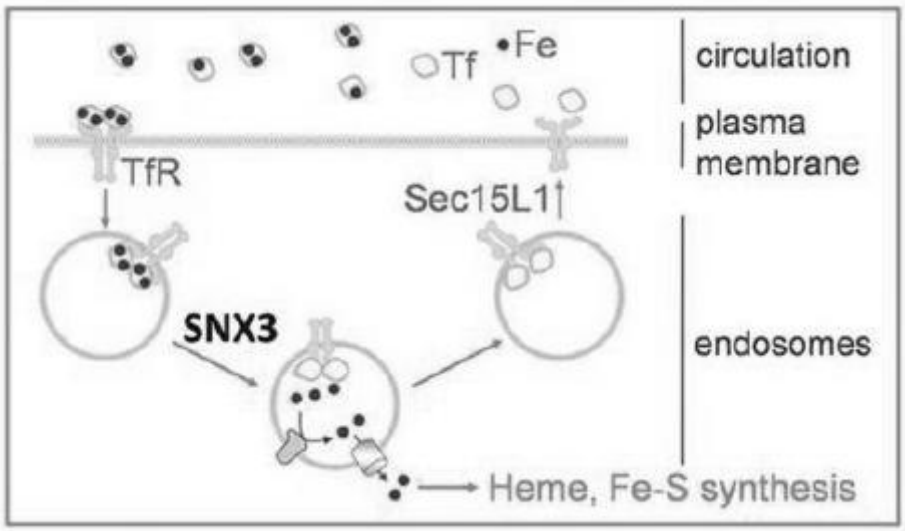
Introduction: Sorting of endocytic ligands and receptors is critical for diverse cellular processes. The functional significance of endosomal sorting proteins in mammalian physiology, however, remains largely unknown.

Methods and Materials: Here we report that Sorting Nexin 3 (Snx3), a cargo-specific retromer component, facilitates the endocytic recycling of transferrin receptor (Tfrc), and thus is required for the proper delivery of iron to erythroid progenitors. Snx3 is highly expressed in hematopoietic tissues of zebrafish and mouse. Silencing of Snx3 results in anemia in zebrafish and hemoglobin defects in mammalian hematopoietic cells due to impaired transferrin (Tf)-mediated iron uptake.

Results: This impaired iron assimilation can be complemented with non-Tf bound iron chelates. We mechanistically show that Snx3 physically interacts with Tfrc to sort Tf-Tfrc complexes to the recycling endosomes.

Conclusion: Our findings uncover a role of Snx3 in regulating Tfrc recycling, cellular iron homeostasis, and erythropoiesis in vertebrates. Thus, the identification of Snx3 provides a new genetic tool for exploring erythropoiesis and human disorders of iron metabolism.

Podium Abstracts



Podium #38
IRON REGULATORY PROTEINS SECURE EFFICIENT DIETARY IRON INTAKE AND DEFINE A BASAL SET POINT FOR THE REGULATION OF IRON ABSORPTION
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Introduction: Mammalian iron metabolism is regulated systemically by the hormone hepcidin, and cellularly by iron regulatory proteins (IRPs) that orchestrate a posttranscriptional regulatory network.
Methods and Materials: Through ligand-inducible genetic ablation of both IRPs in the gut epithelium of adult mice, we demonstrate that IRP deficiency impairs steady-state iron absorption and promotes mucosal iron retention.
Results: This retention appears to result from a ferritin-mediated "mucosal block". We also find that IRPs are dispensable for intestinal sensing of body-iron requirements. However, alteration of basal levels of iron-absorption molecules in IRP deficiency results in inadequate expression in conditions of body-iron loading and erythropoietic stimulation, respectively.
Conclusion: IRPs thus secure sufficient iron transport across absorptive enterocytes by restricting ferritin, and define a basal set point for iron absorption upon which IRP-independent systemic regulatory inputs are overlaid.

Podium #39
A SUPPRESSOR SCREEN USING CAENORHABDITIS ELEGANS TO IDENTIFY GENES REGULATING CELLULAR IRON HOMEOSTASIS
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(Presented By: Elizabeth Leibold)

Introduction: Cellular iron homeostasis is maintained within a narrow physiological range by regulating iron uptake, storage, utilization and export. Significant progress has been made in identifying proteins involved in the transport, utilization and sensing of iron, but fundamental questions remain unanswered. We still do not fully understand how iron is trafficked within cells, how iron is incorporated and released from ferritin and the identity of regulators of iron transport and storage.
Methods and Materials: We are using *C. elegans* as a model organism to address these questions. We chose *C. elegans* because they have a simple anatomy and a rapid life cycle, and mutants are easily obtained by genetic screens. Importantly, the mechanisms regulating mammalian iron metabolism are conserved in *C. elegans.* We recently reported that hypoxia-inducible factor 1 (HIF-1) regulates intestinal iron metabolism in C. elegans. HIF-1 activates *smf-3* encoding a protein similar to mammalian divalent metal transporter-1 (DMT1) and represses *ftn-1* and *ftn-2* (ferritin) transcription during iron deficiency.
Results: We found that *hif-1* null animals develop normally when grown under iron sufficient conditions, but are developmentally delayed when grown under iron limitation. The developmental delay in iron limited *hif-1* animals can be rescued by increasing cytosolic iron through RNAi depletion of FTN-1 and FTN-2. This observation formed the basis for a suppressor screen to identify second-site mutations that suppress the developmental delay of *hif-1* animals on low iron. *hif-1*

animals were exposed to the mutagen EMS. Gravid mutagenized F2 worms were allowed to lay eggs on plates containing the iron chelator 2,2'dipyridyl (BP). F3 generations were grown for 5 days, and scored for size against N2 wildtype and *hif-1* animals. N2 wildtype animals develop normally, whereas hif-1 worms are developmentally delayed and rarely progress beyond the third larval stage. Fifteen mutations that rescued the *hif-1* developmental phenotype were identified of which 4 showed full rescue and 11 showed partial rescue.

Conclusion: We are using a one step whole-genome sequencing single-polymorphism method to identify the molecular lesions of these *hif-1* suppressors. Homologs of the identified suppressors will be tested to determine their roles in mammalian iron homeostasis.

Podium #40

IN A MOUSE MODEL OF CHRONIC INFLMATION, LIVER-SPECIFIC DELETION OF ALK3 (A BMP TYPE I RECEPTOR) PREVENTS THE DEVELOENT OF ANEMIA OF INFLMATION

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(Presented By: Claire Mayeur)

Introduction: Anemia of inflammation (AI) is a common form of anemia in patients, who have inflammatory states such as neoplasia, infection or chronic heart failure. One of the mechanisms contributing to AI is a persistent induction of hepcidin synthesis by cytokines including interleukin 6 (IL-6). Hepcidin is synthesized by the liver and regulates iron metabolism by decreasing iron absorption in the duodenum and iron release by macrophages and hepatocytes. Bone morphogenetic protein (BMP) signaling participates in the regulation of hepcidin gene expression. In a murine model of AI, mice injected with turpentine and treated with LDN-193189 (a BMP type I receptor inhibitor) did not develop AI. Hence, we sought to identify which BMP type I receptor was required for the development of AI.

Methods: Among the four BMP type I receptors, only Alk2 (Acvr1) and Alk3 (Bmpr1a) are expressed at high levels in the liver. We generated mice with a liver-specific deletion of Alk2 or Alk3 using mice homozygous for Alk2 or Alk3 sequences flanked by loxP sites (Alk2fl/fl and Alk3fl/fl, respectively) and a Cre recombinase transgene expressed under the control of the liver-specific albumin promoter (Alb-Cre). At baseline, Alk2fl/fl;Alb-Cre and Alk3fl/fl;Alb-Cre mice have normal hemoglobin levels (Hb = 15.1 ± 0.6 g/dL). We induced chronic inflammation in female Alk2fl/fl;Alb-Cre and Alk3fl/fl;Alb-Cre mice, as well as their corresponding controls, Alk2fl/fl and Alk3fl/fl mice, by administering turpentine subcutaneously (5 mL/kg) once a week for three weeks (beginning when the mice were 8 weeks old). One week after the third injection, blood was collected to measure cells blood counts and serum IL-6 levels. Livers were harvested, and RNA was extracted for measurement of levels of mRNAs encoding hepcidin.

Results: Turpentine administration induced formation of a sterile abscess and systemic inflammation (characterized by splenomegaly, an increase in the number of blood granulocytes and platelets, and an increase in serum IL-6 levels) in all four genotypes. Turpentine challenged Alk2fl/fl and Alk3fl/fl mice developed AI with a reduced hemoglobin level (13.3 ± 0.3 g/dL and 13.0 ± 0.3 g/dL, respectively), compared to untreated controls. Alk2fl/fl;Alb-Cre mice, developed AI (Hb = 13.9 ± 0.8 g/dL). In contrast, turpentine challenge did not induce anemia in Alk3fl/fl;Alb-Cre mice (Hb = 15.3 ± 0.7 g/dL; $p < 0.0001$ vs Alk3fl/fl). After turpentine challenge, hepatic hepcidin mRNA levels were 69% less in Alk2fl/fl;Alb-Cre mice than in Alk2fl/fl mice ($p = 0.04$) whereas hepatic hepcidin mRNA levels were 99.5% less in Alk3fl/fl;Alb-Cre mice than in Alk3fl/fl mice ($p < 0.001$).

Conclusion: Deletion of Alk3 in the liver prevents the development of AI in a murine model. Targeting the BMP type I receptor, Alk3, to prevent the induction of hepcidin gene expression by inflammatory mediators may offer a novel therapeutic approach to the treatment of AI.

Podium #41

INFLMATION REGULATES TMPRSS6 EXPRESSION VIA STAT5 IN HUMAN CELLS AND MICE

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(Presented By: Delphine Meynard)

Introduction: *TMPRSS6* is a regulated gene, with a crucial role in the regulation of iron homeostasis by inhibiting hepcidin expression. Mutations in TMPRSS6 gene result in an Iron Refractory Iron Deficiency Anemia (IRIDA). The liver antimicrobial peptide hepcidin, the main regulator of iron homeostasis, also has a role in immunity, and is upregulated by inflammation.
In this study, we studied whether TMPRSS6 was also regulated by inflammation and explored the mechanism of this potential regulation. In addition, we also determined if the regulation of TMPRSS6 by inflammation was part of the mechanism leading to the increase of hepcidin expression in response to inflammation.

Methods and Materials: We demonstrated that IL-6 treatment of Hep3B cells down-regulates TMPRSS6 expression and matriptase 2 activity. We also showed that LPS injection in wild-type mice leads to a decrease in Tmprss6 expression. In addition, injection of LPS in Hjv-/- mice (BMP-SMAD pathway inhibited) also induced a down-regulation of Tmprss6 expression indicating that the down-regulation of Tmprss6 by inflammation in mice is not dependent on the Bmp-Smad pathway.

Results: Interestingly, in wild-type mice the phosphorylation of Stat5 is decreased by inflammation with the same time course as the decrease in Tmprss6 expression. Moreover, by electrophoretic mobility shift assay, we established that Stat5 binds to a Stat5 element located on the mouse Tmprss6 promoter. We then supported this result with two in vitro assays. First, we showed that over-expression of Stat5 positively regulates Tmprss6 expression by binding to a Stat5 element located on the mouse Tmprss6 promoter, using a mouse wild-type Tmprss6 promoter luciferase reporter construct in comparison with the same construct but with a mutation in the STAT5 element, Second, we demonstrated that silencing of STAT5 expression in Hep3B cells results in a decrease of TMPRSS6 expression. Altogether, these results indicate that TMPRSS6 is regulated by inflammation and that this regulation occurs through a decrease in Stat5 phosphorylation.
Importantly, our results suggest that the inflammatory modulation of TMPRSS6 expression is part of the regulation of hepcidin by inflammation. Indeed, we demonstrated that silencing of TMPRSS6 expression during IL-6 treatment potentiates the increase in HAMP caused by inflammation in Hep3B cells. In addition, we also showed that TMPRSS6 over-expression reduces the increase of HAMP-promoter activity induced by IL-6 treatment in Hep3B cells.
Conclusion: These results indicate that inflammation regulates TMPRSS6 expression, and that in turn TMPRSS6 has a functional role in mediating the inflammatory regulation of hepcidin regulation.

Podium #42

A STRONG ANTI-INFLMATORY SIGNATURE REVEALED BY LIVER TRANSCRIPTION PROFILING OF TMPRSS6-/- MICE

Laura Silvestri, PhD[1], Michela Riba[2], Marco Rausa[1], Melissa Sorosina[3], Davide Cittaro[2], Filippo Martinelli-Boneschi[3], Antonella Nai[1], Alessia Pagani[1] and Clara Camaschella[1]
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(Presented By: Laura Silvestri)

Introduction: The anti-microbial peptide hepcidin provides a critical connection between iron metabolism and the immune response. The type II transmembrane liver serine protease TMPRSS6 is the main negative regulator of hepcidin. We have previously demonstrated (Pagani et al., Blood 2011) that modulation of hepcidin in mice influences the inflammatory response.
Methods and Materials: In line with this observation, Tmprss6 null animals, characterized by chronic iron deficiency with high hepcidin, show a blunted production of pro-inflammatory cytokines and reduced tissue macrophages recruitment after sublethal doses of LPS, when compared with iron deficiency (low hepcidin) mice (IDA). However, the molecular pathway/s that account for the phenotype observed in *Tmprss6* null mice remain undefined. Since the liver plays a crucial role in the response to systemic inflammation we investigated the whole genome transcriptional profiling of total liver of Tmprss6-/- mice in comparison with IDA animals. This approach was performed with the aim of identifying signaling pathway/s activated by chronic hepcidin overexpression or Tmprss6 deficiency, independently of the iron status. The animals used in this study were previously described by Pagani et al. (Blood 2011). Gene expression profile was determined using the MouseWG-6 v2 Expression BeadChips (Illumina®). Normalized gene expression data were analysed by LIMMA Bioconductor package for extraction of differentially expressed genes, considering a factorial design model and pairwise comparisons. Inactivation of *Tmprss6* is associated with the up-regulation of 148 genes involved in the BMP-SMAD pathway, ion transport, protein dephosphorylation, regulation of transcription and cell growth, and the downregulation of 85 genes involved in the immune response, in various metabolic processes, proteolysis and response to stress.
Results: LPS-mediated acute inflammation causes the upregulation of 1764 and the downregulation of 1587 genes in *Tmprss6* null mice. Among upregulated genes, there are genes belonging to the fatty acid and vitamins metabolism, whereas genes belonging to the immunity and TLR-mediated responses are downregulated. In the attempt to distinguish whether the observed transcriptional changes are due to the genetic loss of *Tmprss6* or to the increased hepcidin, we analysed liver gene expression of mice with different iron status and IDA mice, injected or not with hepcidin. We observed that neither chronic nor acute hepcidin modulation induces the transcriptional changes observed in *Tmprss6* KO animals, suggesting a role for *Tmprss6,* and not for hepcidin.
Conclusion: Altogether our results strengthen the proinflammatory role of *Tmprss6*, compatible with the finding of a proinflammatory condition in iron deficiency anemia, which is characterized by TMPRSS6 activation.

Podium #43

HEMOPEXIN DEFICIENCY IN MICE RESULTED IN ENHANCED TH17 DIFFERENTIATION AND EXACERBATED EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

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(Presented By: Giada Ingoglia)

Introduction: Hemopexin (Hx) is the plasma protein with the highest binding affinity to heme and controls heme-iron availability in macrophages and also in T lymphocytes. We previously demonstrated that iron content in lymphocyte, by controlling TfR1 membrane expression, modulates their responsiveness to IFNγ. The latter plays a crucial role in CD4+ T cells

differentiation towards a Th17 phenotype. The aim of this study was to investigate the role of Hx in the development of a T-cell mediated inflammatory autoimmune response.
Methods and Materials: We immunised Wt and Hx -/- mice with 200 micrograms of myelin oligodendrocytes glycoprotein, to induce them the experimental autoimmune encephalomyelitis (EAE), the mouse model of multiple sclerosis. During EAE, Hx content in serum increased and remained high. When EAE was induced in Hx knock-out (Hx-/-) mice, they developed a clinically earlier and exacerbated EAE compared with wild-type mice, associated to an higher amount of CD4+ infiltrating T cells.
Results: The severe EAE developed by Hx-/- mice could be ascribed to an enhanced expansion of Th17 cells accounted for both a higher disposition of naïve T cells to differentiate towards the Th17 lineage and a higher production of Th17 differentiating cytokines IL-6 and IL-23 by antigen presenting cells. We discuss our results by considering the Hx ability to prevent iron loading in macrophages thus controlling the extent of inflammatory response.
Conclusion: Taken together, these data indicate that Hx has a negative regulatory role in Th17-mediated inflammation and prospect its pharmacological use to limit the expansion of this cell subset in inflammatory and autoimmune disease.

Podium #44
IRON RECYCLING AND RETENTION IN MICE WITH A FERRITIN H DELETION IN MACROPHAGES
Claude Schweizer, PhD, Larry Richman and Lukas Kühn, PhD
EPFL - Ecole polytechnique Fédérale de Lausanne, ISREC - Swiss Institute for Experimental Cancer Research
(Presented By: Lukas Kühn)

Introduction: Iron recycling requires the phagocytosis of senescent erythrocytes in macrophages, their lysis and subsequent breakdown of haem to liberate iron, which is then exported and transported to haematopoietic cells by transferrin. Under conditions of inflammation a substantial amount of iron can be retained in macrophages provoking anaemia of inflammation, presumably due to increased hepcidin levels counteracting iron export by ferroportin. For a long time it was hypothesized that ferritin might have a direct function in iron recycling by macrophages.
Methods and Materials: In order to study the role of ferritin in iron recycling and retention in macrophages, we have crossed our ferritin H lox/lox mice with lysozyme-Cre transgenic mice generating mice that showed a 96% inactivation of the ferritin H gene in macrophages. Mice were analyzed for haematological parameters and showed no significant change in the number of macrophages, nor any other haematopoietic cell lineage, except for a 20% increase in platelets.
Results: Notably, deleted mice showed normal haematocrit and haemoglobin levels. This demonstrates that ferritin is not required for erythrocyte iron recycling. Macrophage ferritin H deleted mice showed slightly increased serum iron levels and transferrin saturation compared to control mice. Tissue iron was reduced to 18% in spleen and 55% in liver indicating an important role of macrophages in iron storage of these tissues. Although macrophages had lost their iron storage capacity, they did not show signs of oxidative stress or damage, as previously observed in other tissues deleted of ferritin H. It suggests that excess free iron does not remain in the macrophages, but is rapidly exported.
Conclusion: Iron-loading of mice had no negative effects on the number of macrophages either. We are presently testing whether iron retention after an inflammatory challenge is affected in the macrophage ferritin H deleted mice.

Podium #45
HEME EXPORT THROUGH FLVCR1B IS ESSENTIAL TO PRESERVE MITOCHONDRIAL FUNCTIONALITY
Deborah Chiabrando[1], Carlotta Giorgi[2], Lorenzo Silengo[1], Fiorella Altruda[1], Paolo Pinton[2] and Emanuela Tolosano[1]
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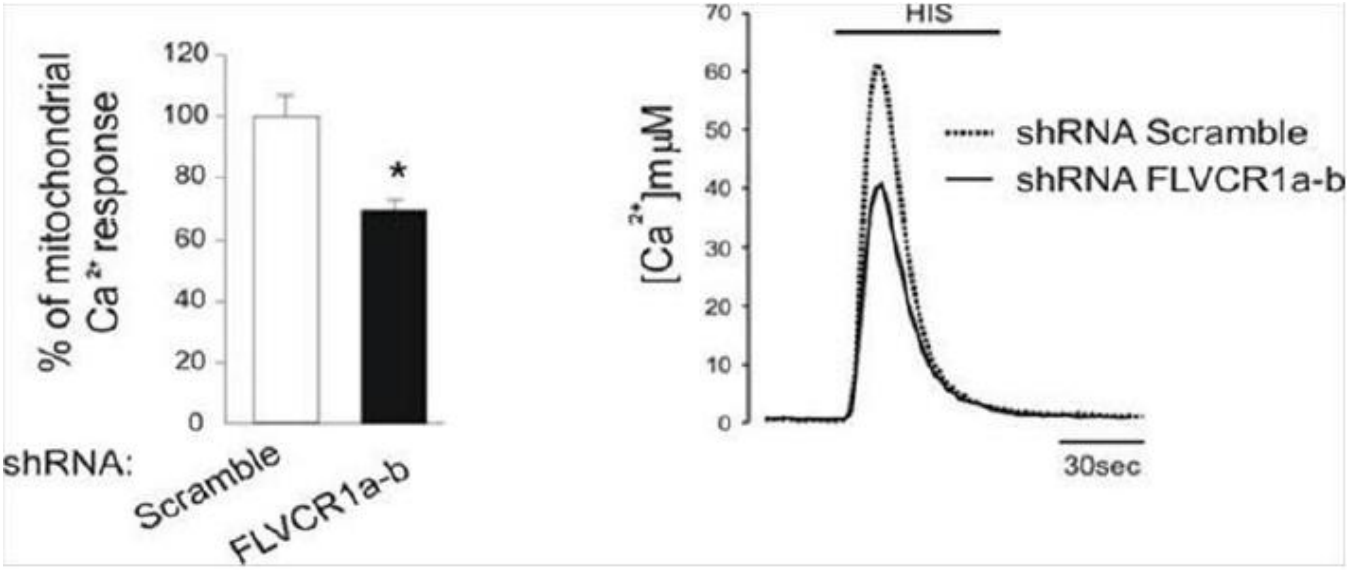
(Presented By: Deborah Chiabrando)

Background: Feline Leukemia Virus subgroup C Receptor 1 (FLVCR1) is a cell membrane heme exporter that contributes to maintain the balance between heme level and globin synthesis in erythroid precursors. Consistently, FLVCR1-null mice died in utero due to a failure of erythropoiesis (*Keel SB et al, Science, 2008*). We previously identified a mitochondrial isoform of FLVCR1, named FLVCR1b, that was able to support fetal murine erythroid differentiation in the absence of the cell membrane isoform (herein called FLVCR1a). We demonstrated that Flvcr1b overexpression promoted heme synthesis and in vitro erythroid differentiation, whereas the silencing of Flvcr1b caused mitochondrial heme accumulation and termination of erythroid differentiation. This findings indicate that FLVCR1b mediates erythroid differentiation by controlling mitochondrial heme efflux (*Chiabrando D et al, JCI, 2012*).
Objectives: The aim of the work was to further investigate the role of FLVCR1b during erythroid differentiation. We previously reported that loss of FLVCR1b impairs erythroid differentiation due to a block of heme export out of mitochondria (*Chiabrando D et al, JCI, 2012*). Now, we want to investigate whether the loss of FLVCR1b and the consequent heme accumulation in mitochondria could affect mitochondrial functionality.
Methods: To assess whether the loss of FLVCR1b affect mitochondrial functionality, the mitochondrial Ca2+ response after agonist stimulation was monitored as a highly sensitive readout of mitochondrial state. It is well known that mitochondrial alterations cause defects in Ca2+ uptake by the organelle (*Pinton P. et al, Methods in Cell Biology, 2007*).

Results: The silencing of FVLCR1b in HeLa cells caused a significant reduction of Ca2+ spike in the mitochondrial matrix evoked by agonist stimulation. These data suggested that in absence of FLVCR1b heme accumulates in mitochondria resulting in the alteration of mitochondrial functionality. The consequences of FLVCR1b loss on mitochondrial membrane potential, ATP synthesis, mitochondrial biogenesis, cell proliferation and apoptosis are still under investigation.
Discussion: All together these results suggested that the impairment of erythroid differentiation observed in the absence of FLVCR1b is due to an impairment of mitochondria functionality consequent to the block of heme export from mitochondria. Mitochondria are essential organelles regulating both cell survival and cell death. We hypothesize that misregulation of FLVCR1b expression could interfere with these processes. These results, linking heme biosynthesis pathway to mitochondrial Ca2+ signalling, will have broad implications in cellular metabolism.



Podium #46
GLYCINE TRANSPORTER 1 PLAYS A CRUCIAL ROLE IN HEMOGLOBINIZATION
Matthias Schranzhofer[1], Richard Bergeron, MD, PhD[2], Daniel Garcia dos Santos, PhD[1] and Prem Ponka, MD, PhD[1]
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(Presented By: Matthias Schranzhofer)

Introduction: Vertebrate heme synthesis requires three substrates: succinyl-coenzyme A, which regenerates in the tricarboxylic acid cycle, iron and glycine. It is well recognized that inadequate delivery of iron to immature erythroid cells leads to a decreased production of heme, but virtually nothing is known about the impact of restricted transport of glycine on the process of hemoglobinization. Two ATP- and Na+-dependent glycine membrane transport systems have been identified in reticulocytes and shown to decrease during maturation to erythrocytes (Weigensberg & Blostein, J Membr Biol 86:37, 1985). However, it is unknown whether the reticulocyte glycine transporters are related to glycine transporter 1 (GlyT1) and 2 (GlyT2) identified more recently in the brain (rev. in Zafra & Giménez UBMB Life 60:810, 2008) and how relevant they are for proper hemoglobinization during erythroid differentiation.
Methods and Materials: To address these issues, we exploited mice in which the gene encoding GlyT1 was disrupted (Tsai et al., PNAS 101:8485, 2004). Since the GlyT1 knockout (GlyT1–/–) mice die during the first postnatal day, we conducted analysis of blood parameters on newborn pups.
Results: As shown in the table below GlyT1–/– animals develop microcytic hypochromic anemia. Additionally, we observed that GlyT1–/– fetuses were typically paler than their GlyT1+/+and heterozygous (GlyT1+/–) counterparts. We next isolated erythroid cells from fetal livers (E 13.5) of GlyT1+/+, GlyT1+/–and GlyT1–/– mice and studied their hemoglobinization using a two-phase liquid culture method. Heme levels and biosynthesis rate were measured spectrophotometrically and by incorporation of [2-14C]glycine into heme, respectively. Erythroid cells from GlyT1–/– mice exhibited a substantial decrease in both heme levels and production rate, as compared to those derived from GlyT1+/– or GlyT1+/+ mice. These observations are congruent with a strong decrease in total uptake of [2-14C]glycine by erythroid cells from GlyT1–/– mice. Moreover, both total and cell surface transferrin receptor levels were decreased in GlyT1–/–erythroid cells. This was accompanied by a severe decrease in cellular iron acquisition from transferrin and its incorporation into heme. Furthermore, treatment of GlyT1+/+ cells with specific inhibitors for GlyT1 resulted in decreased expression of transferrin receptors, diminished cellular iron uptake and reduced iron incorporation into heme. In contrast, a specific inhibitior of GlyT2 had no effect on the expression of transferrin receptors or iron incorporation into heme. Finally, during erythroid differentiation, β-globin mRNA levels correlated with mRNA for GlyT1 but not for GlyT2.

Conclusion: These results provide the first evidence that GlyT1 is essential for proper hemoglobinization of developing erythrocytes in vivo and in vitro. Our finding that curtailed cellular acquisition of glycine restricts heme synthesis suggests that Glyt1 may be a rate limiting step of heme synthesis in erythroid cells. It also needs to be stressed that inhibitors of GlyT1 are currently being considered for the treatment of schizophrenia (Javitt, Curr Opin Drug Discov Devel 12:468, 2009). However, our study provides a warning that such a therapy could lead to the development of hypochromic microcytic anemia.

Table 1: Red blood cell parameters in newborn mice containing both GlyT1 alleles ($^{+/+}$), only one allele ($^{+/-}$) or none ($^{-/-}$)

	RBC ($x10^6/mm^3$)	HGB (g/dL)	HCT (%)	MCV (μm^3)	MCH (pg)
GlyT1$^{+/+}$ (n=10)	3.60 ± 0.18	13.06 ± 0.53	39.24 ± 1.66	109.20 ± 5.45	36.33 ± 1.65
GlyT1$^{+/-}$ (n=18)	3.42 ± 0.30	12.34 ± 0.96*	36.94 ± 2.60*	108.39 ± 4.42	36.16 ± 1.62
GlyT1$^{-/-}$ (n=12)	3.32 ± 0.21*	9.61 ± 0.59***	29.52 ± 1.83***	89.00 ± 2.37***	28.94 ± 1.05***

*=p<0.05; ***=p<0.0001; Statistical analysis was done by using one way ANOVA followed by Dunnett's multiple comparison test.

Podium #47

ABNORMAL BODY IRON DISTRIBUTION AND ERYTHROPOIESIS IN A NOVEL MOUSE MODEL WITH INDUCIBLE GAIN OF IRON REGULATORY PROTEIN (IRP) -1 FUNCTION

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European Molecular Biology Laboratory (EMBL)
(Presented By: Daniela Casarrubea)

Introduction: Disorders of iron metabolism account for some of the most common human diseases. Cellular iron homeostasis is maintained by iron regulatory proteins (IRP) -1 and -2 through their binding to cis-regulatory iron-responsive elements (IREs) in target mRNAs. Mouse models with IRP deficiency have yielded valuable insights into iron biology, but the physiological consequences of gain of IRP function in mammalian organisms have remained unexplored.
Methods and Materials: Here we report the generation of a mouse line allowing conditional expression of a constitutively active IRP1 mutant (IRP1*) using Cre/Lox technology.
Results: Systemic activation of the IRP1* transgene from the Rosa26 locus yields viable animals with gain of IRE-binding activity in all the organs analyzed by band shift assay. IRP1* activation alters the expression of IRP target genes and is accompanied by iron loading in the same organs. Furthermore, mice display macrocytic erythropenia with decreased hematocrit and hemoglobin levels. Flow cytometry analysis of bone marrow derived cells revealed an early blockade in erythroid differentiation. Thus, inappropriately high IRP1 activity causes disturbed body iron distribution and erythropoiesis.
Conclusion: This new mouse model further highlights the importance of appropriate IRP regulation in central organs of iron metabolism. Moreover, it opens novel avenues to study diseases associated with abnormally high IRP1 activity, such as Parkinson's disease or Friedreich's ataxia.

Podium #48

THE IRON-INDEPENDENT REGULATING ROLE OF MACROPHAGES IN STRESS ERYTHROPOIESIS IS ASSOCIATED WITH THE INTEGRIN β1/FOCAL ADHESION KINASE-1 PATHWAY

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(Presented By: Bart J. Crielaard)

Introduction: Macrophages contribute to physiological as well as pathological conditions in which erythropoietic activity is enhanced (see companion abstract from Crielaard *et al.*). We postulated that the "switch" between steady-state and stress erythropoiesis (SE) is mediated by iron-dependent and –independent pathways, and that macrophages contribute to both mechanisms by recycling iron for erythropoiesis as well as interacting directly with erythroblasts (EB) within the erythroblastic islands. It has been suggested that integrin β1 (Itgb1) and focal adhesion kinase-1 (Fak1) might be required in SE. The current study explored the role of the Itgb1/Fak1 pathway in the macrophage-EB interplay and its therapeutic potential in disorders marked by chronic stress erythropoiesis (CSE).
Methods and Materials: For the *in vitro* experiments, macrophages and EBs were isolated from the blood of mice and human volunteers. For the *in vivo* experiments, phlebotomized mice, animals with beta-thalassemia intermedia (Hbbth3/+ or BTI), and conditional knockout BTI mice for Itgb1 (employing the LoxP/Mx.Cre strategy) were analyzed using flow cytometry. Additional conditional knockout BTI mice for Fak1 are presently being generated. Phlebotomy-induced stress erythropoiesis in healthy mice was associated with a strong expansion of the macrophage population defined as *Gr1-/CD155-/F4/80+*, illustrating a central role of macrophages in expanding the eythroid progenitor pool under conditions of enhanced erythropoiesis. Administration of liposomal clodronate eliminated this population. We then generated human and mouse erythroid islands *in vitro* to evaluate proliferation, expansion and differentiation of EBs. Cycling and proliferation of EBs were significantly

increased upon co-culturing with macrophages, while differentiation was reduced. These effects were dependent on direct interactions with the macrophages, as demonstrated by transwell culturing. Similarly, macrophage depletion reduced cell cycling and increased differentiation of thalassemic EB, with a significant amelioration of ineffective erythropoiesis, splenomegaly and spleen architecture.
Results: Human EBs co-cultured with macrophages retained a higher surface expression of Itgb1, even though the expression of other differentiation markers did not change. In addition, association of Fak1 was demonstrated upon immunoprecipitation of Itgb1, identifying the Itgb1/Fak1 pathway as an important candidate regulating the interplay between macrophage and EB. Exposure to a Fak1 inhibitor (Fak1i) decreased the proliferation of EB co-cultured with macrophages *in vitro*, while Fak1i delayed the recovery from anemia and reduced the spleen size in phlebotomized animals *in vivo*. In mice with BTI, administration of FAK1i rapidly reverted splenomegaly and ameliorated anemia, by reducing erythroid expansion and improving EB maturation. However, these effects were not of the same magnitude as when macrophages were depleted. Potential explanations might relate to the observation that administration of Fak1i had no effects on additional players involved in this process such as serum iron and transferrin saturation, which further contribute to erythroid cell cycle and differentiation. Finally, preliminary data using conditional knockout mice showing reduced Itgb1 expression suggested similar positive effects on erythroid maturation and splenomegaly in BTI, suggesting that the Itgb1/Fak1 pathway might mediate the interaction between macrophages and EBs. Further analyses to identify additional partners of these proteins are in progress.
Conclusion: In conclusion, we identified a new mechanism contributing to SE and CSE, which may have critical scientific and therapeutic implications in the near future.

Podium #49
RETINAL IRON DYSREGULATION IN SYSTEMIC OR CONDITIONAL KNOCKOUT MOUSE MODELS OF HUMAN DISEASE: PROTECTION BY IRON CHELATION
Joshua Dunaief, MD, PhD[1], Majda Hadziahmetovic, MD[1], Natalie Wolkow[1], Ying Song, MD[1], Paul Hahn, MD, PhD[1], Yafeng Li[1], John Connelly, PhD[2], Michael Spino, PharmD[2], Sophie Vaulont, PhD[3] and Marie-Paule Roth[4]
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(Presented By: Joshua Dunaief)

Introduction: Iron toxicity has been implicated in several diseases, including age−related macular degeneration (AMD), aceruloplasminemia, and intraocular iron foreign body. The retina's high concentration of oxygen and easily oxidized polyunsaturated fatty acids make it especially sensitive to iron toxicity. Because iron regulation is important for retinal health, we have studied mechanisms of retinal iron homeostasis, created mouse models of iron-induced retinal degeneration, and tested iron chelation therapy.
Methods: We have studied iron overload and retinal degeneration in systemic ceruloplasmin (Cp)/hephaestin (Heph) knockout, Cp/retina-specific Heph KO, hepcidin (Hepc) KO and bone morphogenic protein 6 (Bmp6) 6 KO mice, and in WT mice following intraocular iron injection, using histology, electron microscopy, Perls' staining, and atomic absorption spectrophotometry. Labile iron levels were measured indirectly through qPCR measurement of TfR mRNA. Oxidative stress was measured by quantification of isoprostane F2−alpha. The iron chelator deferirpone was orally administered to test for protection against retinal degeneration induced by iron in the above models as well as by bright light or the chemical oxidant sodium iodate.
Results: All of the mutant mice listed above have age−dependent retinal degeneration. The degenerations are morphologically similar: the photoreceptors die over time, while the retinal pigment epithelial cells become autofluorescent, hypertrophic, and laden with iron−containing lysosomes. Male Bmp6 KO mice are affected while females are not. Administration of the iron chelator deferiprone decreases retinal labile iron levels and provides effective protection against retinal degeneration induced by iron, bright light, and sodium iodate.
Discussion: These results demonstrate the importance of Cp, Heph, Bmp6 and Hepc in retinal iron regulation and maintenance of retinal health, and indicate the possibility of iron chelation therapy for protection against retinal degeneration.
Conclusions: Iron homeostasis is important for retinal health, and many of the genes involved in systemic iron regulation are expressed by the retina and play a local role in control of retinal iron levels. In situations where iron dysregulation may occur, as with gene mutation, chronic inflammation, or retinal bleeding, iron chelation may help restore healthy iron levels.

Podium #50
A NOVEL RELATIONSHIP BETWEEN β-AMYLOID PROTEIN PRECURSOR AND TAU IN ALZHEIMER'S DISEASE RELATED IRON DISRUPTION
James Duce, PhD[1], Peng Lei, PhD[2], Andrew Tsatsanis, BSc[1], Bruce Wong, PhD[2], Linh Lam, BSc[2], Scott Ayton, PhD[2], Jack Rogers, MD, PhD[3], David Finkelstein, PhD[2] and Ashley Bush, MD, PhD[2]
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(Presented By: James Duce)

Introduction: Alzheimer's disease (AD) is complicated by pro-oxidant neuronal Fe2+ elevation adjacent to neurofibrillary tangles composed of the microtubuleassociated protein tau and extracellular zinc accumulation in β-amyloid containing plaques. Cellular iron homeostasis is tightly regulated as unbound iron catalyzes the production of toxic reactive oxygen

species. Ferroxidases oxidize Fe2+ to Fe3+ and are essential for maintaining intracellular iron homeostasis, partly through their ability to bind to the iron exporter ferroportin and the cell surface. Correlated with increased iron, ferroxidase activities may be disrupted with age and exacerbates various neurodegenerative diseases. While the main ferroxidase within the body is known to be ceruloplasmin (CP), this protein is not expressed within neurons.

Methods and Materials: While β-amyloid protein precursor (APP) has historically been associated with AD due to the prevalence of the APP derived amyloid-β (Aβ) peptide within insoluble extracellular 'plaques' deposited within the AD brain, the function of APP has remained largely unknown. However, we recently discovered that APP possesses a conserved H-ferritin-like ferroxidase domain with activity similar to CP.

Results: Our findings now support a new role for APP in iron homeostasis and similar to other ferroxidases, APP facilitates the efflux of iron from the neuron via its transport to the cell surface where it interacts with the iron exporter; ferroportin. Deletion of APP induces intracellular iron accumulation. However, zinc also directly inhibits APP ferroxidase activity and abnormal cortical zinc buffering, via Aβ transfer in the AD brain, may link amyloid pathology with neuronal iron accumulation.

Iron dyshomeostasis may also be exacerbated through tau's role in APP trafficking to the cell surface. Deletion of tau from primary neurons decreased cell surface APP expression, causing an iron export lesion that resulted in an age-dependent neuronal iron accumulation paralleling studies with APP deficient cell cultures and brain tissue.

Conclusion: Results are consistent with APP being able to modify the intracellular labile iron pool but that tau is required to transport the enzyme to its correct location. Mislocalization of either protein as well as zinc have major implications on disrupted APP ferroxidase activity with neurodegenerative diseases such as AD. Collectively, these data indicate a new relationship between two characteristic AD-related proteins and how disruption in either or both functions can lead to pro-oxidant neuronal Fe2+ elevation.

Podium #51

IRON ACCUMULATION IN THE CHOROID PLEXUS AND OTHER BRAIN BARRIER COMPONENTS IN MOUSE MODELS OF HEMOCHROMATOSIS

Elizabeth Milward[1], Moones Heidari, MSc[1], Bulent Acikyol, BSc[1], Ross Graham, PhD[2], Anita Chua, PhD[3], Roheeth Delima, BSc[3], Michael House, PhD[3], Debbie Trinder, PhD[3], John Olynyk, MD, PhD[4], Pablo Moscato, PhD[1] and Daniel Johnstone, PhD[5]

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(Presented By: Elizabeth Milward)

Introduction: The choroid plexus consists of a layer of epithelial cells that prevents the free exchange of solutes between the blood and cerebrospinal fluid (CSF). It forms part of the barrier structure that has traditionally been considered to protect the brain from damage in disorders such as hereditary hemochromatosis involving excessive iron loading elsewhere in the body.

Methods and Materials: We performed Perls' staining enhanced with diaminobenzidine (DAB) on fixed brain tissue from four different mouse models of iron loading and wildtype control mice (all 13 wks of age) to explore the effects of iron overload disorders on iron distribution and accumulation within the brain. The four models were wildtype AKR mice fed a short-term iron-supplemented diet (2% carbonyl iron for 3 weeks), AKR mice with disruption of the *Hfe* gene (*Hfe-/-*) or transferrin receptor 2 gene (*Tfr2Y245X*) and AKR mice with simultaneous disruption of each gene (*Hfe-/-*x*Tfr2Y245X*), all on normal chow. All four models have significantly increased hepatic iron concentrations relative to wildtype controls (3-fold to 4-fold increases, $p<0.05$). However only the *Hfe-/-*x*Tfr2Y245X* model shows increased brain levels of total iron (by inductively coupled plasma-atomic emission spectroscopy) and non-heme iron (>1.4-fold increase, $p<0.025$), accompanied by higher levels of ferritin (2.3-fold increase, $p=0.0005$).

Results: At 3 months of age, Perls' staining was observed in the choroid plexus lining all ventricles in wild-type AKR mice and all models of iron loading. Staining intensity was lowest in wild-type AKR mice, and slightly higher in dietary iron-supplemented mouse. Staining intensity was greater in both single mutant models, with intensity in *Tfr2Y245X* mice appearing slightly higher than in *Hfe-/-* mice. The *Hfe-/-*x*Tfr2Y245X* mice displayed greatest intensity. Staining was sometimes seen in cells lining blood vessels within choroid plexus invaginations however this was typically less intense than in the choroid plexus epithelial cells. At 1 year of age, staining within the choroid plexus of *Hfe-/-*x*Tfr2Y245X* mice was so intense that cellular structure was obscured. At both times, there was also staining of other cells within the brain, with widespread staining across many regions by 1 year of age.

Conclusion: These observations show that both dietary iron loading and genetic iron loading are accompanied by accumulation of iron within barrier structures lining the brain. This may help protect the brain against damage when body iron levels are high. The choroid plexus transports a range of solutes both into and out of the brain. So the findings may reflect excess iron uptake into the choroid plexus from the blood or CSF, saturation of iron export mechanisms out of the choroid plexus or both. In any event, the staining observed in cells within the brain in younger and older animals suggests that the various brain barriers do not fully protect the brain in iron loading disorders.

Podium Abstracts

Podium #52

TARGETING BRAIN CHELATABLE IRON AS THERAPEUTIC STRATEGY FOR PARKINSON'S DISEASE. TRANSLATIONAL AND CLINICAL STUDIES

Caroline Moreau, MD, PhD[2], Jean-Christophe Devedjian, Jerome Kluza, Charlotte Laloux, Aurélie Jonneaux, Maud Petrault, Gilles Ryckewaert, Kathy Dujardin, MD, PhD, Guillaume Garçon, Nathalie Rouaix, Alain Duhamel, Patrice Jissendi, Bernard Sablonniere, Jean-Christophe Corvol, Christian Rose, Luc Defebvre, Philippe Marchetti, Ioav Cabantchik[3], Regis Bordet and David Devos[1]

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(Presented By: David Devos)

Introduction: Focal iron accumulation and oxidative damage in dopaminergic neurons of the substantia-nigra (SN)-pars-compacta are characteristic features of Parkinson's disease (PD). The possible involvement of labile iron in reactive oxygen species (ROS) formation and neuronal damage in PD have rendered it a target for chelation and disease attenuation but also posed the major challenge of sparing the organism from systemic iron deprivation by non-specific chelation.

Methods and Materials: Using deferiprone's siderophore properties (Sohn et al., 2008) we devised regimens of chelation that while sparing the erythron and plasma iron they afford dissipation of brain iron foci, reduce dopamine auto-oxidation and significantly reduce the motor handicap features of PD. Firstly tested in dopaminergic LHUMES cells and in human lymphocytes (metabolism, labile iron, ROS and viability) and then translationally in the MPTP-mouse PD model (motor function, MRI of dopamine, oxidative stress parameters) and neurons derived from mouse brain, we setup the first randomized, double−blind, placebo−controlled, parallel−group, single−center trial using a delayed onset paradigm whereby PD patients (early stage with adjusted dopaminergic treatment) received either placebo for 6 months followed by deferiprone (30 mg/kg/day in 2 doses) for 6 months or deferiprone straight through for 12 months.

Results: The UPDRS motor and the Clinical Global Impression scores reflected a significant attenuation of disease progression from 0 to 6 to 12 months concomitant with reduction in the SN R2* relaxation rates (3T magnet MRI) and 2D fast field echo multishot sequence. The 1-year conservative oral deferiprone regimen that demonstrably attenuated disease progression (UPDRS score) had the advantage of sparing plasma iron levels or blood cell production.

Conclusion: Thus this work describes for the first time a conservative mode of iron chelation as a promising therapeutic strategy for PD and possibly other mitochondrial-oxidative-stress-related pathologies.

Supported by PHRC grants from the French Ministry of Research.

Sohn YS, Breuer W, Munnich A, Cabantchik ZI. Redistribution of accumulated cell iron: a modality of chelation with therapeutic implications. Blood. 2008;111:1690-9.

Podium #53

A NOVEL DISEASE ENTITY CHARACTERIZED BY IDIOPATHIC GENERALIZED SEIZURES AND L-FERRITIN DEFICIENCY

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(Presented By: Sonia Levi)

Introduction: Genetic ablation of the H-ferritin gene in animal models reveals its critical importance for development, while the role of the L-ferritin gene in vertebrates has not been addressed.

Methods and Materials: Here we describe a case of a 23-year old female patient affected by a homozygous loss of function mutation in the L-ferritin gene and idiopathic generalized seizures during infancy. The patient is hallmarked by a homozygous nucleotide substitution (G310T) in exon 3 of the L-ferritin gene, which results in a stop codon at amino acid 104 (E104X), generating a peptide unable to fold in a ferritin full cage. We characterized parameters of iron homeostasis and oxidative status in primary fibroblasts that were derived from a patient's skin biopsy, as well as in dopaminergic neurons from reprogrammed fibroblasts. We show that the L-chain ferritin in E104X fibroblasts is undetectable and that the ferritin molecule is composed only of H-chains. In accordance with the enhanced capacity of the H-ferritin homopolymer to incorporate iron, the LIP is decreased (~ 4 fold), which may have caused increased cellular iron uptake (~1.5 fold) via TfR1 (~1.4 fold increase). Moreover reduced iron availability caused cytosolic ROS scavengers (catalase and SOD1 protein were reduced by ~ 4 and ~ 2.5-fold, respectively).

Results: Despite cytosolic iron starvation, ROS production was enhanced and higher levels of oxidized proteins were detected. Furthermore, ferritin showed an accelerated degradation rate in E104X cells (half-life was ~ 5 h compared to ~18 h in controls). The increase in ROS and TfR1 protein levels was further confirmed in reprogrammed dopaminergic neurons from E104X fibroblasts that were obtained with an efficiency of about 10%.

Conclusion: Our results highlight for the first time the pathophysiological consequences of human L-ferritin deficiency and contribute to the definition of a new disease entity characterized by the absence of L-peptide in ferritin and idiopathic generalized seizure.

Podium Abstracts

Podium #54
EFFECT OF H63D-HFE ON CHOLESTEROL METABOLISM, BRAIN ATROPHY, AND COGNITIVE IMPAIRMENT: IMPLICATIONS FOR ALZHEIMER DISEASE
Fatima Ali-Rahmani, PhD, Patricia Grigson, PhD, Sang Lee, PhD, Elizabeth Neely, BX, Brian Kinsman, BX, James Connor, PhD and Cara-Lynne Schengrund, PhD
Pennsylvania State University College of Medicine
(Presented By: James Connor)

Introduction: The H63D variant of the hemochromatosis (HFE) gene, when expressed in carriers of the apolipoprotein E4 (APOE4) allele, is implicated as a risk factor for earlier onset of Alzheimer disease (AD).
Methods and Materials: We tested the hypothesis that like expression of APOE4, expression of H63D-HFE disrupts cholesterol metabolism contributing to an increase in neurodegeneration and memory deficits. Analysis of SH-SY5Y human neuroblastoma cells transfected to stably express either wild type (WT-) or H63D-HFE indicated about a 50% reduction in cholesterol content in cells expressing H63D-HFE. This was accompanied by a significant decrease in expression of 3-hydroxy-3-methyl-glutaryl-CoA reductase, and a significant increase in expression of cholesterol hydroxylase.
Results: Consistent with these studies, H67D-HFE (homologous to human H63D-HFE) knock-in mice, showed a greater age dependent decline in brain cholesterol than WT-HFE animals and changes in expression of proteins regulating cholesterol metabolism. Brains of aged H67D-HFE mice also exhibited a significant decrease in expression of synapse and myelin proteins, significant increase in caspase-3 expression, and cortical and hippocampal neurodegeneration relative to WT-HFE controls. H67D-HFE mice also had greater reduction in brain volume and poorer recognition and spatial memory than WT-HFE mice, symptoms associated with AD.
Conclusion: These findings indicate that H63D-HFE could significantly disrupts brain cholesterol homeostasis and through this process could contribute to development of AD.

Podium #55
OVER-EXPRESSION OF HEPATIC SMAD7 IN MICE CAUSES HEMOCHROMATOSIS
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(Presented By: Maja Vujic Spasic)

Introduction: The regulation of hepcidin expression, the key iron hormone, is mainly controlled by the activity of the iron-signaling Bmp6/Smad pathway. The same pathway governs the transcription of Smad6 and Smad7, two inhibitory Smad proteins which act in a negative feedback loop to block the overshooting signalling triggered by the cytokines of the Tgf-β family members. Smad6 effectively inhibits Bmp6/Smad signalling and only weakly affects the response to Tgf-β, contrasting the role of Smad7, which efficiently inhibits both signalling pathways.
Methods and Materials: We postulated that when overexpressed, inhibitory Smads may decrease hepcidin expression by blocking the activity of the Tgf-β/Bmp6/Smad, or alternatively, Smad6 and Smad7 may directly bind to the promoter regions of hepcidin and control its transcription. To test this hypothesis, we overexpressed Smad6 or Smad7 in primary hepatocytes by adenovirus-mediated gene delivery. Our data show significant hepcidin mRNA suppression, whereby Smad7 overexpression caused a more pronounced reduction likely due to blocking the activity of both Tgf-β and Bmp6/Smad signaling pathways.
Results: In order to address whether the observed findings are operational in vivo, we first generated Smad7 transgenic mice and bred them to albumin-Cre transgenic mice to create double transgenic mice with hepatocyte-specific Smad7 overexpression. At the age of 12 weeks and 6 months, these mice show significantly decreased hepatic hepcidin mRNA expression and hepatic iron overload, two hallmarks of hemochromatosis in mice and humans.
Conclusion: Collectively, these data identify Smad7 as a critical regulator of systemic iron homeostasis in vivo and warrant further investigations into the molecular mechanisms of Smad7-mediated hepcidin suppression and its relevance for liver diseases.

Podium #56
ANALYSIS OF TISSUE SPECIFIC KNOCKOUTS OF HEPCIDIN
Sara Zumerle, Jacques Mathieu, Stéphanie Delga, Lydie Viatte, Sophie Vaulont and Carole Peyssonnaux
INSERM, U1016, Institut Cochin, Paris, France, CNRS, UMR8104, Paris, France, Université Paris Descartes, Sorbonne Paris Cité, Paris, France
(Presented By: Sara Zumerle)

Introduction: Hepcidin is a 25 amino acid peptide demonstrated to be the iron regulatory hormone capable of blocking iron absorption from the duodenum and iron release from macrophages. Its expression is induced by iron accumulation and diminished in situations of iron needs (anemia, hypoxia). Hepcidin controls serum iron levels by binding to ferroportin, the only known iron exporter, and inducing its degradation. Mutations affecting hepcidin regulators or hepcidin gene itself cause hemochromatosis, a common genetic disorder. Accordingly, hepcidin knockout (KO) mice are iron overloaded. Hepcidin is produced mainly by the liver, but many tissues have been described as being capable of expressing hepcidin: macrophages,

brain, heart, retina, kidney, adipocyte, pancreas… However, detection of the protein has been hampered by the lack of good antibodies and the contribution of these tissues to circulating hepcidin is currently unknown. In addition, the impact of hepcidin deficiency in different tissues with regards to iron homeostasis remains to be investigated.
Methods and Materials: To address these questions, we sought to generate mice with tissue-specific ablation of the hormone. For that, we created a floxed hepcidin1 allele (flanking exons 2 and 3 of the gene) with the classical Cre-loxP strategy. Using the ubiquitous Cre-deleter mice (E2a-Cre mice), we have validated our targeting strategy and reproduced the iron overload seen in the classical hepcidin KO mice. We are first studying the effect of hepcidin deficiency in the liver by crossing the floxed hepcidin mice (Hepclox/lox mice) with transgenic mice expressing the hepatocyte-specific Albumin Cre recombinase (Alb-Cre mice). Liver-specific KO mice have been compared to wild type and total KO mice (E2a-Cre), and the iron status of the animals has been monitored.
Results: We show that at the age of 2 months the liver-specific KO mice fully recapitulate the severe iron overload phenotype observed in the total KO mice, with increased plasma iron and severe parenchymal iron accumulation, demonstrating that hepatocyte constitutes the predominant reservoir for systemic hepcidin, the other tissues being unable to compensate for the severe iron overload. Accordingly, hepcidin expression in tissues other than liver has been measured by qPCR, and no significant variations between wild type and liver-specific KO mice has been outlined. Iron-related genes expression in the duodenum showed an increase of iron absorption genes on the apical membrane (Duodenal Cytochrome B - DCYTB, Divalent Metal Transporter 1 – DMT1) and iron exporter on the basolateral membrane (Ferroportin), which is observed in total KO as well.
Conclusion: Overall, our results show that hepatic hepcidin is fundamental for the maintenance of iron systemic homeostasis in basal conditions. The role of hepatic hepcidin will also be studied in conditions of altered iron homeostasis (hypoxia, inflammation, iron-rich or deficient diet). We are currently generating other tissue specific knockout of hepcidin (for example, LysM-Cre mice) to elucidate the effect of hepcidin deficiency in different tissues, especially in sites with poor perfusion where circulating hepcidin is scanty.

Podium #57
SURVIVAL AND CAUSES OF DEATH IN A COHORT OF 1086 TREATED C282Y HFE HOMOZYGOUS PATIENTS
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(Presented By: Edouard Bardou-Jacquet)

Introduction. Due to various methodological limitations (retrospective diagnosis, small sample size, lack of *HFE* testing, inaccurate control group, imprecise data on iron burden and/or on long-term therapy), previous studies on the impact of *HFE* hemochromatosis on survival have led to conflicting results. Our aim was to assess survival and causes of death in a large and well-defined prospective cohort of C282Y homozygotes.
Methods. Since 1989, the clinical, biological, imaging and histological data collected at diagnosis and during follow-up in all C282Y homozygotes (n = 1875) referred to our tertiary reference are prospectively recorded. The 1086 patients diagnosed from 1996, year of availability of *HFE* testing, were selected for the present study. Venesection therapy was advised when serum ferritin levels were greater than 300 µg/L (200 µg/l in women) or, in absence of hyperferritinemia, when transferrin saturation was elevated and/or when functional symptoms attributable to hemochromatosis were present. After low body-iron levels (serum ferritin <50 µg/L) were achieved, the patients were advised to continue phlebotomy therapy in order to maintain serum ferritin <50 µg/L in the long term. End of follow-up was fixed at December 31, 2009. Vital status and causes of death were obtained from the French INSEE and INSERM institutes and compared to the national statistical tables published for the French general population. Standardized Mortality Ratios (SMR) were determined.
Results: Mean follow-up was 8.7 (±3.9) years. Overall mortality of patients (SMR: 0.94, CI: 0.69-1.24) was similar to that of the general population. Serum ferritin >2000µg/L at diagnosis was associated with higher mortality (SMR: 2.83, CI: 1.79-4.24) while serum ferritin <1000µg/L was associated with lower mortality than the general population (SMR: 0.27, CI: 0.12-0.54). Deaths were related to liver disease in 51% of cases, mainly from hepatic cancer (76%). There was no increase in cardiovascular or extra-hepatic cancer mortality. Serum ferritin <1000µg/L at diagnosis was associated with lower cardiovascular (SMR: 0.17, CI: 0-0.93) and extra-hepatic cancer mortality (SMR: 0.09, CI: 0-0.52). In a multivariate Cox regression analysis, age at diagnosis, diabetes, alcohol consumption and the Guyader score for fibrosis were independently associated with the risk of death.
Conclusion: Contrary to some previous studies, we found that overall mortality in patients with *HFE* hemochromatosis is similar to that of the general population. However, patients with severe iron overload have an increased mortality, mainly related to primary liver cancer, and patients with slight or mild iron overload have a lower mortality due to lower cardiovascular and extra hepatic cancer mortality. Sustained iron removal is beneficial to hemochromatosis patient, especially in case of low iron burden, which supports an early diagnosis and should be kept in mind when advocating the lightening of venesection therapy.

Podium Abstracts

Podium #58

PRIMARY HEPATIC IRON OVERLOAD IN EXTREME OBESITY IS COMMON AND NOT ASSOCIATED WITH METABOLIC ABNORMALITIES

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(Presented By: Glenn Gerhard)

Introduction: Mild to moderate hepatic overload in obese patients is considered a secondary form of iron overload either as part of the dysmetabolic iron overload syndrome or in association with non-alcoholic steatohepatitis (NASH). However, only a fraction of patients at metabolic risk or with NASH demonstrate histological evidence of iron overload and the pathophysiological mechanisms and clinical significance are not well delineated.
Methods and Materials: Large-scale unbiased studies of hepatic iron are problematic because biopsy is the gold standard for the assessment of iron stores in the liver, which are difficult to obtain on an elective basis in large numbers of individuals. We have recruited over 2000 patients of mixed European ancestry enrolled in a bariatric surgery program from whom liver biopsies were obtained as part of clinical standard of care. The median BMI of the cohort was about 47 kg/m2, the average age about 47 years, with approximately 80% of patients female. Intra-operative wedge biopsies of the liver were processed, formalin fixed, paraffin embedded, sectioned, and stained with H&E for routine histology, Masson's trichrome for assessment of fibrosis, and Perls'/Prussian Blue stain to determine iron status. All liver biopsies were read by experienced pathologists as part of the standard clinical care.
Results: Iron deposition was reported as hepatocyte, kupffer cell, or a mixed pattern. The pathology data on each biopsy, including NASH findings for fibrosis and iron status, were obtained from pathology reports and entered into a SAS database. Over 15% of patients had evidence of iron overload, with slightly more patients with hepatocyte staining than with kupffer cell or mixed patterns. A large number of clinical variables including results of laboratory tests, co-morbid disease diagnoses, and medication usage related to the common co-morbid medical conditions associated with extreme obesity were evaluated using logistic regression against the presence or absence of each of the three histological patterns of iron deposition adjusting for age, gender, and BMI. Serum ferritin was the most strongly associated variable with each of the three patterns, consistent with the presence of increased iron stores. Total iron binding capacity was also strongly associated with the three patterns. The variables related to the hepatocyte and mixed patterns largely overlapped and included red blood cell indices such as hemoglobin, transferrin saturation, and total bilirubin. In contrast, the kupffer cell pattern was associated with serum creatinine, suggesting that it may be a distinct clinical-pathological condition.
Conclusion: The relatively high prevalence of hepatic iron overload in this large population of obese patients unselected for liver disease, that was not associated with specific metabolic abnormalities, suggests that these histological patterns may represent one or more novel forms of primary hepatic iron overload.

Podium #59

THE USE OF NEXT-GENERATION SEQUENCING TO SCREEN FOR MUTATIONS IN 16 GENES INVOLVED IN IRON HOMEOSTASIS AS AN AID TO GENETIC DIAGNOSIS FOR PATIENTS WITH IRON OVERLOAD OR IRON DEFICIENCY

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(Presented By: Patricia Bignell)

Introduction: Next-generation sequencing (NGS) is quickly becoming a proven and valuable tool in many clinical applications. Recent improvements in the accuracy and cost optimisation of NGS and exon capturing platforms have widened the application of whole exome sequencing to molecular diagnostic laboratories and the identification of variants to explain the heritability of complex diseases.
Methods and Materials: We sought to demonstrate the clinical utility of NGS by sequencing key genes within iron metabolism using a designed TruSeq Custom Amplicon panel. The OXIRON TSCA panel has 340 amplicons which covers 16 genes this includes the 5 genes we screen routinely by Sanger sequencing, HFE, HFE2, HAMP, SLC40A1 and TFR2. The additional genes on the panel are TMPRSS6, SLC11A2, CP, TF, HEPH, ALAS2, FTL, FTH1, SEC23B, CYBRD1 and FLVCR1. This provides a one step sequencing platform including atypical haemochromatosis, aceruloplaminamemia, hyperferritinaemia cataract syndrome, iron refractory iron deficiency anaemia (IRIDA) and atransferrinaemia. NGS on gene panels hold many advantages over other biomarker detection methodologies. Through deep, targeted sequencing, NGS can obtain similar sensitivity of proven methods, while interrogating thousands of loci in parallel. This, along with the ability of NGS to discern individual reads, allows for the quantification of allele frequency and gives these systems the power to resolve complex mutations.
Results: We were able to successfully sequence multiple genes in parallel, with 250 nanograms of gDNA. TSCA is amenable to a wide range of samples with the ability to generate up to 1536 amplicons per reaction and integrated indices to support up to 96 samples per MiSeq run. TSCA provides an unprecedented level of sample multiplexing, offers a fully integrated DNA data solution, including convenient online probe design and ordering, a streamlined workflow and automatic data analysis. The entire TSCA process takes only 4 days to go from gDNA to data.Whole exome sequencing analysis is emerging as a cost effective method for the clinical diagnosis of not only multigene disorders but also of single large gene disorders. The cost for

running the TSCA OXIRON panel is ~£450 per sample whereas to screen the 5 common genes, HFE2, HFE, HAMP, SLC40A1 and TFR2 by Sanger sequencing would be £750 per sample.We have performed validation studies and the NGS demonstrated 100% concordance with Sanger sequencing results.
Conclusion: Overall these studies directly demonstrate the fundamental advantages of NGS in clinical mutational screening.

Podium #60
IDENTIFICATION OF GENES AND VARIANT ALLELES ASSOCIATED WITH IRON OVERLOAD IN HEMOCHROMATOSIS HFE C282Y HOMOZYGOTES
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(Presented By: Christine E. McLaren)

Introduction: Variability in iron overload severity among homozygotes for the *HFE* C282Y polymorphism is an important unexplained feature of hereditary hemochromatosis (HH). We conducted exome sequencing of DNA from C282Y homozygotes with markedly increased iron stores (cases) and C282Y homozygotes with normal or mildly increased iron stores (controls) to identify rare and common causal variants associated with variability of disease expression in HH.
Methods: Criteria for cases included serum ferritin >1000 µg/L at diagnosis, and (a) mobilized body iron >10 g by quantitative phlebotomy, and/or (b) hepatic iron concentration >236 µmol/g dry weight. Criteria for controls included (a) serum ferritin <200 µg/L for females and <300 µg/L for males, or (b) age ≥50 y with ≤3.0 g iron removed by phlebotomy or age ≥40 y with <2.5 g iron removed by phlebotomy to achieve serum ferritin <50 µg/L. Deep sequencing of the full exome was performed in 33 cases and 15 controls. After quality control filtering, the dataset included 82,068 SNPs and 1,403 insertions/deletions (indels). Our analytical strategy included examination of common and rare alleles. An allele burden test compared the cumulative number of rare coding variants within each gene among the case and control samples. An association test was conducted for individual variants. A Bonferroni multiple test correction based on the number of variants analyzed was applied to the individual variant association analysis p-values; a correction based on the number of genes analyzed was applied to the allele burden test p-values.
Results: All study participants were male; 47 were Caucasians and one was African-American. Mean (SD) ages at presentation were 54 (11.0) y and 56 (9.4) y for cases and controls, respectively. Median serum ferritin was 2788 µg/L in those with increased iron stores and 302 µg/L in those with normal or mildly increased iron stores. The median transferrin saturation (94%) was greater in cases than in the comparison group (70%). Nine genes showed statistically significant associations ($p<4.2\times10^{-6}$) between the cumulative number of low-frequency and rare coding missense, stop-gain/loss, and frameshift alleles and case-control status in the allele burden test. These genes were *ALDH3B2* ($p=5.32\times10^{-15}$),*TSPEAR/KRTAP10-7* ($p=1.76\times10^{-12}$), *FAM189A1* ($p=7.01\times10^{-12}$), *P2RY4* ($p=8.55\times10^{-10}$), *GANC* ($p=1.17\times10^{-7}$), *PLA2G4D* ($p=3.90\times10^{-7}$), *UNC93A* ($p=5.34\times10^{-7}$), *BOD1L* ($p=1.81\times10^{-6}$) and *OR8B4* ($p=2.05\times10^{-6}$). *OR8B* met statistical significance in an analysis restricted to variant alleles predicted to be possibly damaging ($p=2.05\times10^{-6}$) and probably damaging ($p=7.06\times10^{-6}$). Several individual variants contributed to the allele burden test statistic because their frequency was low. After correction for multiple testing, previously known iron-related candidate genes that were considered did not reach statistical significance.
Summary and Conclusions: Some genes associated with case-control status in this analysis are novel, not well characterized, and not previously associated with iron overload disorders. One example is *UNC93a*, a gene that encodes a multi-pass transmembrane protein that may be a transporter. These data indicate associations between iron status in *HFE* C282Y homozygotes and genes that may modify severity of disease expression. Our results identify candidate genes for expanded studies that would examine their functional significance to iron absorption and metabolism.

Podium #61
IDENTIFICATION OF HEPARINS WITHOUT ANTICOAGULANT ACTIVITY WHICH INHIBIT HEPCIDIN IN VIVO
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(Presented By: Maura Poli)

Introduction: There is an increasing interest in the development of pharmacological agents able to modulate hepcidin, the peptide hormone that critically regulates iron homeostasis in response to iron and inflammation. In particular, hepcidin antagonist may have a therapeutic role in the anemia of chronic diseases, where hepcidin levels are often increased by pro-inflammatory cytokines.
Methods and Materials: We previously demonstrated that heparin is a potent inhibitor of hepcidin expression in hepatic cell lines, probably by interfering with BMP/HJV/SMAD signalling, and that it was also effective in reducing hepcidin expression in mice (Poli M, Blood 2011; 117:997-1004). The therapeutic use of heparin for hepcidin modulation is hampered by its strong anticoagulant activity, thus we were interested in evaluating modified heparins without such activity. In fact the affinity binding to antithrombin is abolished by chemical treatments involving reduction and oxidation (RO) which cause glycol splitting and make the heparin chains more flexible.

Results: Various types of RO heparins were obtained which differed in molecular weight, degree of sulfation and of acetylation. All of them showed anti-hepcidin activity, with two glycol-split molecules being as potent as commercial unfractionated heparin. These two molecules suppressed BMP/SMAD signalling in HepG2 cells, in the presence or absence of BMP6 stimulation, at pharmacological concentrations with maximum inhibition after 6 hours. The same was observed in primary mouse hepatocytes. More important, single subcutaneous injections of 20-60 mg/kg strongly reduced liver pSMAD, hepcidin mRNA and serum hepcidin, while increasing serum iron. Also a week-long treatment with daily injection s of RO-heparins reduced hepcidin expression and serum level, without evident adverse effects on coagulation. Moreover, heparin treatments strongly reduced hepcidin induction caused by LPS, a model of acute inflammation.
Conclusion: In conclusion, we identified some non-anticoagulant heparins with strong anti-hepcidin activity both in vitro and in vivo, which might represent promising hepcidin antagonist with potential therapeutic applications.

Podium #62

IRON MOBILIZATION AND PHARMACODYNIC MARKER MEASUREMENTS IN NON-HUMAN PRIMATES FOLLOWING ADMINISTRATION OF PRS-080, A NOVEL AND HIGHLY SPECIFIC ANTI-HEPCIDIN THERAPEUTIC

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(Presented By: Andreas Hohlbaum)

Rationale: Pharmacological blockade of hepcidin/ferroportin interactions might provide a novel point of therapeutic intervention for treating anemia of chronic disease by addressing the underlying functional iron deficiency. The initial discovery and characterization of a highly selective and potent specific inhibitor against hepcidin thereby blocking the interaction between hepcidin and the ferroportin receptor (PRS−080, a small molecular weight Anticalin therapeutic protein) has been described recently (ASH#687, ASN#P0838). Single dose PK/PD data in non−human primates (NHP) and an Anticalin specific PK/PD model provided a rationale for selecting a drug candidate for clinical development with 'fit for purpose' potency and half−life. We have now assessed dose response relationships and safety upon repeated doses in non−human primates to further refine our PK/PD model, characterize the pharmacodynamic signature, and inform the clinical development of our drug candidate.
Methods: Single dose and repeat dose PK/PD, and safety studies were performed in NHP at dose levels ranging from 0.5 to150 mg/kg using i.v. and s.c. administration. The concentration−time profiles of total and free Anticalin were measured and correlated with PD markers (hepcidin, serum iron, TSAT, CHr) and safety parameters such as hematology, clinical chemistry and histopathology.
Results and Discussion: A hepcidin−specific, dose−dependent and significant iron mobilization was observed in Cynomolgus monkeys following a single i.v. bolus of PRS−080 at dose levels of 3mg/kg and above starting as early as 6 hours. Safety studies with supra−pharmacological doses up to 150 mg/kg indicated that the Anticalin−induced hyperferremia plateaued at 65µM total serum iron (Cmax). This plateau was reached at dose levels starting at 3 mg/kg suggesting a physiological regulation that prevents the release of non−physiological amounts of iron from tissue stores that could potentially generate non transferrin−bound iron (NTBI). In addition, the iron response following high doses of PRS−080 (tested up to150 mg/kg) was transient and reversible. There were no detectable differences in terms of onset, magnitude, and duration of the iron response following a single dose of either s.c. or i.v. administration providing further insights into our understanding of the kinetics of iron response following hepcidin inhibition. Furthermore, a similar iron response was observed after multiple doses (i.v. and s.c.) suggesting a lack of tolerance and/or counter−regulatory mechanisms modulating the pharmacological effect of PRS−080. A comprehensive evaluation of PD biomarkers will be described. The Anticalin was also safe and well−tolerated as no test article related clinical or macroscopic observations occurred up to 150mg/kg when administered 5 times Q2D in a preliminary dose range finding study.
Conclusions: Our results describe the preclinical pharmacological efficacy and safety of PRS−080, a hepcidin−specific Anticalin in healthy primates and provide additional insights into the impact of hepcidin inhibition on iron mobilization. An E.coli−based GMP process for cost effective production has been established and a GLP TOX program is on track to support a First In Man clinical trial in 2013. PRS−080 has a novel mechanism of action compared to ESAs, IV iron, and HIF−PH inhibitors, thereby presenting a potential for transforming anemia supportive care.

Podium Abstracts

Podium #63

RANDOMIZED DOUBLE BLIND PLACEBO CONTROLLED PK/PD STUDY ON THE EFFECTS OF A SINGLE INTRAVENOUS DOSE OF THE ANTI-HEPCIDIN SPIEGELMER NOX-H94 ON SERUM IRON DURING EXPERIMENTAL HUMAN ENDOTOXEMIA

Lucas van Eijk, MD, MSc, Dorine Swinkels, MD, PhD, Aaron John, Frank Schwoebel, PhD, Frank Fliegert, MD, Luciana Summo, PharmD, PhD, Stéphanie Vauleon, PhD, Coby Laarakkers, BSc, Kai Riecke, MD and Peter Pickkers, MD, PhD
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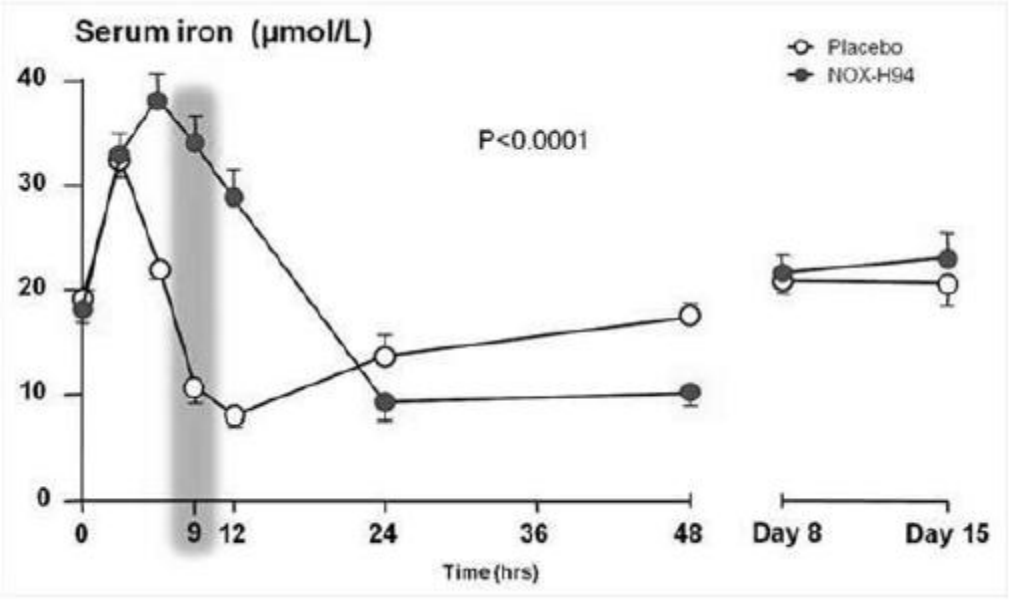
(Presented By: Lucas van Eijk)

Introduction: Anemia of chronic disease (ACD) is generally attributed to increased hepcidin production. The first−in−class hepcidin antagonist NOX−H94, a PEGylated anti−hepcidin L−RNA oligonucleotide, is in development for targeted treatment of ACD. We investigated whether NOX−H94 prevents the inflammation−induced serum iron decrease during experimental human endotoxemia. Effects on the innate immune response were studied as secondary endpoints.

Materials and Methods: This randomized, double−blind, placebo−controlled trial was carried out in 24 healthy young men. At T=0 hours (h), 2 ng/kg E. coli endotoxin (LPS) was administered intravenously (i.v.), followed by 1.2 mg/kg NOX−H94 or placebo i.v. at T=0.5h. Blood was drawn serially for 24h and at day 3, 8 and 15 after endotoxin administration for measurements of inflammatory parameters, cytokines, NOX−H94 pharmacokinetics, total hepcidin−25 (free and NOX−H94−bound), and iron parameters. The difference of serum iron change from baseline at T=9h was defined as primary endpoint. Data are expressed as mean±SD.

Results: LPS administration led to flu−like symptoms peaking at T=1.5h post administration, irrespective of the treatment group. Peak CRP, the rise in leucocytes and plasma levels of TNF−α, IL−6, IL−10, and IL−1RA peaked markedly and similarly in both treatment groups. NOX−H94 plasma concentrations peaked at 0.7±0.4h after the start of administration, after which they declined according to a two−compartment model, with a T½ of 22.5 ± 4.28h. Total hepcidin−25 rose to a peak of 23.0±5.2 nM at T=5.5±0.05h post LPS in control subjects and normalized at about T=24h. In the NOX−H94 treated group, total hepcidin−25 concentrations, which include hepcidin−25 bound to NOX−H94, rose to 559±112 nM at T=9.5±1.5h post-LPS and normalized slowly until day 15. The observed serum iron concentrations are shown in the figure: In the placebo group, serum iron increased from 19.0±7.6 µmol/L at baseline to a peak at T=3h post LPS, returned close to baseline at T=6h and decreased under the baseline concentration at T=9h reaching its lowest point at T=12h. In the NOX−H94 group, serum iron concentrations rose until T=6h and then slowly declined until T=24h. From 6 to 12 h post LPS, the serum iron concentrations in NOX−H94 treated subjects were significantly higher than in placebo treated subjects (P<0.0001, ANCOVA). At T=24 h and 48 h, serum iron was lower in NOX−H94 treated subjects than in placebo controls.

Discussion and conclusion: Experimental human endotoxemia induced a robust inflammatory response with hepcidin release and a subsequent decrease in serum iron. Treatment with NOX−H94 had no effect on innate immunity, but effectively prevented the inflammation−induced drop in serum iron concentrations. These findings demonstrate the clinical potential of the anti−hepcidin drug NOX−H94 for further development to treat patients with ACD.



Podium Abstracts

Podium #64

LOW DOSE MINIHEPCIDIN PEPTIDE CAUSES IMPROVEMENT OF ANEMIA BY INCREASED ERYTHROPOIETIC EFFICIENCY IN A MOUSE MODEL OF THALASSEMIA INTERMEDIA

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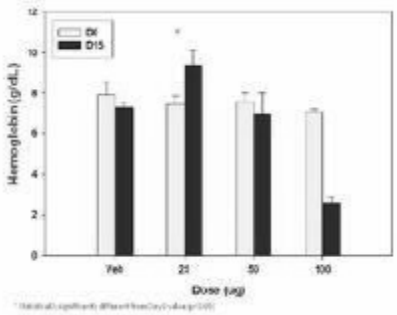
(Presented By: C. Casu)

Introduction: In beta-thalassemia excess alpha globin chains combine with excess free heme iron to form hemichromes in erythroid cells leading to oxidative damage, premature cell death and anemia. The resultant ineffective erythropoiesis is critical in the development of iron overload by decreasing hepcidin levels, further increasing the amount of heme iron available for erythropoiesis.

Methods and Materials: These features are well reproduced in the th3/+ mouse model of thalassemia intermedia (Non-Transfusion Dependent Thalassemia or NTDT), which shows increased erythropoiesis, splenomegaly and iron overload due to low levels of hepcidin (Gardenghi et al, 2010). In these animals, increasing hepcidin levels has been demonstrated to reverse these cellular changes and to improve anemia. It is hypothesized that this beneficial effect of hepcidin is caused by reducing the availability of iron to match the impaired hemoglobin generation, thus reducing the amount of free heme iron to cause oxidative damage to and death of late stage erythroid cells.

Results: Modified peptide analogs of the 9 N-terminal amino acids of hepcidin have been demonstrated to reproduce the biological effects of hepcidin (Preza et al, 2011, Ramos et al, 2012). One of these minihepcidin peptides (M004), which reduced serum iron in the C57BL/6 mouse by >70% for at least 24 h after a single dose of 175 µg, has been evaluated in the th3/+ mouse by daily subcutaneous injection. In animals treated with a high dose of M004 (100 µg/day for 14 days) severe iron restriction was observed (Tf sat = 6%) that caused a marked reduction in MCV and MCH and a significant exacerbation of anemia (Hb reduction of 4.5 g/dL (-63.4%) compared to baseline values). In these animals accumulation of immature erythroid precursors was observed in flow cytometry studies, reflecting the erythroid maturation block caused by lack of iron for hemoglobin generation. In animals treated with low dose M004 (25 µg/day) iron restriction was observed (Tf sat = 15%). Flow cytometry studies demonstrated an increase in the proportion of mature rather than immature erythroid cells. Consistent with the flow cytometry findings a 30% increase in peripheral red cells was observed associated with a 30% reduction in reticulocyte count and a reduction in spleen size of >50%, reflecting improved erythropoietic efficiency. At this dose Hb increased by 1.8 g/dL (+24.0%; see figure). Reduced ROS as well as diminished apoptosis suggests that the beneficial hematological effects of low dose minihepcidin therapy are the result of decreased oxidative damage and improved erythroid morphology, leading to improved survival of both erythroid precursors and erythrocytes.

Conclusion: These data indicate that minihepcidin peptides may have therapeutic benefit in the treatment of beta thalassemia by inducing a moderate level of iron restriction that reduces the cellular damage caused by hemichrome formation without further impairment of hemoglobin synthesis. Importantly a therapeutic window between a dose that produces beneficial effects on erythropoiesis and the dose that exacerbates anemia by causing excessive iron restriction has been established. It is likely that increased hepcidin activity produced by minihepcidin therapy will also have a preventative effect on the development of iron overload by reducing dietary iron absorption as well as by reducing transfusion requirements as anemia improves.



Podium Abstracts

Podium #65
RNAI TARGETING OF TMPRSS6 DECREASES IRON OVERLOAD IN A MOUSE MODEL OF HEREDITARY HEMOCHROMATOSIS AND ELIORATES ANEMIA AND IRON OVERLOAD IN MURINE β-THALASSEMIA INTERMEDIA

Paul Schmidt, PhD[1], Iva Toudjarska, PhD[2], Anoop Sendamarai, PhD[1], Tim Racie, BS[2], Stuart Milstein, PhD[2], Brian Bettencourt, PhD[2], Julia Hettinger[2], David Bumcrot, PhD[2], James Butler, PhD[2] and Mark Fleming, MD, DPhil[1]
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(Presented By: Paul Schmidt)

Introduction: Mutations in *HFE* are the primary cause of hereditary hemochromatosis (HH) in humans. Loss of functional HFE protein leads to inappropriately high iron uptake from the diet and subsequent tissue iron loading. β-thalassemia is a congenital anemia caused by partial or complete loss of β-globin synthesis causing ineffective erythropoiesis and anemia, leading to secondary iron overload. The elevated iron loading noted in both diseases is caused by inappropriately decreased hepatic expression of the central iron regulatory hormone hepcidin. TMPRSS6, also known as matriptase-2, is a membrane-bound serine protease expressed in hepatocytes that negatively modulates hepcidin production. TMPRSS6 is postulated to regulate hepcidin production by cleaving Hemojuvelin (*HJV*), a bone morphogenetic protein (BMP) co-receptor and key modulator of hepcidin expression, from the hepatocyte surface. Previous work has demonstrated that genetic ablation of *Tmprss6* in mouse models of HH (*Hfe-/-*) or β-thalassemia intermedia (*Hbbth3/+*) leads to increased hepcidin expression, decreased iron loading, and also reduces ineffective erythropoiesis in *Hbbth3/+* animals.
Methods and Materials: On this basis, we hypothesized that treatment of mouse models of HH (*Hfe-/-*) and β-thalassemia intermedia (*Hbbth3/+*) with Tmprss6 siRNA packaged in lipid nanoparticles (LNP) that are preferentially taken up by the liver would increase hepcidin expression. Here we demonstrate that LNP-Tmprss6 siRNA treatment of *Hfe-/-* and *Hbbth3/+* mice induces hepcidin and diminishes tissue and serum iron levels. Furthermore, LNP-Tmprss6 siRNA treatment of *Hbbth3/+* animals substantially improved the anemia by altering RBC survival and diminishing ineffective erythropoiesis.
Results: Thus, our observations indicate that pharmacological manipulation of Tmprss6 with RNAi therapeutics is a practical approach to treating iron overload diseases associated with diminished hepcidin expression and may well have efficacy in modifying disease-associated morbidities of other globinopathies.
Conclusion: Future work will seek to test the efficacy of the *Tmprss6* RNAi therapeutic in other mouse models of human disease and better understand the mechanism by which elevated hepcidin diminishes ineffective erythropoiesis in *Hbbth3/+* mice.

Podium #66
IDENTIFICATION OF NONFERRITIN MITOCHONDRIAL IRON DEPOSITS IN A MOUSE MODEL OF FRIEDREICH ATAXIA

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(Presented By: Des R. Richardson)

Introduction: There is no effective treatment for the cardiomyopathy of the most common autosomal recessive ataxia, Friedreich ataxia (FA). This disease is due to decreased expression of the mitochondrial protein, frataxin, which leads to alterations in mitochondrial iron (Fe) metabolism. The identification of potentially toxic mitochondrial Fe deposits in FA suggests Fe plays a role in its pathogenesis. Studies using the muscle creatine kinase (MCK) conditional frataxin knockout mouse that mirrors the disease have demonstrated frataxin deletion alters cardiac Fe metabolism. Indeed, there are pronounced changes in Fe trafficking away from the cytosol to the mitochondrion, leading to a cytosolic Fe deficiency.
Methods and Materials: Considering Fe deficiency can induce apoptosis and cell death, we examined the effect of dietary Fe supplementation, which led to body Fe loading and limited the cardiac hypertrophy in MCK mutants.
Results: Furthermore, this study indicates a unique effect of heart and skeletal muscle-specific frataxin deletion on systemic Fe metabolism. Namely, frataxin deletion induces a signaling mechanism to increase systemic Fe levels and Fe loading in tissues where frataxin expression is intact (i.e., liver, kidney, and spleen). Examining the mutant heart, native size-exclusion chromatography, transmission electron microscopy, Mössbauer spectroscopy, and magnetic susceptibility measurements demonstrated that in the absence of frataxin, mitochondria contained biomineral Fe aggregates, which were distinctly different from isolated mammalian ferritin molecules.
Conclusion: These mitochondrial aggregates of Fe, phosphorus, and sulfur, probably contribute to the oxidative stress and pathology observed in the absence of frataxin.

Podium Abstracts

Podium #67

BRAIN IRON AND MRI IN ALZHEIMER'S DISEASE, PARKINSON'S DISEASE, AND MULTIPLE SYSTEM ATROPHY

Joanna Collingwood[1], Mary E. Finnegan[1], Naomi P. Visanji[2], Emily House[3], Lili-Naz Hazrati[4], Vijay Antharam[5], John-Paul Bullivant[5], Martin R. Lees[1], Albina Mikhailova[5], Christopher Batich[5], Mark R. Davidson[5] and Jon Dobson[5]
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(Presented By: Joanna Collingwood)

Introduction: Iron from multiple brain regions in Alzheimer's disease (AD), Parkinson's disease (PD), and Multiple System Atrophy (MSA) is compared with healthy controls. Altered iron regulation has been observed in various forms in many neurodegenerative disorders; its contribution to disease progression remains an active topic of research. Dysregulation of metal elements besides iron is implicated in various pathological processes, but tracemetals analysis in human brain almost invariably occurs post-mortem. Iron is unusual; it holds particular scope for clinical detection by magnetic resonance imaging (MRI). Iron's influence on certain MRI parameters is well-demonstrated, but clinical attribution of MRI signal to iron requires validation in context. By testing relationships between regional brain iron in human tissue, and relevant MRI parameters, we are exploring the potential to differentiate between neurodegenerative disorders on the basis of brain iron status. Some iron changes arguably precede the extensive degeneration that is clinically observed in later stages of disease.
Methods and Materials: Following initial analysis of AD hippocampus, we present data from substantia nigra (SN), pons, caudate nucleus (CN), and putamen. For each case, the regions of interest are dissected on dry ice from tissue that has been stored at -80 °C. Protocols are designed to avoid iron contamination. Three cases are included in each group (AD, PD, MSA, and control). Each tissue sample is divided: one piece is for analysis by MRI and subsequent synchrotron microfocus X-ray fluorescence (μXRF) analysis; the adjacent piece is used for superconducting quantum interference device (SQuID) magnetometry analysis of isothermal remanent magnetisation (IRM) and total iron analysis. R2 and R2* maps are obtained using high-field (9.4 and 14.1 T) MRI. Tissue is held at 2 °C for measurement, enabling imaging while preserving tissue structure. Tissue is then re-frozen and cryosectioned for iron mapping by μXRF, and subsequent histology. R2 and R2* values are correlated with iron maps obtained from corresponding regions in each tissue block, testing for i) a relationship between iron concentration, R2 and R2*, and ii) disease-specific differences in these parameters. In the adjacent block, IRM is measured at several temperatures (including above and below the blocking temperature for ferritin), to obtain information about the iron oxide particulate fractions in the tissue; the block is then digested for determination of total iron.
Results: Disease-dependent differences in the direct iron measures, and in the MRI parameters, are observed. These include differences in grey/white matter contrast in R2* maps, and region-specific elevations in IRM at 5 K that correlate with total iron. Findings include evidence for elevated iron in the MSA pons, the PD SN, and the AD CN, compared to healthy controls.
Conclusion: Interpretation of findings for the SN, pons, and putamen is supported by quantitative analysis of ferritin, ferroportin, and transferrin. μXRF mapping provides evidence of disease-specific iron elevation in individual cells in certain regions of interest, including neuronal cell bodies in the AD pons. The emerging differences between groups support the contention that MRI measurement of brain iron changes may assist with differential diagnosis of neurodegenerative disorders.

Podium #68

RESPIRATORY CHAIN DEFICIENCY DUE TO FE-S CLUSTER DEFICIENCY IN SKELETAL MUSCLE LEADS TO A SYSTEMIC STARVATION RESPONSE CHARACTERIZED BY INCREASED FGF-21 SECRETION AND UP-REGULATION OF KETOGENIC ENZYME HMGCS2 IN SKELETAL MUSCLE FIBERS OF ISCU MYOPATHY PATI

Daniel Crooks, PhD[1], Thanemozhi Natarajan, PhD[2], Chuming Chen, PhD[3], Hongzhan Huang, PhD[3], Manik Ghosh, PhD[4], Wing-Hang Tong, PhD[4], Ronald Haller, MD[5], Cathy Wu, PhD[3] and Tracey Rouault, MD[4]
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(Presented By: Daniel Crooks)

Introduction: ISCU Myopathy, a disease characterized by life-long exercise intolerance and impaired mitochondrial oxidative metabolism, is caused by deficiency of the Fe-S cluster scaffold protein ISCU.
Methods and Materials: We performed gene expression analysis on muscle biopsies from ISCU Myopathy patients to elucidate which molecular processes were transcriptionally remodeled in response to impaired Fe-S cluster assembly.
Results: We found that ISCU depletion led to increased expression of the mitochondrial iron importer MFRN2 and the rate-limiting heme biosynthetic enzyme ALAS1. Gene expression and histologic studies demonstrated that patient muscle composition was shifted towards fewer glycolytic muscle fibers, more oxidative fibers, and increased capillary abundance. Paradoxically, mitochondrial fatty acid uptake and oxidation genes were coordinately up-regulated in patient muscles despite dramatic impairments of aconitase and succinate dehydrogenase activities. The ketogenic enzymes HMGCS2 and BDH1 were also significantly up-regulated, as was the secreted starvation response hormone, FGF-21.
Conclusion: We propose that ketogenesis may be initiated to restore free coenzyme A levels and shunt fatty acid oxidation products to distal respiration-competent tissues when TCA cycle and/or respiratory chain function is sufficiently impaired in affected patient muscle fibers. Moreover, our work shows that plasma FGF21 is a sensitive non-invasive biomarker of ISCU Myopathy, in addition to other mitochondrial myopathies.

Podium Abstracts

Podium #69

BONE MARROW IRON ACCUMULATION IN SICKLE CELL DISEASE, PAROXYSMAL NOCTURNAL HEMOGLOBINURIA AND THALASSEMIA PATIENTS ASSESSED BY MRI

Lucía Gutiérrez[1], Michael J. House[1], Nisha Vasavda[2], Emma Drašar[2], Austin Kulasekararaj[2], Swee L. Thein[2] and Tim G. St Pierre[1]

[1]The University of Western Australia; [2]King's College London

(Presented By: Tim G. St Pierre)

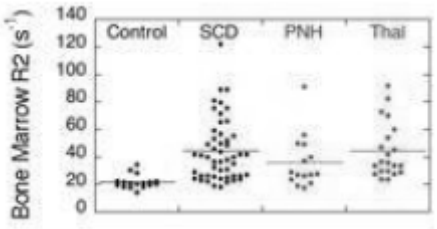
Introduction: Bone marrow iron accumulation, and its relationship to other organs, has not been extensively studied in diseases associated with iron imbalance owing to the difficulties in accessing this organ for quantitative measurements. The proton transverse relaxation rate (R2) has been shown to correlate with tissue iron concentration. In this study we have compared bone marrow R2 in patients with sickle cell disease (SCD), paroxysmal nocturnal hemoglobinuria (PNH), β-thalassemia, and a control group.

Methods: Analysis of magnetic resonance images was retrospectively performed in patients that had already had an assessment of hepatic iron loading as part of their clinical care programme. There were 40 SCD patients (25 females, 15 males, 26.9 ± 8.8 years), 15 PNH patients (7 females, 8 males, 45.5 ± 15.7 years), 13 thalassemia patients (7 females, 6 males, 38.3 ± 14.8 years), and 17 healthy control participants (4 females, 13 males, 37 ± 7.7 years). Axial images of the abdomen covering part of the thoracic and lumbar vertebrae were obtained from clinical MRI scanners operating at 1.5 T.

Spin density projection assisted R2-MRI (FerriScan®) [St Pierre, T.G., et al., Blood, 2005. 105(2): p. 855-61] were used to assess liver iron concentration (LIC) in the participants. R2 values for the bone marrow were derived from pixel-wise mono-exponential fits to image data acquired with a single spin-echo sequence (FerriScan®). Group means were compared using Student's t test, with p=0.05 as the threshold for significance.

Results: Mean bone marrow R2 values for SCD (43.2 ± 21.1 s-1, p <0.0001), PNH (36.1 ± 19.2 s-1, p = 0.0059) and Thalassemia patients (44.3 ± 20.0 s-1, p < 0.0001) were significantly higher than control mean bone marrow R2 values (21.8 ± 4.9 s-1) (Figure). Median LIC and ranges for the these groups were: controls = 1.1 mg/g, range 0.4 – 1.8 mg/g; SCD = 4.7 mg/g, range 0.6 – > 43 mg/g; PNH = 7.6 mg/g, range 1.8 – > 43 mg/g and Thalassemia = 10.8 mg/g, range 0.6 – 43 mg/g.

Discussion and Conclusions: These results indicate that R2 measurements of bone marrow can be used to quantitatively assess bone marrow iron deposition in diseases such as SCD, PNH and Thalassaemia. This technique opens up the opportunities to assess the effect of chelators on bone marrow iron overload.



Podium #70

BIO-MARKERS OF IRON TRAFFICKING AND DISTRIBUTION IN TRANSFUSIONAL OVERLOAD: INSIGHTS FROM COMPARING DIOND BLACKFAN ANEMIA WITH SICKLE CELL DISEASE AND THALASSEMIA (MCSIO PILOT STUDY)

John Porter, MD[1], Patrick Walter, PhD[2], Lynne Neumayr, MD[2], Patricia Evans, PhD[1], Marcela Weyhmiller, PhD[2], Paul Harmatz, MD[2], John Wood, MD, PhD[3], Jeff Miller, MD[4], Guenter Weiss, MD[5], Quing Yuan, PhD[6], Zhiyue Wang, PhD[6], Regine Grosse, MD[7], Bjoern Schoennagel, MD[7], Joerg Meerpohl, MD[8], Roland Fischer, PhD[7], Peter Nielsen, MD, PhD[7], Markus Seifert, BSc[5] and Elliott Vichinsky, MD[2]

[1]University College London; [2]Children's Hospital & Research Center Oakland; [3]Children's Hospital Los Angeles; [4]National Institute of Diabetes and Digestive and Kidney Diseases; [5]Medical University of Innsbruck; [6]University of Texas Southwestern Medical Center Dallas; [7]University Medical Center Hamburg-Eppendorf; [8]University Medical Center Freiburg

(Presented By: John Porter)

Background: Transfused thalassemia major (TM), Sickle Cell Disease (SCD), and Diamond Blackfan Anemia (DBA) patients are at risk for organ injury due to iron overload. A Multicenter Study of Iron Overload (MCSIO) is exploring how key candidate factors affect iron distribution; including inflammation, ineffective erythropoiesis, level of iron overload, and hepcidin synthesis. Plasma non−transferrin bound iron (NTBI) could be a key mechanism by which iron is delivered to tissues.

Methods: 15 iron−overloaded patients (5 from each group of TM, SCD, and DBA) with ferritin > 1500 g/dl or LIC > 7 mg/g dry wt, age >16, age 0 to 9 at initiation of transfusion and 10 to 20 years of transfusion exposure were enrolled from 3 sites in the US and Europe. Five non−transfused healthy controls were also enrolled. A detailed medical, transfusion and chelation history were obtained. Fasting, early morning blood samples were obtained prior to transfusion. Chelation was held for 72 hours prior.

Results: DBA patients had the highest NTBI despite having the lowest ferritin and LIC levels. SCD had the lowest LPI and NTBI levels and this paralleled the lowest expression levels monocyte ferroportin. GDF15 levels were highest in TM. EPO

levels were nearly >100x higher in DBA than TM or SCD despite similar pre-transfusion Hb values. In DBA, GDF15 values were approximately three times controls, while soluble transferrin receptors (sTfR) values are almost undetectable.
Conclusions: High NTBI in DBA cannot be explained by IE, as markers for this were no higher than in SCD (GDF15) or were virtually absent (sTfr); consistent with erythron maturation arrest in DBA. High hepcidin (and hepcidin/ferritin ratio) and high EPO in DBA are consistent with a lack of hepcidin suppression by IE or by EPO. The lack of transferrin iron clearance by the erythron in DBA is likely to account for the high NTBI values. Low NTBI and LPI in SCD relative to the degree of iron overload are likely to be a consequence of: lower IE in SCD than in TM: secondly, decreased egress on macrophage iron due to low ferroportin expression. As hepcidin levels when corrected for iron overload (hepcidin/ferritin ratio) did not differ between SCD and TM, the possibility of a local inflammatory mediator of ferroportin expression needs to be considered.

Pre-transfusion Median Values	TM, n=5	SCD, n=5	DBA,n=5	Con,n=5
HB (g/dl)	10.90	9.40	9.20	13.5
Ferritin (µg/L)	3251	12000	2150	32
NTBI (uM)	1.86	1.91	9.39	-1.83
LPI (uM)	1.30	0.05	0.86	0.01
EPO (mIU/ml)	41.0	28.0	2004	7.0
GDF15 (pgm/ml)	5504	538	789	279
solubleTfr (nmol/l)	11.65	8.40	<0.05	3.2
CRP (mg/l)	0.9	2.2	1.8	0.24
Hepcidin (nM)	3.97	24.3	28.7	0.81
Hepcidin/ferritin	0.0017	0.0016	0.0124	0.0232
Ferroportin (mRNA/RPL27)	0.0410	0.0252	0.0882	0.0564
LIC [mg/gd.w.] from R2*	18.3	29.6	6.1	<2.2

Podium #71
IRON METALLODRUGS: STABILITY, REDOX ACTIVITY AND TOXICITY AGAINST ARTEMIA SALINA
Hector Aguilar Vitorino[1], Flavia Zanotto, PhD[2], Luca Mantovanelli[2] and Breno Pannia Espósito, PhD[1]
[1]University of São Paulo – Institute of Chemistry; [2]University of Sao Paulo-Institute of Biosciences
(Presented By: Hector Aguilar Vitorino)

Introduction: Iron metallodrugs comprise mineral supplements, anti-hypertensive agents and, more recently, magnetic nanomaterials that have been proposed as contrast agents for magnetic resonance imaging or antitumor hyperthermia therapy. These materials have been designed for specific biological activities, which leads to concern regarding their incorrect disposal in the environment.
Methods and Materials: In this work, we evaluated the stability of iron supplements (basically iron-saccharide derivatives) from different suppliers, as well as ferrocene-derivatives (3,5,5-trimethylhexanoyl, TMH), in buffered medium against the fluorescent probe calcein. The amount of ascorbate-induced redox-active iron in these formulations has been studied by measuring the rate of oxidation of the probe dihydrorhodamin. The effect of the compounds on the viability of Artemia salina, a model of aquatic ecotoxicology, was also studied.
Results: Our results indicate that iron-saccharide derivatives have a relatively low (4.5 – 7.0 %) amount of redox-active iron, however TMH-ferrocene and (TMH)2-ferrocene (suggested for iron supplementation) displayed a considerably higher percentage of redox-active iron (8.5 and 17.5 % respectively), increasing with the level of substitution on the cyclopentadiene ring.
Conclusion: The parent ferrocene molecule displayed higher stability and lower redox-active iron in comparison with the other compounds, and was the most toxic for A. salina (40% of lethality at 10 µM), suggesting that toxicity results from the absorption of the whole compound followed by cell injury rather than from absorption of “labile” iron.

Podium #72

THE NEUROTROPHIN SPAETZLE5 UPREGULATES HEME BIOSYNTHESIS VIA NITRIC OXIDE SIGNALING TO INCREASE STEROID HORMONE PRODUCTION IN DROSOPHILA

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(Presented By: Krist King-Jones)

Introduction: Steroid hormones are ancient signaling molecules that play fundamental roles in development and disease. In *Drosophila*, pulses of the steroid hormone ecdysone govern all major developmental transitions, including larval molts and the transformation of a larva to a pupa. Ecdysone is mainly produced in the larval prothoracic gland, and while we have a relatively good understanding of the enzymatic steps that synthesize the hormone, comparatively little is known about the complex signaling pathways that control the formation, timing and amplitude of an ecdysone pulse. In a genetic screen for novel regulators of ecdysone biosynthesis, we identified *spaetzle5*, a neuropeptide related to vertebrate neurotrophins. Loss-of-*spaetzle5* function triggers porphyria-like phenotypes in the prothoracic gland, namely substantial accumulation of mitochondrial protoporphyrins, concomitant with strong upregulation of *ALAS*. These phenotypes cause larval lethality due to severely reduced steroid hormone production. Remarkably, these phenotypes are indistinguishable from disrupting nitric oxide (NO) production in the prothoracic gland, which is corroborated by the fact that loss-of-*spaetzle5* results in no detectable NO levels. Furthermore, we have identified a transcription factor that acts as a putative heme sensor, and is responsible for *ALAS* induction.

Methods: We are currently examining whether NO is required as a developmental signal to increase iron availability in prothoracic glands when demand for ecdysone is high. In a parallel approach, we have now screened ~8,000 RNAi lines for porphyria-like phenotypes, and have isolated an additional 21 genes that are critical for heme biosynthesis and its regulation.

Conclusion: Our studies have identified a novel pathway critical for the regulation of heme biosynthesis in ecdysone-producing tissues, paving the way for identifying common principles underlying the regulation of heme biosynthesis and steroid hormone production in all higher eukaryotes.

Poster Abstracts

Poster #1

THE ROLES OF HEPCIDIN AND FERROPORTIN IN AUTOCRINE REGULATION OF IRON LEVELS

Samira Lakhal-Littleton, BSc, DPhil, Daniel Biggs, BSc, MSc, Rebecca Diaz, BSc, PhD, Ana Santos, BSc, PhD, Chris Preece, BSc, PhD, Benjamin Davies, BSc, PhD, Peter Robbins, MA, DPhil, BM, BCh
Oxford University
(Presented By: Samira Lakhal-Littleton)

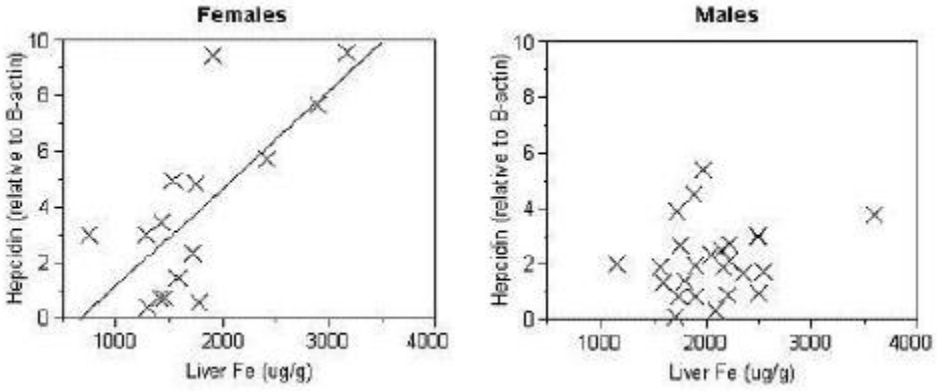
Introduction: Liver-derived hepcidin Hamp regulates iron homeostasis by downregulating ferroportin Fpn expression at the sites of iron absorption (duodenal enterocytes), recycling (reticuloendothelial macrophages) and storage (liver). However, Fpn and Hamp are also expressed in tissues not associated with systemic iron handling, such as the heart, skeletal muscle, lung and brain, raising the possibility of autocrine iron regulation in these tissues. Current mouse models of ubiquitous gene deletion do not enable us to address this hypothesis due to the confounding effects of ubiquitous loss of
Hamp and Fpn on systemic iron levels.
Methods and Materials: To overcome this limitation, we have used the LoxP Cre technology to enable tissue-specific manipulation of Hamp and Fpn expression in the mouse. Here we describe, for the first time, the generation and biochemical validation of three mouse models: 1) tissue-specific Hamp knockout, 2) tissue-specific Fpn knockout and 3) tissue-specific Fpn C326Y knock-in (hamp-resistant).
Conclusion: Our animal models are valuable tools for micro-dissecting iron regulation at a single-tissue level in health and in disease. They will help achieve the mechanistic understanding necessary for the development of evidence-based iron manipulation therapies for diseases where iron dysregulation contributes to pathogenesis.

Poster #2

SEX DIFFERENCES IN HEPATIC HEPCIDIN SIGNALING IN THE HEMOJUVELIN KNOCKOUT MOUSE

Casey Brewer, BSc; Maya Otto-Duessel, PhD; Ruth Wood, PhD; John Wood, MD, PhD
Children's Hospital Los Angeles
(Presented By: Casey Brewer)

Introduction: In humans, there is a two-fold greater risk of mortality for males with iron overload compared to females with the disease; the cause for this is unknown. Marked sex differences in cardiac iron loading have also been observed in hemojuvelin knockout (HJV KO) mice. The protein hemojuvelin (HJV) is essential for proper hepcidin expression in response to high systemic iron levels. HJV mutations result in juvenile hemochromatosis, a condition marked by low hepcidin levels despite severe systemic iron overload.
Methods and Materials: We hypothesized that hepatic hepcidin signaling during iron overload is sexually dimorphic, providing females with a survival advantage compared to males. HJV KO mice on a high iron diet were used as our model of
iron overload. Mice were gonadectomized at four weeks of age and received a Silastic hormone implant; males received an implant containing either testosterone (n=7), dihydrotestosterone (n=7), estrogen (n=7), or a cholesterol control (n=12). Female mice received either estrogen (n=7) or a cholesterol control (n=7). A group of intact males (n=7) and females (n=7) had a sham surgery and a cholesterol implant as a control. Physiologic dosing of hormone levels was confirmed by measuring the seminal vesicles of males and the uteri of females. Hepatic hepcidin signaling was measured via RT-PCR of the following targets: hepcidin, bone morphogenetic protein 6 (BMP6), and the bone morphogenetic protein type II receptors Activin Receptor II A (ACTRIIA) and Activin Receptor II B (ACTRIIB).
Results: Hepcidin expression was decreased in HJV KO compared to wild type ($p<0.0001$), but more effectively suppressed in HJV KO males compared to HJV KO females ($p<0.05$). There was no effect of steroid treatment on hepcidin expression in HJV KO males. Among females, intact HJV KO females expressed the most hepcidin ($p<0.001$) while gonadectomized
HJV KO females implanted with estrogen expressed the least ($p<0.05$); gonadectomized HJV KO females implanted with a cholesterol control expressed an intermediate amount of hepcidin. Not only were hepcidin levels 1.8 times higher on average in females than in males ($p<0.05$), but females also retained a positive correlation between liver iron concentration and hepcidin expression (Figure, $R2=0.51$, $p<0.01$). This positive correlation between liver iron concentration and hepcidin expression was abrogated in male HJV KO mice (Figure). In both male and female HJV KO mice, BMP6 expression was positively correlated with liver iron concentration ($R2=0.23$, $p<0.02$ and $R2=0.39$, $p<0.01$, respectively) indicating that
iron sensing was preserved. However, coupling of BMP6 signaling to hepcidin expression was only maintained in females ($R2=0.42$, $p<0.01$). Of note, female HJV KO mice expressed 27% more ACTRIIA and 49% more ACTRIIB than males ($p<0.04$ and $p<0.03$, respectively); BMP type II receptors ACTRIIA and ACTRIIB play a key role in BMP signaling. In particular, ACTRIIB was strongly correlated with hepcidin expression in HJV KO females ($R2=0.63$, $p<0.001$), but not males.
Conclusion: In conclusion, hepcidin signaling in response to liver iron overload is incompletely disrupted in female HJV KO mice. It is possible that this could provide a survival advantage to females with iron overload compared to males. Elucidation of the mechanism responsible for sexually dimorphic hepcidin regulation may also impact the choice of hormonal contraceptives used in countries where thalassemia syndromes are endemic.



Poster #3

HEPATOCYTE GROWTH FACTOR ACTIVATOR INHIBITOR TYPE 2 (HAI-2) MODULATES HEPCIDIN EXPRESSION BY INHIBITING CELL SURFACE PROTEASE MATRIPTASE-2

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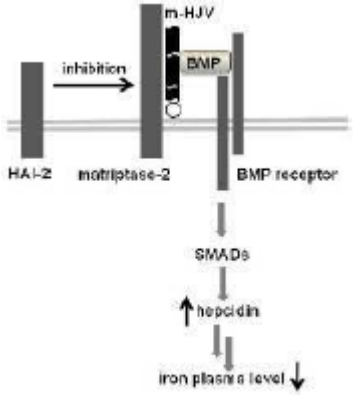
University of Bonn
(Presented By: Eva Maurer)

Introduction: Matriptase-2 is encoded by the *TMPRSS6* gene and mainly expressed in the liver as a multi-domain, type II transmembrane serine protease (TTSP). Mutations of matriptase-2 lead to inappropriate high levels of the systemic iron regulatory protein hepcidin, which cause iron-refractory iron deficiency anemia (IRIDA). In cleaving the bone morphogenetic protein (BMP) co-receptor hemojuvelin (HJV) matriptase-2 modulates BMP/SMAD signaling and suppresses the expression of the hepcidin-encoding gene HAMP. With regard to its important function in iron metabolism, matriptase-2 activity can be expected to be tightly regulated by endogenous inhibitors. The activities of trypsin-like serine proteases are regulated by serpins and the Kunitz-type serine protease inhibitors, HAI-1 and HAI-2. These inhibitors modulate the proteolytic activity of the close homologue matriptase *via* the formation of complexes.

Methods and Materials: In this study, matriptase-2 and the Kunitz-type inhibitor HAI-2 have been co-expressed, and a high inhibitory potential of HAI-2 against matriptase-2 was observed at the cell surface as well as in conditioned media by measuring the proteolytic activity of matriptase-2 with the chromogenic substrate Boc-Gln-Ala-Arg-p-nitroanilide. Additionally, immunoblot analysis demonstrated that matriptase-2 is present in a stable complex, resistant to SDS but not to boiling and reducing conditions. Using immunoaffinity chromatography, the complex was isolated from conditioned media, and subsequently performed LC-MS/MS analysis revealed that the complex consists of peptides matching matriptase-2 and HAI-2. Moreover, dual luciferase assays using a reporter gene construct that harbors 2700 bp 5'-upstream of human *HAMP* gene plus its 5'UTR (71 bp) and qRT-PCR revealed that HAI-2 modulates the expression of *HAMP* probably by inhibiting the proposed matriptase-2-catalyzed cleavage of HJV.

Results: This study characterized HAI-2 as the first endogenous inhibitor of matriptase-2 and reveals that HAI-2 may be a new player in the complex regulatory mechanism of iron metabolism in that it regulates hepcidin expression. The putative mechanism of how HAI-2 may modulate hepcidin synthesis and iron metabolism is illustrated in Figure 1. HAI-2 may inhibit the activity of matriptase-2, and abrogates in this way the processing of membrane-bound HJV. The expression of the hepcidin-encoding gene *HAMP* increases *via* BMP/SMAD signalling, and the iron plasma levels decrease. The regulation of matriptase-2 activity by HAI-2 in iron metabolism may have clinical importance for patients with primary or secondary hemochromatosis in thalassemia who exhibit inappropriate low hepcidin levels. Low levels of HAI-2 leading to upregulated matriptase-2 activity and decreased hepcidin synthesis may be linked to imbalanced iron regulation and diseases such as hemochromatosis. In contrast, elevated HAI-2 levels might lead to increased hepcidin synthesis and to low iron plasma levels with a phenotype similar to IRIDA.

Figure 1: Schematic model of the putative role of HAI-2 in the regulation of iron homeostasis. HAI-2 inhibits the proteolytic activity of matriptase-2 and abrogates the processing of HJV. In turn, *HAMP* expression is induced *via* HJV/BMP/SMAD signaling. BMP, bone morphogenetic protein; CM, Cell Membrane; CP, Cytoplasma; HJV, Hemojuvelin; SMADs, Sons of Mothers Against Decapentaplegic homologues.



Poster #4
THE CELL SURFACE PROTEASE MATRIPTASE-2 CLEAVES AHSG (FETUIN A): A NEW LINK BETWEEN IRON HOMEOSTASIS AND DIABETES
Marit Stirnberg, PhD; Eva Maurer, MSc; Katharina Arenz; Verena Lülsdorff; Michael Gütschow, Prof
University of Bonn
(Presented By: Marit Stirnberg)

Introduction: AHSG (α2-Heremans-Schmid glycoprotein; also known as Fetuin A) is highly expressed in the liver and secreted into the circulation. It has a physiological role in biomineralization of bone. Interestingly, AHSG also mediates insulin resistance by binding directly to the insulin receptor, thereby blunting its intrinsic activity, as well as to toll-like receptor 4 activating an inflammatory signaling cascade leading to lipid-induced insulin resistance. The dominant form of AHSG in human plasma is a two-chain disulfide linked protein. The 40 residue 'connecting peptide' spanning from amino acid 301 to amino acid 340 is removed by proteolytic cleavage. The unprocessed single-chain form of the protein was shown to shut down the insulin receptor activity. Matriptase-2 is a type II transmembrane serine protease (TTSP) controlling the expression of the hepcidin-encoding gene *HAMP* through the suppression of BMP/SMAD signaling probably by cleaving hemojuvelin. So far, the only known putative substrates of matriptase-2 are hemojuvelin and matriptase-2 itself as this protease undergoes complex auto-processing resulting in activation and cell surface release of the enzyme in transfected cell systems.
Methods and Materials: In order to explore new substrates of matriptase-2 we performed a pull down assay and identified AHSG as a potential interaction partner of matriptase-2. In this study, AHSG was further characterized as a putative matriptase-2 substrate. Transiently transfected HEK and Huh-7 cells expressing matriptase-2 and/or AHSG were analyzed by western blotting for cleavage of AHSG by matriptase-2. In both transfected cell systems AHSG was processed by matriptase-2. Additionally, a protein interaction between AHSG and matriptase-2 was observed by isolating matriptase-2 from lysates co-expressing matriptase-2 and AHSG-Myc by immunoaffinity chromatography using an immobilized anti-c-Myc antibody.
Results: Site directed mutagenesis of Arg residues located within the connecting peptide of AHSG suppressed matriptase-2-mediated AHSG processing. Importantly, downregulation of matriptase-2 in Huh-7 cells resulted in a stabilization of unprocessed AHSG demonstrating that endogenous matriptase-2 is expressed at levels sufficient to modulate AHSG processing. In order to characterize the significance of AHSG processing by matriptase-2 *HAMP* promoter activity was analyzed by a reporter gene assay after transfection of Huh-7 cells with AHSG and/or matriptase-2. AHSG was able to modulate *HAMP* promoter activity whereas co-expression of matriptase-2 abolished this effect.
Conclusion: In conclusion, AHSG was identified as a new potential substrate of matriptase-2. By processing AHSG, matriptase-2 might modulate its activity which contributes to the complex regulation of *HAMP* expression. Future investigations are necessary to analyze which signaling cascade leading to an altered *HAMP* expression is influenced by the action of different processed forms of AHSG. AHSG is supposed to be a potential therapeutic target for type 2 diabetes in that it mediates insulin resistance. Our data demonstrate that AHSG might be involved in iron homeostasis providing a new link between iron control and diabetes.

Poster #5
DEFINING THE MECHANISM OF HEPCIDIN-INDUCED FERROPORTIN ENDOCYTOSIS
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University of California, Los Angeles, Center for Iron Disorders
(Presented By: Sharraya Aschemeyer)

Introduction: Iron is transported into plasma by the only known cellular iron exporter, ferroportin, which is expressed on enterocytes absorbing dietary iron, macrophages recycling aged erythrocytes, and hepatocytes which store iron. Ferroportin is the hepcidin receptor: hepcidin binds to ferroportin, causing its endocytosis and degradation, thus inhibiting the entry of iron into plasma. Ligand-induced receptor endocytosis is usually triggered by a conformational change followed by phosphorylation

and ubiquitination. Our lab determined that the binding of hepcidin to ferroportin causes its ubiquitination and degradation in lysosomes, but the ubiquitin ligases and the molecular machinery involved in ferroportin endocytosis are not known.
Methods and Results: We used siRNAs (clathrin and caveolin-1) to knockdown key proteins involved in clathrin-mediated or caveolin-mediated endocytosis in a ferroportin -inducible human embryonic kidney cell line. First these cells were treated with siRNA, and then doxycycline was added to induce ferroportin. Hepcidin was later added to trigger ferroportin endocytosis and degradation. The total amount of ferroportin before and after hepcidin treatment was quantified by western blot. The knockdowns were also confirmed via western blot. Knockdown of clathrin using siRNA hindered total ferroportin degradation in the presence of hepcidin whereas caveolin-1 knockdown did not. For unbiased identification of ferroportin-interacting proteins after hepcidin treatment, we used mass spectrometric proteomic analysis. We analyzed immunoprecipitated ferroportin from HEK293 cells overexpressing the tagged protein and treated with hepcidin for 30 min. Mass spectrometry confirmed that ferroportin was ubiquitinated and identified candidate interactors prominently including an E2 ubiquitin-conjugating enzyme and an E3 ubiquitin ligase as well as vesicle-associated proteins. Characterization of the ferroportin-interacting proteins is ongoing.
Conclusion: Our data indicate that ferroportin is endocytosed in a process dependent on ubiquitination and clathrin-coated pits. Because hepcidin-related iron disorders are caused by alterations in the rate of ferroportin endocytosis, understanding the details of hepcidin-mediated ferroportin endocytosis could identify potential targets for development as novel therapeutics for iron disorders.

Poster #6
REGULATION OF HEPCIDIN TRANSCRIPTION BY REACTIVE OXYGEN SPECIES AND HYPOXIA
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University of Nebraska Medical Center
(Presented By: Duygu Dee Harrison-Findik)

Introduction: Increases in oxidative stress correspond to the severity of liver diseases. Alcohol or hepatitis C viral protein-induced oxidative stress suppresses hepcidin expression in the liver leading to an increase in intestinal iron absorption, which is inhibited by antioxidants.
Methods and Materials: The aim of this study is to investigate the role of reactive oxygen species and hypoxia in the regulation of liver hepcidin transcription. Transgenic mice lacking the expression of different antioxidant enzymes or aryl hydrocarbon receptor nuclear translocator (ARNT /HIF-1β) were administered plain water (as control) or 10 % ethanol in the drinking water for 1 week. As determined by electron paramagnetic resonance spectroscopy, MnSOD+/-mice, lacking the expression of mitochondrial manganese superoxide dismutase (MnSOD) enzyme on one allele and fed with plain water, displayed a two-fold increase in superoxide production, which was elevated to four-fold after alcohol administration. However, the level of mitochondrial superoxide production, as detected by mitoSox, was similar in control and alcohol-fed MnSOD+/- mice. The basal level of liver hepcidin expression was unaltered in MnSOD+/- mice compared to control littermates.
Results: The decrease in liver hepcidin expression following alcohol exposure was similar in MnSOD+/- and negative littermate mice. The antioxidant enzymes, catalase, which is primarily expressed in cytosol, and glutathione peroxidase-1 (gpx-1), which is expressed both in mitochondria and cytosol, eliminate hydrogen peroxide. The basal liver hepcidin expression was unaltered in homozygous catalase, but was decreased in homozygous gpx-1 knockout mice. Similar to control mice, alcohol inhibited hepcidin expression in catalase knockout mice. In contrast, alcohol-fed homozygous gpx-1 knockout mice displayed an increase in hepcidin expression. Alcohol exposure induced HIF-1α and HIF-2α protein expression in ARNT floxed (control), but not in liver-specific ARNT knockout mice, which lack HIF-1β expression on one allele. Similarly, alcohol could not inhibit hepcidin expression in ARNT knockout mice, compared to ARNT floxed mice. However, chromatin immunoprecipitation studies did not show HIF-1α or HIF-2α binding to hepcidin promoter in untreated or alcohol-fed ARNT knockout and control mice.
Conclusion: In summary, our studies suggest a role for hydrogen peroxide, but not superoxide free radicals, in the regulation of hepcidin expression in the liver. The effect of hypoxia on hepcidin transcription does not involve the hypoxia-inducible transcription factors and might be regulated by indirect mechanisms. A better understanding of hepcidin regulation by oxidative stress and hypoxia is important to elucidate the mechanisms of iron-induced liver injury.

Poster #7
DIFFERENTIAL RESPONSE OF THE HEPCIDIN REGULATORY PATHWAY TO ORAL VERSUS PERENTERAL IRON IN MICE LACKING HFE AND TFR2
Cameron McDonald, Daniel Wallace, Lesa Ostini, Nathan Subramaniam
Queensland Institute of Medical Research
(Presented By: Cameron McDonald)

Introduction and Aims: The hemochromatosis-associated proteins Hfe and Transferrin Receptor 2 (*Tfr2*) are key upstream regulators of hepcidin (*Hamp1*) in response to body iron status. While some studies have suggested that Hfe and Tfr2 form an iron-sensing complex, there is mounting evidence to suggest that this is not the case, and that their precise roles are still unclear. As iron status increases, *Bmp6* expression is up-regulated and signalling occurs through the HJV:BMPR complex

leading to phosphorylation of Smad1/5/8 (pSmad1/5/8). Phosphorylated Smad1/5/8 then complexes with pSmad4, and translocates to the nucleus where *Hamp1* up-regulation occurs. This study aims to further elucidate the roles of Hfe and Tfr2 in hepcidin regulation.

Methods: Wild type, *Hfe*-/-, *Tfr2*$^{-/-}$, and *Hfe*$^{-/-}$/*Tfr2*$^{-/-}$ mice were subjected to iron loading via dietary or parenteral routes. Animals were fed a 2% carbonyl iron diet for 2 weeks prior to sacrifice at 5 weeks of age, or injected intra-peritoneally with 0.3 mg/g Fe-dextran 4 days prior to sacrifice. Iron status and localization were examined. Expression of hepcidin regulatory genes, and Smad1/5/8 phosphorylation were assessed to isolate the influence of iron route and genotype.

Results: Dietary iron accumulated exclusively in hepatocytes, while parenteral iron accumulated predominantly in non-parenchymal cells. *Bmp6* upregulation was blunted in *Hfe*$^{-/-}$/*Tfr2*$^{-/-}$ mice in response to dietary iron, but was not affected in response to parenteral Fe-dextran. Phosphorylation of Smad1/5/8 relative to *Bmp6* was unaffected in all animals. In contrast to *Bmp6* regulation, *Hfe*$^{-/-}$/*Tfr2*$^{-/-}$ mice fed iron showed only a mild reduction in *Hamp1* relative to pSmad1/5/8, while Fe-dextran injected *Hfe*$^{-/-}$/*Tfr2*$^{-/-}$ mice showed an almost complete loss of *Hamp1* induction by pSmad1/5/8. Finally, *Hfe*-/- single null mice showed no loss of *Bmp6* regulation, while *Tfr2*$^{-/-}$ single null mice had a blunted *Bmp6* response equal to that of *Hfe*$^{-/-}$/*Tfr2*$^{-/-}$.

Conclusion: This study highlights the separate roles of Hfe and Tfr2 in the regulatory responses to iron administered by different routes, and distributed in different cell types. Significantly, it shows that Tfr2, but not Hfe, is required for iron-induced up-regulation of Bmp6, and suggests that Hfe asserts its regulatory influence on *Hamp1* expression downstream of *Bmp6* induction.

Poster #8

ATOH8 REGULATES HEPCIDIN TRANSCRIPTION, CELLULAR PSMAD1,5,8 LEVELS AND IS SUPPRESSED BY ERYTHROPOIETIC ACTIVITY

Andrew McKie, BSc, PhD[1]; Neeta Patel, BSc[2]; Joe Varghese, MD, PhD[3]; Patarabutr Masaratana, MD, PhD[4]; Molly Jacob, MD, PhD[3]; Oluyemisi Latunde-Dada, BSc, PhD[2]; Robert J. Simpon, BSc, PhD[2]

[1]Kings College London; [2]Kings College London, UK, Division of Diabetes and Nutritional Sciences; [3]Christian Medical College Vellore, India, Department of Biochemistry; [4]Faculty of Medicine Siriraj Hospital, Bangkok, Thailand, Department of Biochemistry

(Presented By: Andrew McKie)

Introduction: Little is known about how changes in erythropoietic state regulate hepcidin. ATOH8, a member of the basic helix loop helix (bHLH) family of transcription factors, has been previously shown to be induced in liver of iron-loaded mice however its role in iron metabolism has not been established1.

Methods and Materials: ATOH8 expression in HEK293 cells resulted in dose dependent increase in hepcidin promoter activity as well as endogenous Hamp levels. Mutation of E-box or SMAD response elements within the hepcidin promoter significantly reduced the effects of ATOH8, suggesting that ATOH8 acts directly on the hepcidin promoter itself as well as through BMP signalling. In support of the latter, ATOH8 expression led to a dose dependent increase in pSMAD1,5,8 levels in HEK293 cells.

Results: As shown by others1,2 we found that liver Atoh8 levels were increased by 'straightforward' liver iron loading. However, in conditions where erythropoeitic activity was increased including: haemolytic anaemia;
hypotransferrinaemia and hypoxia, liver ATOH8 mRNA and protein levels were markedly down-regulated. In the case of hemolytic anaemia and hypotransferrinaemia the decrease in ATOH8 occurred despite very marked increases in liver iron demonstrating that ATOH8 is regulated in the same direction as hepcidin and likely responds to changes in transferrin saturation rather than iron stores. Under a number of varied conditions in vivo, in freshly isolated primary hepatocytes and in HepG2 cells Atoh8 and Hamp1 levels significantly correlated.

Conclusion: In summary, ATOH8 is a novel transcriptional regulator of hepcidin and provides a molecular mechanism by which hepcidin levels can be regulated by erythroid activity.

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Poster #9

FERROPORTIN1 IS ESSENTIAL FOR IRON ABSORPTION IN SUCKLING MICE BUT IS NOT DOWN-REGULATED BY HEPCIDIN

Sarah Wilkins, Deepak Darshan, David Frazer, Greg Anderson
Queensland Institute of Medical Research
(Presented By: Sarah Wilkins)

Introduction: Intestinal iron absorption is exceptionally high in suckling mammals. Our previous studies in rats suggest that iron absorption during the suckling period is refractory to stimuli that would normally reduce absorption in weaned animals. In adults, this down-regulation is mediated by an increase in the expression of hepcidin, which binds to the iron export protein ferroportin1, causing its degradation. To better understand the regulation of iron absorption during the suckling period, we

used mice to extend our previous studies and characterised the relationship between hepdicin, ferroportin1 and iron absorption during this crucial stage of life.
Methods: We treated 15 and 25 day old mice with either 300mg/kg iron dextran or 0.1mg/kg LPS and studied them 4 days or 10 hours later respectively. Blood was collected for serum iron analysis, and liver tissue and duodenal enterocytes were collected for gene and protein expression profiles. Intestinal iron absorption was measured by assessing the whole body retention of oral 59Fe. To determine whether ferroportin1 was involved in iron absorption during the suckling period, absorption was measured in intestine-specific ferroportin1 knockout mice. The ability of hepcidin protein to affect ferroportin1 activity was examined by administering synthetic hepcidin (1µg/g body weight) intraperitoneally to 15 and 25 day old mice. The animals were sacrificed 24 hours later and the duodenum was examined for ferroportin1 expression by western blotting and immunofluorescence.
Results: In agreement with our previous studies in rats, there was very little change in absorption in 15 day old mice injected with either iron dextran or LPS, with the absorption of a dose of 59Fe remaining above 74% in all cases. This was despite a large increase in the expression of hepatic *hepcidin* mRNA. However, absorption was decreased significantly (to less than 20% of littermate control levels) in 15 day old ferroportin1 knockout mice, indicating that this iron export protein is responsible for the majority of the iron absorbed at this time. The injection of synthetic hepcidin supported these results with no decrease in the expression of ferroportin1 protein in 15 day old mice and a significant decrease in 25 day old animals. Immunofluorescent localisation of ferroportin1 showed that the protein localised to the basolateral membrane of duodenal enterocytes in both 15 and 25 day old mice. Basolateral expression of ferroportin1 decreased in response to hepcidin administration in 25 day old animals but not in 15 day old mice.
Discussion and Conclusions: These data demonstrate that the high iron absorption that occurs during the suckling period is mediated by ferroportin 1. However, ferroportin1 is not responsive to hepcidin mediated degradation at this time, despite being expressed on the basolateral membrane. This suggests that alterations to ferroportin1 that prevent hepcidin binding and/or changes to the ferroportin1 degradation pathway during suckling allow iron absorption to remain high regardless of hepcidin expression levels.

Poster #10
CHARACTERIZATION OF FERRITIN IN THE HEPATIC CIRCULATION
Jeffrey Cooper, MD[2], Feng Qi, MD, Abdul Waheed, PhD and Robert Fleming[1]
[1]Saint Louis University School of Medicine; [2]Saint Louis University/Pediatrics
(Presented By: Robert Fleming)

Introduction: Iron signaling to hepcidin is mediated by signals reflecting circulating iron and tissue iron stores. Evidence supports a role for ferritransferrin in mediating the circulatory iron signal. The mechanism through which tissue iron stores regulates hepcidin remains unclear; however liver hepcidin expression correlates with liver iron concentrations (LIC), liver ferritin (Ft) levels, and circulating ferritin concentrations. While ferritin in the systemic circulation is iron-deplete, ferritin in the hepatic circulation has not been characterized. We characterized transferrin and ferritin iron content in serum samples obtained from hepatic, portal, and systemic circulations of mice with variation in dietary iron intake, and measured associated changes in LIC, and liver hepcidin and Bmp6 expression.
Design/Methods: Adult mice were placed on diets containing <20 ppm, 200 ppm, or 25,000 ppm iron for 1 week. Mice were anesthetized and blood obtained from the systemic circulation (cardiac puncture), hepatic vein, or portal vein. Serum samples and tissue (liver, duodenum) homogenates underwent native PAGE followed by enhanced Perls' staining for iron and/or immunoblotting. Samples also underwent SDS-PAGE followed by immunoblotting. Immunoblots were reacted with antibodies to H and L ferritins. Liver hepcidin and Bmp6 expression was measured by real-time RT-PCR.
Results: Ferritin from duodenum was predominantly H-type, while that from liver tissue was predominantly L-type. Ferritin from the serum regardless of source was L-type, but with a migration pattern in native PAGE distinct from liver tissue ferritin. The concentration of iron-replete ferritin was greater in the intrahepatic than portal serum. The concentrations of iron-replete ferritin in the intrahepatic circulation were increased with dietary iron loading, in association with LIC and liver hepcidin and Bmp6 expression.
Conclusion: The concentration of iron-replete ferritin in the intrahepatic circulation varies with dietary iron intake, and is associated with LIC and hepcidin expression. These observations indicate that iron-replete ferritin is released into the hepatic circulation where it could potentially contribute to iron redistribution and/or participate in local signaling of iron stores in the regulation of hepcidin.

Poster #11

IRON ACTIVATION OF HEPCIDIN IN HEMOJUVELIN KNOCKOUT MICE PREFERENTIALLY TARGETS SPLENIC BUT NOT INTESTINAL FERROPORTIN

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Lady Davis Institute for Medical Research and McGill University
(Presented By: Kostas Pantopoulos)

Introduction: Hemojuvelin (Hjv) is a bone morphogenetic protein (BMP) co-receptor involved in the control of systemic iron homeostasis. Functional inactivation of Hjv leads to severe iron overload in humans and mice due to marked suppression of the iron-regulatory hormone hepcidin.
Methods and Materials: To elucidate the role of Hjv in iron-sensing pathways, Hjv-/- mice were backcrossed in pure C57BL/6 background and analyzed for their responses to dietary iron manipulations.
Results: The animals developed iron overload under all regimens. Transferrin (Tf) was highly saturated regardless of the dietary iron content, while liver iron deposition was proportional to it. Surprisingly, hepcidin mRNA expression responded to dietary iron intake. Qualitatively, irondependent regulation of hepcidin in Hjv-/- mice was similar to isogenic wild type controls, but more than an order of magnitude lower. Iron signaling via the BMP/Smad pathway was preserved but substantially attenuated. The intestinal iron transporters DMT1 and ferroportin were overexpressed. While DMT1 was highly regulated by iron, ferroportin responded to iron only in the spleen but not the duodenum.
Conclusion: Our data demonstrate that Hjv is dispensable for iron sensing and rather acts as an enhancer for hepcidin expression. In addition, they suggest a crucial contribution of duodenal DMT1 in iron overload under conditions of Hjv deficiency, and indicate the existence of tissue-specific mechanisms for regulation of ferroportin.

Poster #12

HEPCIDIN TRANSCRIPTION AND PEPTIDE SECRETION ARE GOVERNED BY DIFFERENT REGULATORY MECHANISMS

Kosha Mehta, PhD[2]; Vinood Patel, PhD[2]; Mark Busbridge, PhD[3]; Derek Renshaw, PhD[2]; Mohammed Zariwala, PhD[2], Robert Evans, PhD[4]; Sebastien Farnaud, PhD[1]
[1]University of Bedfordshire; [2]University of Westminster; [3]Imperial College; [4]University of Brunel
(Presented By: Sebastien Farnaud)

Introduction: The role of hepcidin in maintaining systemic iron homeostasis in human and animal models has been described whereby an increase in body iron level leads to an increase in hepcidin expression. The expected similar mechanism has been shown in primary cells, but could not be replicated consistently in immortal cells. In vitro studies with HepG2 cell lines have shown an unexpected inverse relationship between iron increase and Hamp mRNA expression. The differences in iron sensing between hepatoma cell lines and human primary hepatocytes have been studied and proposed elsewhere, but in the present work the experimental challenge is being revisited.
Methods and Materials: In the present study, the experimental conditions have been redefined and unlike previous studies, levels of hepcidin levels were measured pre- and post-translationally.
Results: Results showed an increase in both Hamp mRNA expression and hepcidin peptide secretion within the first 4h of diferric human transferrin treatment, after which the increased peptide secretion was maintained whereas a decrease was observed for Hamp mRNA expression. The simplicity of the experimental set up, together with the gradual decrease in hepcidin peptide levels observed in the absence of diferric transferrin, suggests that in this instance, the increase in hepcidin expression is independent of FCS or other external parameters and can be attributed solely to the overdose of diferric human transferrin.
Conclusion: However, the different variations observed between the Hamp mRNA expression and the corresponding hepcidin peptide expression, with a continuous increase in peptide secretion but a gradual decrease in RNA expression after 4h, suggests the existence of two different regulatory mechanisms.

Poster #13

IMPROVED MASS SPECTROMETRY ASSAY FOR PLASMA HEPCIDIN: DETECTION OF A NOVEL HEPCIDIN ISOFORM AND ITS CLINICAL CONSEQUENCES

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[1]Radboud University Medical Center; [2]Pieris AG, Freising, Germany
(Presented By: Dorine Swinkels)

Introduction: Hepcidin-25 is a hepatocyte-produced peptide hormone that regulates systemic iron homeostasis. Under physiological conditions N-terminal truncated hepcidin-20 and -22 peptides have been observed in the urine, but not or at low concentrations, in plasma. These smaller hepcidin isoforms mostly occur in plasma in diseases that are associated with increased hepcidin concentrations, such as sepsis and kidney failure (KF).

Methods and Materials: Thus far we and others measured hepcidin using a Weak Cation Exchange Time-of-flight Mass Spectrometry (WCX-TOF-MS) method, with the synthetic hepcidin-24 analogue spiked into the sample as an internal standard for quantification. We updated this assay by replacing this internal standard by a stable isotope heavy hepcidin-25 (+41 Da). Intra-run CV's (n=8) of hepcidin-25, 24, 22 and 20 were 2.2-3.5%, 2.9-8.2%, 7.9-8.6% and 4.2-7.6%, respectively. Inter-run CV's (n=8) were 6.4%, 16.8%, 11.6% and 13.7%, respectively. This hepcidin assay improvement was clinically evaluated using samples from various patient groups. Its relevance was underscored for fresh samples from KF patients by the observations of a peak with low intensity at m/z 2673.9 that resembles the theoretical mass of hepcidin-24 with four intramolecular disulphide bridges. The identity of this peak was confirmed by its specific disappearance from the mass spectrum by pre-incubation of these plasma samples with anti-hepcidin molecules.
Results: Consequently, the use of heavy hepcidin-25 as internal standard in samples from KF patients, revealed slightly higher hepcidin-25 concentrations compared to those obtained with hepcidin-24 as an internal standard. Next, we assessed whether the observed hepcidin-24, -22 and -20 isoforms could be derived from N-terminal processing of hepcidin-25 in the circulation. In plasma samples from intensive care patients (n=10) that were kept at room temperature for 1 and 7 days, hepcidin-25 gradually decreased (mean: -12% and– 52%, respectively), whereas the smaller hepcidin isoforms increased during this period (hep24, +22% and +13%; hep22, +25% and +42%; hep20, +59% and +75%). Plasma hepcidin-25 processing was highly sample dependent, could partly be blocked by protease inhibitors, and did not occur at -20°C. Finally, we quantified the bioactivity of synthetic hepcidin-24 relative to hepcidin-25,- 20 and -22 (all purchased from Peptides International) by assessing their ability to internalize and degrade green fluorescent protein-fused ferroportin in a cell-based assay. The EC50 for hepcidin-24 was 10- fold higher than that of hepcidin-25. The activities of the shorter hepcidin-22 and -20 isoforms was even lower than that of hepcidin-24. These findings indicate that at relative physiological concentrations the smaller hepcidin isoforms are unlikely to contribute to hepcidin bioactivity in iron metabolism.
Conclusion: In conclusion, we here report the existence of hepcidin-24, and show that this and other hepcidin isoforms can originate from N-terminal processing of circulating hepcidin-25. This phenomenon may be important in patients with increased hepcidin-25 levels and impaired renal clearance as an alternative system to reduce its activity on ferroportin. Our current improved insights in pre-analytical sample handling and the implementation of the hepcidin-25 isotope as internal standard will further improve our understanding of circulating hepcidin-25 and its isoforms and as such pave the way towards further optimization and standardization of plasma hepcidin assays.

Poster #14
NEOGENIN INTERACTS WITH MATRIPTASE-2 TO FACILITATE HEMOJUVELIN CLEAVAGE
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(Presented By: An-Sheng Zhang)

Introduction: Hemojuvelin (HJV) and matriptase-2 (MT2) are co-expressed in hepatocytes, and both are essential for systemic iron homeostasis. HJV is a glycosylphosphatidylinositol-linked membrane protein that acts as a co-receptor for bone morphogenetic proteins to induce hepcidin expression. MT2 regulates the levels of membrane-bound HJV in hepatocytes by binding to and cleaving HJV into an inactive soluble form that is released from cells. HJV also interacts with neogenin, a ubiquitously expressed transmembrane protein with multiple functions.
Methods and Materials: In the present study, we showed that neogenin interacted with MT2 as well as with HJV and facilitated the cleavage of HJV by MT2. In contrast, neogenin was not cleaved by MT2, indicating some degree of specificity by MT2. Down-regulation of neogenin with siRNA increased the amount of MT2 and HJV on the plasma membrane, suggesting a lack of neogenin involvement in their trafficking to the cell surface. The increase in MT2 and HJV upon neogenin knockdown was likely due to the inhibition of cell surface MT2 and HJV internalization.
Results: Analysis of the Asn-linked oligosaccharides showed that MT2 cleavage of cell surface HJV was coupled to a transition from high mannose oligosaccharides to complex oligosaccharides on HJV.
Conclusion: These results suggest that neogenin forms a ternary complex with both MT2 and HJV at the plasma membrane. The complex facilitates HJV cleavage by MT2, and release of the cleaved HJV from the cell occurs after a retrograde trafficking through the TGN/Golgi compartments.

Poster #15
DIFFERENCES IN ACTIVATION OF MOUSE HEPCIDIN BY DIETARY IRON AND PARENTERALLY ADMINISTERED IRON DEXTRAN: COMPARTMENTALIZATION IS CRITICAL FOR IRON SENSING
Alina Daba, MD, PhD; Konstantinos Gkouvatsos, MD, PhD; Giada Sebastiani, MD; Kostas Pantopoulos
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(Presented By: Kostas Pantopoulos)

Introduction: The iron regulatory hormone hepcidin responds to both oral and parenteral iron. Here, we hypothesized that the diverse iron trafficking routes may affect the dynamics and kinetics of the hepcidin activation pathway.
Methods and Materials: To address this, C57BL/6 mice were administered an iron-enriched diet or injected i.p. with iron dextran and analyzed over time. After one week of dietary loading with carbonyl iron, mice exhibited significant increases in

serum iron and transferrin saturation, as well as in hepatic iron, Smad1/5/8 phosphorylation and bone morphogenetic protein 6 (BMP6) and hepcidin mRNAs.
Results: Nevertheless, hepcidin expression reached a plateau afterwards, possibly due to upregulation of inhibitory Smad7, Id1 and matriptase-2 mRNAs, while hepatic and splenic iron continued to accumulate over 9-weeks. One day following parenteral administration of iron dextran, mice manifested elevated serum and hepatic iron levels and Smad1/5/8 phosphorylation, but no increases in transferrin saturation or BMP6 mRNA. Surprisingly, hepcidin failed to appropriately respond to acute overload with iron dextran, and a delayed (after 5-7 days) hepcidin upregulation correlated with increased transferrin saturation, partial relocation of iron from macrophages to hepatocytes and induction of BMP6 mRNA.
Conclusion: Our data suggest that the physiological hepcidin response is saturable, and are consistent with the idea that hepcidin senses exclusively iron compartmentalized within circulating transferrin and/or hepatocytes.

Poster #16
EFFECT ON HFE GENOTYPE ON NATIVE CIRCULATING HEPCIDIN-25 IN MEN AGED 30-52 YEARS
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(Presented By: Palle Lyngie Pedersen)

Introduction: Hepcidin is a small multifunctional peptide involved in regulation of iron uptake as well as being a part of the antimicrobial defense system. Hence, regulation of Hepcidin gene expression includes a wide range of stimulators from the iron metabolism as well as the inflammatory system.
Aim: Here we evaluate the effects of both hereditary hemochromatosis (HFE) genotype and inflammatory regulation on circulating serum Hepcidin. In addition we present a new novel HPLC-MS/MS method to detect serum Hepcidin-25 with intact cysteine disulphide bonds.
Methods: All male residents in Næstved and Vordingborg cities, Zealand, Denmark, aged 30-50 years (n = 10,993) were invited to a two-step screening for HFE. Firstly, all participants were screened for common HFE gene variants (C282Y, H63D, S65C). Secondly, all participants with HFE gene variants and a wild type control group were invited for a biochemical screening for iron overload. In total of 6,020 complete HFE genotypes were obtained and biochemical data was available in 1,453 (24 %) of which additional data were available for 1,295 (22 %) individuals who had completed a comprehensive questionnaire. The Hepcidin assay is based on detection and quantification of serum Hepcidin with intact cystein bonds relative to that of a stable isotope labeled Hepcidin internal standard with a known concentration. Hepcidin was measured on a HPLC MS/MS (Waters Acquity – Quattro Micro MS/MS) instrument. Data presented in this abstract include the first 281 measurements of Hepcidin. The hepcidin-25 method is linear over the concentration range 0.5 – 500 nmol/L. The LOQ was set to 0.5 nmol/L and using QA samples (6.8 nmol/L) over three months a total variation of 11% was assessed. Serum iron, transferrin and ferritin was measured using Dimension® RxL Clinical Chemistry System with heterogeneous Immunoassay Module (Siemens) Interleukin-8 (IL-8), interleukin-1β, interleukin-6, interleukin-10, tumor necrosis factor, and interleukin-12p70 were determined using the BD Cytometric Bead Array (CBA) Human Inflammatory Cytokines Kit on a FACS Canto II flow cytometer (BD Bioscience).
Results: HFE genotype have no influence on serum Hepcidin levels (Kruskal Wallis test, p=0.95). However, pro-inflammation, judged by increased serum IL-8 seem to elevate serum Hepcidin (IL-8 > 15 pg/l, r=0.32, p=0.02).
Conclusion: We will present a novel method for detection of Hepcidin-25 with intact cysteine disulphide bonds. HFE have been proposed to have a contributory role in Hepcidin response to iron intake. The aim of this study was to evaluate whether HFE genotype contribute to the Hepcidin expression. This cannot be confirmed in the 281 preliminary men investigated.

Poster #17
ENHANCED HEPCIDIN EXPRESSION IN THE RAT DUODENUM AND THE ROLE OF IRON IN EXPERIMENTAL DUODENAL ULCERATION
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(Presented By: Gordon McLaren)

Introduction: We recently demonstrated that an increased duodenal iron concentration potentiates carrier-mediated uptake of the duodenal ulcerogen cysteamine and that co-localization of iron and cysteamine create conditions favorable to oxidative stress reactions and duodenal ulceration (Arch Biochem Biophys 2012;525:60-70). We hypothesized that an enhanced iron concentration in the proximal duodenum during ulcer formation may affect hepcidin expression as a regulator of duodenal iron absorption. Several pathways are involved in the regulation of hepcidin expression. IL-6 induces hepcidin upregulation via induction of STAT3 phosphorylation. We previously demonstrated cysteamine-induced STAT3 phosphorylation in rat duodenum (Gastroenterology 2009;136:A23). Hepcidin expression is also controlled by activation of BMP signaling through the SMAD1/5/8 pathway.

Methods: Sprague-Dawley rats were gavaged with water or cysteamine-HCl (25 mg/100g x 3 at 4 h intervals) and euthanized 0.5, 2, 6, 12, or 24 h later. Groups of rats were also treated with a second ulcerogen, mepirizole (40 mg/100g in 1% methylcellulose subcutaneously). The expression of hepcidin, IL-6, IL-1α and BMP6 in duodenum was measured by real-time PCR. Western blot assays were used for detection of SMAD5 and phosphorylated SMAD1,5,8. IL-6 in serum was quantified by ELISA.
Results: The level of hepcidin mRNA in control rat duodenum was low but cysteamine administration at 2, 12 and 24 h markedly increased hepcidin mRNA expression by 6.3-, 11.9- and 43.3-fold, respectively. Mepirizole administration at 2, 12 and 24 h upregulated hepcidin mRNA expression by 6.0-, 19.0- and 35.5-fold, respectively. Hepcidin mRNA in liver was also enhanced, reaching a maximum of 3.2-fold 6 h after cysteamine treatment. IL-6 was not detectable in control rat blood and up to 12 h after cysteamine administration but became detectable at 24 h when duodenal ulcers were almost formed. IL-6 mRNA was not detectable in rat duodenal mucosa up to 12 h after cysteamine treatment but was markedly elevated at 24 h. IL-1α mRNA expression was almost undetectable in control duodenum but was elevated after cysteamine administration to 7.5-, 45- and 169-fold at 2, 12 and 24 h, respectively. Cysteamine did not affect duodenal BMP6 mRNA expression. Western blot assays demonstrated that cysteamine administration elevated SMAD5 protein levels and induced phosphorylation of SMAD proteins in the rat proximal duodenum in a time-dependent manner.
Conclusions: Our results demonstrate hepcidin expression in duodenal enterocytes. The duodenal ulcerogens cysteamine and mepirizole induced hepcidin expression in the rat proximal duodenum with involvement of STAT3 phosphorylation and activation of BMP signaling through the SMAD1/5/8 pathway. These molecular changes appear to represent newly-discovered mechanisms of tissue-specific injury involving iron and leading to duodenal ulceration

Poster #18
THE HEPCIDIN/FERROPORTIN REGULATORY CIRCUITRY IN LUNG (PATHO)PHYSIOLOGY
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(Presented By: Joana Neves)

Introduction: The lung is hallmarked by a thin epithelial layer and high vascularization to allow for gas exchange from the atmosphere into the blood stream. In addition, it exerts critical functions in host defense at the interface between environment and body. As all other organs, the lung needs to prevent excess of iron which causes cellular damage due to the generation of reactive oxygen species (ROS). The risk for oxidative damage in the lung is exacerbated by its continuous exposure to high oxygen levels. To avoid oxidative damage, iron is detoxified within epithelial cells. Deregulation of lung iron homeostasis by either endogenous or exogenous factors can cause tissue injury and contribute to pathogenesis. Furthermore, increased availability of iron as a nutrient for pathogens promotes pulmonary infections and subsequent inflammation.
Methods and Materials: To study the consequences of unbalanced iron homeostasis in the lung, work is in progress to analyze a mouse model of non-classical ferroportin disease hallmarked by a disrupted hepcidin/ferroportin regulatory circuitry and severe systemic iron overload.
Results: Biochemical and histological analysis of the murine lung revealed an increase in pulmonary iron content that is restricted to defined pulmonary cell types, demonstrating that the disruption of hepcidin binding to ferroportin affects the pulmonary iron content.
Conclusion: Experiments are ongoing to (1) identify the pulmonary cell types affected by iron overload, (2) identify pathological effects of iron accumulation (e.g. markers for emphysema or fibrosis) and (3) analyze if iron levels in alveolar macrophages are altered and whether this may affect cytokine expression and neutrophil recruitment to the bronchoalveolar space in response to inflammation.
Supported by the Bundesministerium für Bildung und Forschung, BMBF (DZL 82DZL00401)

Poster #19
SEX HORMONES DIFFERENTLY REGULATE HEPCIDIN EXPRESSION AND IRON HOMEOSTASIS IN VIVO
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(Presented By: Elena Corradini)

Introduction: Hepcidin is the central regulator of iron homeostasis. Synthesized mainly by the liver, it acts by binding ferroportin, the sole iron exporter present at the surface of duodenal enterocytes, macrophages, placental cells, and hepatocytes, leading to its degradation. Thereby, when hepcidin levels increase iron is trapped into cells and iron influx from the gut is reduced, whereas when hepcidin decreases, iron is released from cells to the bloodstream and dietary iron absorption increases. We and others have shown that BMP(6)-SMAD signaling is a main regulatory pathway acting in hepcidin regulation and iron homeostasis. Several stimuli are known to control hepcidin expression in the liver: iron levels, inflammation, ER stress, erythropoietic needs, and hypoxia. Sex hormones influence erythropoiesis, but the underlying mechanisms remain poorly understood. Recent work in humans suggests that higher testosterone levels are associated with reduced circulating hepcidin, indicating that hepcidin may be involved in the androgenic stimulation of erythropoiesis through changes in bone marrow iron supply. On the other hand, data from in vitro and animal studies on the effects of sex hormones

on hepcidin transcription and iron homeostasis are controversial. Based on these premises, the aim of this work is to study the effects of sex hormones (androgens and estrogens) on the regulation of hepcidin expression and systemic iron status in vivo.
Methods and Materials: Male and female C57BL/6 wildtype mice underwent orchiectomy or ovariectomy respectively. Six weeks after surgery, orchiectomized mice showed higher hepcidin (Hamp), Bmp6 and Id1 (a target gene of BMPs) mRNA expression in the liver, and lower liver iron content (LIC) in comparison to sham-operated male mice. In contrast, ovariectomy did not change Hamp, Bmp6, Id1 mRNA expression, and LIC. Furthermore, C57BL/6 male wildtype mice were treated with subcutaneous injections of androgens once a day for three consecutive days, a treatment schedule that did not appreciably affect red blood cell parameters.
Results: Testosterone propionate administration was associated with a 50% decrease of Hamp mRNA levels and with a trend towards lower Id1 expression in the liver, with consequent significant spleen iron depletion, while hepatic Bmp6 mRNA was unchanged. Administration of dihydrotestosterone, which cannot be aromatized into estradiol, elicited the same effects as testosterone propionate, although to a minor extent.
Conclusion: Collectively, our data suggest that male sex hormones influence hepatic hepcidin expression and BMP6-SMAD signaling pathway activation, and may act on hepcidin transcription also through a direct inhibitory mechanism without significant changes in red blood cell parameters. Further studies are ongoing to dissect the mechanistic aspects underlying the inhibitory effect of testosterone on hepcidin transcription.

Poster #20
IDENTIFICATION AND CHARACTERIZATION OF A NOVEL MURINE ALLELE OF TMPRSS6
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(Presented By: Tom Bartnikas)

Background: Mutagenesis screens can establish mouse models of utility for the study of critical biological processes such as iron metabolism. Such screens can produce mutations in novel genes or the establishment of novel alleles of known genes, both of which can be useful tools for study.
Design and Methods: To identify genes of relevance to hematologic as well as other phenotypes, we performed N-ethyl-N-nitrosourea mutagenesis in C57BL/6J mice. An anemic mouse was identified and a putative mutation was characterized by mapping, sequencing and in vitro activity analysis. The mouse strain was backcrossed for ten generations then phenotypically characterized with respect to a previously established null mouse strain. Potential modifying loci were identified by quantitative trait locus (QTL) analysis.
Results: Mapping and sequencing in an anemic mouse termed hem8 identified an I286F substitution in Tmprss6, a serine protease essential for iron metabolism; this substitution impaired in vitro protease activity. After backcrossing to C57BL6/J for ten generations, the hem8-/- strain exhibited a phenotype similar in some but not all aspects to Tmprss6-/- mice. The hem8 and Tmprss6-null mutations were allelic. Both hem8-/- and Tmprss6-/- mice responded similarly to pharmacologic modulators of bone morphogenetic protein signaling, a key regulator of iron metabolism. QTL analysis in the hem8 strain identified potential modifying loci on chromosomes 2, 4, 7 and 10.
Conclusions: The hem8 mouse is a novel allele of Tmprss6. Potential uses for this strain in the study of iron metabolism are discussed.

Poster #21
ISOLATED HEPATIC IRON DEFICIENCY DESPITE ABUNDANT SYSTEMIC IRON IN MDR2-/- MICE: INTEGRITY OF THE BILIARY TRANSPORT SYSTEM IS IMPORTANT IN LIVER IRON HOMEOSTASIS
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(Presented By: Amy Sobbe)

Introduction: Hepatic iron accumulation occurs in up to sixty per cent of patients with advanced hepatocellular liver disease and is related to reduced hepcidin synthesis. However, iron accumulation in liver diseases of biliary origin is very uncommon and occurs in less than eight per cent of affected subjects[1]. In order to explain this observation, we postulated that cholestasis alters hepatic iron metabolism and we investigated iron homeostasis in *Mdr2*$^{-/-}$ mice, an animal model of cholestasis and progressive hepatic fibrosis.
Methods: Wild type and *Mdr2*$^{-/-}$ mice were studied at 3, 5, 8, 12 and 16 weeks of age. ^{59}Fe absorption was measured in additional 5 week old animals. Further wild type and *Mdr2*$^{-/-}$ mice were challenged with either an iron deficient diet from 3-9 weeks of age or a 1% carbonyl iron diet from 5-9 weeks of age. Serum iron indices were measured and full blood counts were performed. Liver and spleen sections were stained with Perls' Prussian Blue and hepatic and splenic iron concentrations were determined. qRT-PCR and Western immunoblotting were used to assess the expression of iron-regulatory genes and proteins respectively.

Results: Serum iron and total iron-binding capacity were approximately 2-fold higher in *Mdr2*$^{-/-}$ mice than in wild types from 3 to 16 weeks of age. Conversely, *Mdr2*$^{-/-}$ mice had a significantly lower hepatic iron concentration (HIC) than wild types, and remaining iron was localized in reticuloendothelial cells. Similar age-dependent increases in stainable splenic iron were seen in wild type and *Mdr2*$^{-/-}$ mice with no significant differences between genotypes at the same age. *Hamp1* and prohepcidin expression were lower in *Mdr2*$^{-/-}$ mice (relative prohepcidin expression: 3wk 131±29 vs 37±3, 8wk 166±21 vs 72±18, 16wk 189±7 vs 118±5 for wild type and *Mdr2*$^{-/-}$ respectively) and 59Fe absorption was higher in *Mdr2-/-* mice at 5 weeks of age (P=0.04). Hepatic transferrin receptor 1 (TFR1) protein levels were increased 4- and 8-fold in *Mdr2-/-* mice at 8 and 16 weeks respectively. Hepatic iron accumulation was attenuated in *Mdr2*$^{-/-}$ mice fed 1% carbonyl iron (HIC: wild type 57±4 vs *Mdr2*$^{-/-}$ 23±2 µmol Fe/g dry wt) despite 3- fold higher serum iron than in wild types. Wild type and *Mdr2-/-* mice fed an iron-deficient diet had similar hepatic iron concentrations (wild type: 2.1±0.1 vs *Mdr2-/-*: 2.2±0.1 µmol Fe/g dry wt).
Discussion and Conclusions: *Mdr2*$^{-/-}$ mice have reduced hepatic iron stores despite lower *Hamp1* and prohepcidin expression, increased duodenal iron absorption, abundant circulating iron and increased hepatic TFR1 expression. *Mdr2*$^{-/-}$ mice were resistant to hepatic iron accumulation when challenged with an iron-loaded diet. These data suggest impaired hepatocyte uptake of iron in *Mdr2*$^{-/-}$ mice. This work may explain the paucity of iron loading in biliary cirrhosis and suggests that iron homeostasis depends upon an appropriately functioning biliary system.
Reference: 1. Stuart KA et al. Hepatology 2000;32(6):1200-7.

Poster #22
INCREASED HEPCIDIN MRNA LEVELS AND REDUCTION OF MEMBRANE HEMOJUVELIN IN TMPRSS6 -/- PRIMARY HEPATOCYTES
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(Presented By: Anne Lenoir)

Introduction: Tmprss6, encoding the liver specific serine protease matriptase-2 (MT2), has recently been shown as an important regulator of hepcidin expression. Mutations found in this gene have been linked to familial cases of Iron-Refractory Iron Deficiency Anemia (IRIDA), with increased levels of hepcidin leading to iron deficiency anemia. *Ex vivo* experiments led to the hypothesis that MT2 inhibits hepcidin expression by proteolytic cleavage of hemojuvelin (HJV) and thus down-regulation of the BMP/SMAD pathway (Silvestri *et al*., 2008). Further studies on double knockout (KO) mice (Hjv and Tmprss6, Truksa *et al*., 2009 ; Finberg *et al*., 2010; Bmp6 and Tmprss6, Lenoir *et al*., 2011) showing that downregulation of BMP/SMAD signaling by MT2 is required for regulation of hepcidin expression and systemic iron homeostasis, have supported this hypothesis. However, recently Krijt *et al.* (2011) demonstrated that, unexpectedly, membrane HJV is decreased in the liver of Tmprss6 KO mice. Due to these *in vitro* and *in vivo* discrepancies, the molecular mechanism of MT2-induced hepcidin repression remains puzzling.
Methods and Materials: In this study, we worked on isolated primary hepatocytes from MT2 KO mice and WT littermates, to bypass systemic effects which could affect hepcidin expression *in vivo.* Primary hepatocytes were isolated from MT2 KO and WT mice using collagenase and cultured for 48 hours. Hepcidin and Id1 (another transcriptional target of BMP/HJV/SMAD pathway) expression were analyzed by real time PCR. HJV content was analyzed using real time PCR and western blot on membrane protein fraction. Furthermore, the activity of HAMP promoter was assessed by luciferase assay, in hepatocytes transfected with plasmids containing firefly luciferase under control of HAMP promoters carrying proximal (0,9kb) or both proximal and distal (2,7kb) BMP-RE. Hepatocytes were also transfected with firefly luciferase under control of a promoter containing the BMP-RE of Id1 promoter, to follow the activation of the BMP/HJV/SMAD pathway.
Results: We found that 48 hours after plating, hepcidin mRNA levels are 10 to 15 times more important in MT2 KO compared to WT hepatocytes, demonstrating a cell autonomous increase of hepcidin expression in KO hepatocytes. Hepcidin overexpression is associated with reduced membrane HJV, as found in total liver from MT2 KO mice. Id1 mRNA levels are also significantly increased in KO hepatocytes. However, using a luciferase assay on transfected hepatocytes (yield of transfection is 10-15%), we find that HAMP promoter and Id1 BMP-RE are not activated at basal state in MT2 KO isolated hepatocytes, and are only partially responsive to BMP6 as compared to WT hepatocytes.
Conclusion: In conclusion, hepcidin overexpression is maintained in MT2 KO compared to WT primary hepatocytes, with a fold of increase higher than the one observed in total liver, suggesting a systemic regulation occurring *in vivo* to avoid an excess of hepcidin-induced iron deficiency. Membrane HJV protein content is strongly decreased in MT2 KO isolated hepatocytes, demonstrating that this decrease is due to MT2 deficiency *per se*. MT2 might also have an effect on post-transcriptional level to regulate hepcidin mRNA levels.

Poster #23

LIVER HEMOJUVELIN AND TRANSFERRIN RECEPTOR 2 PROTEIN CONTENT IN MATRIPTASE-2-DEFICIENT MASK MICE

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(Presented By: Jan Krijt)

Introduction: The serine protease matriptase-2, encoded by the *Tmprss6* gene, is a negative regulator of hepcidin expression. *In vitro*, matriptase-2 has been shown to cleave hemojuvelin protein. *In vivo*, however, there is so far no evidence for matriptase-2-mediated hemojuvelin cleavage, as liver hemojuvelin protein levels are decreased, rather than increased, in *Tmprss6*-mutant mice. Thus, the *in vivo* target of matriptase-2 still remains to be identified. The aim of the present study was to confirm the decrease of hemojuvelin protein in the livers of *mask* mice, which lack the proteolytic domain of matriptase-2, and to determine the possible effect of matriptase-2 deficiency on other membrane proteins participating in iron metabolism.
Methods: *Mask* mice livers and wild-type controls were kindly provided by Dr. Pauline Lee and Dr. Xin Du, La Jolla, CA, USA. Microsomal fraction was obtained by ultracentrifugation and proteins were determined by immunoblotting under reducing conditions. *Id1* mRNA content was determined by real-time PCR.
Results: Confirming our previous data obtained in another strain of *Tmprss6*-mutant mice, hemojuvelin protein content in *mask* mice was markedly decreased. The decrease in hemojuvelin was observed in the microsomal fraction, as well as in whole liver homogenates. Transferrin receptor 2 (Tfr2) protein content and neogenin protein content was slightly decreased. Levels of the type I bone morphogenetic protein receptor Alk3 (Bmpr1a) and the type II receptor Acvr2a were unchanged. Attempts to determine Hfe and Bmp6 protein content were unsuccessful, levels of Bmp2 and Bmp4 were unchanged. Liver *Id1* mRNA content was increased in *mask* mice.
Discussion: Lack of matriptase-2 activity results in iron deficiency anemia, suggesting that matriptase-2 cleaves some protein which stimulates hepcidin expression. An obvious target for matriptase-2 would be the Bmp6/Smad pathway, of which hemojuvelin is an important component. This concept is further strengthened by increased expression of *Id1*, as Id1 is a known target of the Bmp6/Smad pathway. However, the observed decrease of hemojuvelin protein content in the livers of *mask* mice apparently argues against hemojuvelin as a direct substrate of matriptase-2, although, on the other hand, it indicates some specific interaction between the two proteins. In addition to the Bmp6/Smad pathway, hepcidin expression is also controlled by other proteins, such a Hfe, Tfr2 and possibly neogenin. The observed slight decrease of Tfr2 and neogenin protein levels in *mask* mice confirms that Tfr2 and neogenin are not physiological substrates for matriptase-2. Tfr2 protein levels are reportedly influenced by plasma iron levels, so the observed decrease in Tfr2 protein could possibly reflect the iron-deficient status of *mask* mice.
Conclusion: The presented study confirms decreased levels of hemojuvelin protein in matriptase-2-mutant mice, which suggests that, *in vivo*, matriptase-2 could actually target other proteins than the hepatocyte membrane components of the Bmp6/Smad pathway.

Poster #24

IMPLICATION OF THE TRANSCRIPTION FACTOR NRF2 IN THE IRON MEDIATED UPREGULATION OF THE IRON EXPORTER FERROPORTIN IN LIPID RAFT FROM MACROPHAGES

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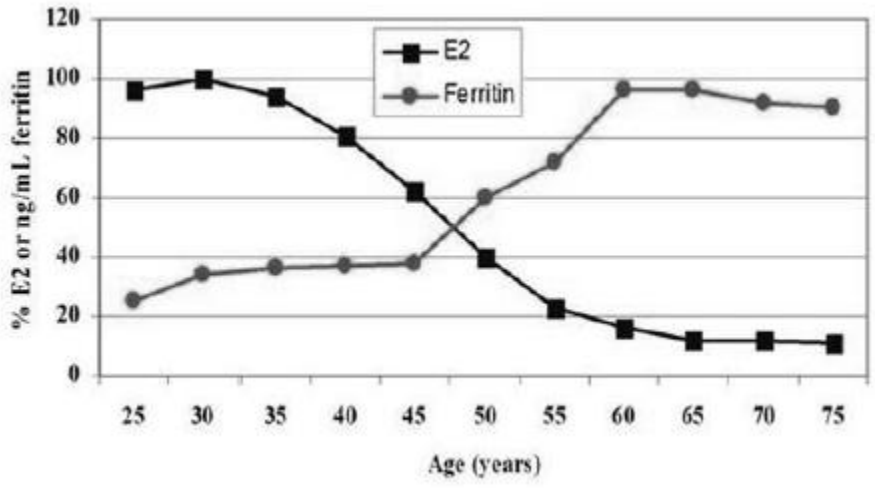
INSERM U1043
(Presented By: Canonne-Hergaux François)

Introduction: Macrophages play a key role in iron homeostasis through the recycling of heme iron from red blood cells during the process of erythrophagocytosis. This process involves the only known mammalian iron exporter ferroportin (FPN) that is expressed at the cell surface of macrophages. Upon iron treatment, FPN is strongly expressed in lipid rafts at the cell surface of macrophages.
Methods and Materials: In order to gain insight into this regulation, we studied the changes of lipid raft proteome of macrophages after iron-NTA treatment using detergent−resistant (Triton X−100) membranes (DRM) containing Fpn, iTRAQ labeling and LC−MS/MS. Using ProteinPilot analyses, the resulting spectra were used to identify and to quantify the proteins present in the sample.
Results: We identified 66 proteins showing more than a 5−fold increased expression in DRM after Fe−NTA treatment. Among those proteins and accordingly to our biochemical analysis, FPN was showed to increase importantly with Fe-NTA (ratio of 25). Some of these proteomic changes (increase of HMOX1, PRDX1, G6PD, HSPA5) indicated a putative cellular response to iron mediated oxidative stress and *in silico* pathway analysis proposed the involvement of the transcription factor Nrf2 in this process. To confirm this hypothesis, J774a1 and Bone Marrow derived macrophages (BMDM) were treated with iron (Fe-NTA and FAC) and with the prototypical Nrf2 activators tBHQ and cadmium (Cd). In all treated macrophages, both Nrf2 and FPN protein expression were upregulated with strong accumulation of Nrf2 into the nucleus. In addition, in BMDM isolated from Nrf2 null mice, the basal expression of FPN at both mRNA and protein was dramatically decreased and the upregulation of FPN by iron was abrogated. These results indicate that elevated macrophage iron activates Nrf2 which contribute to the up-regulation of FPN mRNA and protein. Such observations led us to study the *in vivo* expression of FPN in spleen and liver of

Nrf2 null mice. Contrasting with our *in vitro* experiment, FPN protein expression was increased in both liver and spleen of Nrf2 null mice when compared to wildtype littermate.
Conclusion: These results suggest that other positive regulators of FPN in Nrf2 null mice exist and counteract the lack of Nrf2. A more precise exploration of the iron metabolism in Nrf2 deficient mice is under investigation in order to get insight into the regulation of FPN in these mice.

Poster #25
SYSTEMIC INTERACTION OF IRON WITH ESTROGEN IN POSTMENOPAUSAL WOMEN
Xi Huang, PhD, Qing Yang, PhD; Jinlong Jian, PhD; Stuart Katz, MD; Steven B. Abramson, MD
NYU School of Medicine
(Presented By: Xi Huang)

Introduction: Estrogen (E2) and iron (Fe) are two of the most important growth nutrients in a female's body development. E2 affects the growth, differentiation, and function of tissues such as breasts and bone. Fe is essential for oxygen transport, DNA synthesis, as well as energy production. Although accumulated exposure to E2 is a well-established risk factor for breast cancer and E2 deficiency is considered a major cause of osteoporosis, development of the two diseases cannot be solely explained by E2 alone. We have previously shown that in young women, E2 level is high but Fe level is low. Conversely, in older postmenopausal women, the reverse is true with low E2 and high Fe (Fig. 1). Increasing experimental evidence demonstrates that variations in Fe levels from deficiency in young women to oversufficiency in older postmenopausal women may play important roles in the two diseases. Yet, whether Fe interacts with E2 at systemic level is unknown and clarification of the mechanism may have important clinical implications. In the present study, we first investigate whether high E2 levels lead to low Fe levels in young women by affecting expression of hepcidin, a negative regulator of iron uptake.
Methods and Materials: We examined the sequence of the 5'-flanking region of the hepcidin gene and found four estrogen responsive elements (ERE) in the promoter. Using chromatin immunoprecipitation, deletion, and electrophoretic mobility gel shift assays, we identified a functional ERE half site that is located between −2474 and −2462 upstream from the start of transcription of the hepcidin gene. E2 downregulated hepcidin in HepG2 cells as well as in wild type and Hfe gene knockout mice. Subsequently, we investigate whether high Fe in the form of ferritin, an iron storage protein, contributes to E2 deficiency in older women by altering cytochrome P450 enzymes. It is known that 95% of serum E2 in premenopausal women is derived from ovarian secretion and then distributed through the bloodstream in an endocrine fashion. Almost all of E2 in postmenopausal women are from extragonadal conversion of C19 steroid precursors, such as testosterone. The conversion CYP19A1 occurs locally in tissues such as bone marrow and breast, and thus, E2 acts in a paracrine fashion. Estrogens are eliminated from the body by metabolic conversion to estrogenically inactive metabolites catalyzed by CYP1A1 and CYP1B1.
Results: We have found that ferritin, at a dose of 10 ng/ml culture medium, or equivalent to 100 ng/ml serum, significantly increased CYP1A1 and 1B1 but not CYP19A1 mRNAs, proteins, and activities. Together, our data indicate that high E2 in young women inhibiting hepcidin is to increase Fe uptake, a mechanism to compensate Fe loss during menstruation. In contrast, increased Fe enhancing CYP1A1 and 1B1 activities is to accelerate E2 metabolism, contributing to E2 deficiency in postmenopausal women.
Conclusion: Our results suggest that decreasing iron level may prolong half-life of natural E2 in older women and, thus, may provide a safer option than hormone replacement therapy in improving symptoms and diseases associated with menopause.



Poster Abstracts

Poster #26

NOVEL MUTATIONS IN THE FERRITIN-L IRON-RESPONSIVE ELEMENT THAT ONLY MILDLY IMPAIR IRP BINDING CAUSE HEREDITARY HYPERFERRITINAEMIA CATARACT SYNDROME

Sara Luscieti, Ms[1]; Gabrielle Tolle, Ms[2]; Jessica Aranda, Ms[1]; Carmen Benet-Campos, MD[3]; Frank Risse, MD[4]; Erica Morán, PhD[1]; Martina U. Muckenthaler, PhD[2]; Mayka Sanchez, PhD[1]
[1]IMPPC; [2]MMPU-EMBL, University Hospital of Heidelberg; [3]Hospital Arnau de Vilanova, Valencia, Spain; [4]Praxis für Hämatologie- Onkologie Rhein Ahr, Remagen, Germany
(Presented By: Mayka Sanchez)

Introduction: Hereditary Hyperferritinaemia Cataract Syndrome (HHCS) is a rare autosomal dominant disease characterized by increased serum ferritin levels and early onset of bilateral cataract. The disease is caused by mutations in the Iron-Responsive Element (IRE) located in the 5' untranslated region of LFerritin (FTL) mRNA, which post-transcriptionally regulates ferritin expression.
Methods: We describe two families presenting high serum ferritin levels and juvenile cataract with novel mutations in the L-ferritin IRE. The mutations were further characterized by in vitro functional studies.
Results: We have identified two novel mutations in the IRE of L-Ferritin causing HHCS: the Badalona +36C>U and the Heidelberg +52G>C mutation. Both mutations conferred reduced binding affinity on recombinant Iron Regulatory Proteins (IPRs) in EMSA experiments. Interestingly, the Badalona +36C>U mutation was found not only in heterozygosity, as expected for an autosomal dominant disease, but also in the homozygous state in some affected subjects.
Conclusions: The Badalona +36C>U and Heidelberg +52G>C mutations within the L-ferritin IRE only mildly alter the binding capacity of the Iron Regulatory Proteins but are still causative for the disease.
Funding: This work was supported by the grant PS09/00341 from "Instituto de Salud Carlos III", Spanish Health Program, grant SAF2012-40106 from Ministry of Economy and Competitivity (MINECO) and grant "Ayudas a proyectos de Investigación en Ciéncias de la Vida Fundación Ramón Areces" to M.S. M.U.M. acknowledges funding from the E-RARE/BMBF project 01GM1005 and the Dietmar Hopp Stiftung as well as support from the Center For Rare Diseases, Medical Center University of Heidelberg.

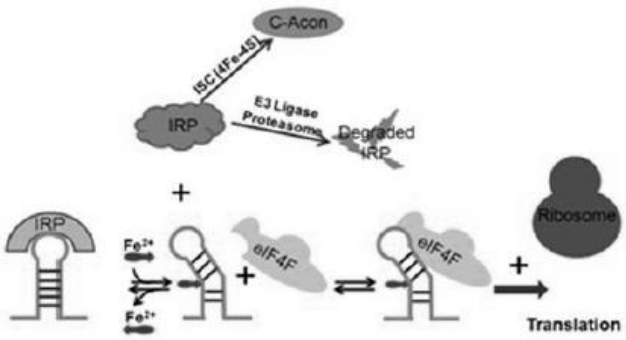
Poster #27

HOW FE2+ ACTIVATES IRE-MRNA: A DUET WITH IRP AND EIF4F

Elizabeth Theil, PhD[1]; Jia Ma, BS[2]; Suranjana Haldar, PhD[1]; Mateen Khan, PhD[2]; Dixie Goss, PhD[2]
[1]CHORI; [2]Hunter-CUNY
(Presented By: Elizabeth Theil)

Introduction: Iron either activates IRE-mRNA by enhancing ribosome binding near the IRE riboregulator, e.g. ferritin, ferroportin and mt-aconitase mRNAs, or inactivates IRE- mRNA by increasing mRNA degradation, e.g. transferrin receptor mRNA.
Methods and Materials: Using ferritin mRNA, the evolutionarily oldest IRE, as the model IRE-riboregulator, we observed:
1. Deleting the IRE-riboregulator decreases ferritin mRNA translation in vitro.
2. Fe2+ increases ferritin mRNA translation in vitro.
3. Fe2+ binds to IRE-RNA, and changes the conformation in the C bulge and terminal loop.
4. Fe2+ binding weakens IRP binding to IRE-RNA.
5. Fe2+ strengthens eIF-4F binding to IRE-RNA; eIF4F is translation factor containing subunits that bind mRNA cap (eIF4E), ribosomes (eIF4G) and mRNA (eIF4A).
Results: Thus, the physiological iron signal is ferrous ion, which changes the IRE riboregulator conformation. The two effects of Fe2+ binding, weaker IRP repressor binding and stronger eIF4F enhancer binding, activate IRE-mRNA for translation (Figure 1). Control of regulator binding (IRP and eIF4F) by the metabolite (Fe2+) is a type of feedback regulation common to riboregulators.
Conclusion: Dual recognition of the IRE-RNA for two regulatory proteins, one a negative regulator (IRP) and one a positive regulator (eIF4F) is a novel regulatory mechanism. (Part support:NIH-DK20251; CHORI Partners; NSF- MCB 1157632).

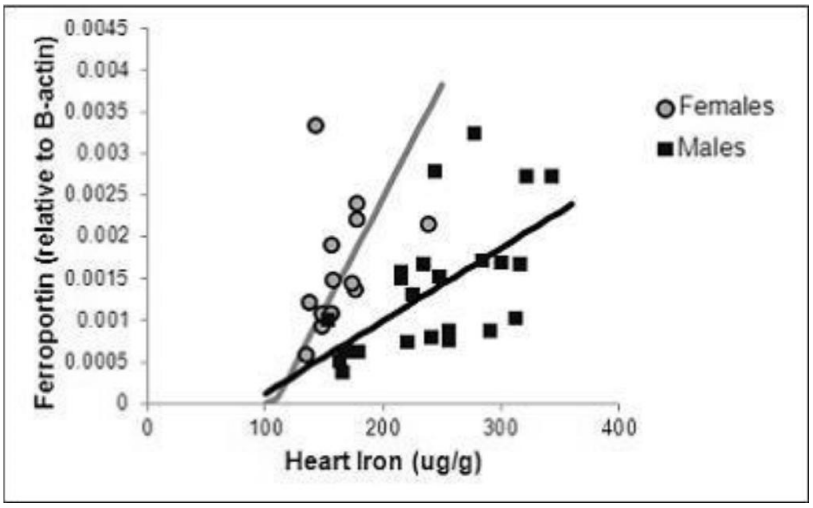
Figure 1. Fe2+ binding to IRE-RNA releases IRP repressor protein and attracts eIF4F translation enhancer protein. (From Ma et al. PNAS ¬109:8417, 2012). Blue-IRP; Green-c-acon (IRP + 4Fe-4S); grey- degraded IRP; yellow-eIF-4F; brown-ribosome.



Poster Abstracts

Poster #28
SEX DIFFERENCES IN THE REGULATION OF CARDIAC IRON TRANSPORTERS
Casey Brewer, BSc; Maya Otto-Duessel, PhD; Ruth Wood, PhD; John Wood, MD, PhD
Children's Hospital Los Angeles
(Presented By: Casey Brewer)

Introduction: Iron cardiomyopathy remains the leading cause of death for patients with iron overload and the mortality rate is twice as great for men as it is for women. We are exploring possible explanations for these observations in hemojuvelin knockout mice (HJV KO), a model of juvenile hemochromatosis. These animals develop four-fold greater cardiac iron than wild-type mice and the myocyte iron deposition mimics patterns observed in human iron cardiomyopathy, including male predominance. We hypothesized that sex hormones alter the expression of cardiac iron transporters in these animals, producing sex-differences in cardiac iron loading.
Methods and Materials: HJV KO mice were gonadectomized at four weeks of age and implanted with a Silastic hormone implant; males received either testosterone (n=7), dihydrotestosterone (n=7), estrogen (n=7), or a cholesterol control (n=12) while females received either estrogen (n=7) or a cholesterol control (n=12). There was also a group of intact males (n=7) and females (n=7) that had a sham surgery and a cholesterol control implant. Expression of cardiac iron transporters was measured by RT-PCR; the transporters analyzed were L-type calcium channels, T-type calcium channels, Zip14, ferroportin, and divalent metal transporter 1 (DMT1).
Results: The L-type, T-type, and Zip14 channels are all putative transporters of non-transferrin bound iron; ferroportin and DMTI are traditional iron transporters whose levels can be regulated by cellular iron via the Iron Regulatory Element/Iron Regulatory Protein (IRE/IRP) system. We did not see an effect of sex or steroid treatment on L-type, T-type, or Zip14 channels, nor did we see a correlation between these channels' expression and heart iron concentration. DMTI
expression was 45% lower in males than females ($p<0.0001$). Lower DMT1 levels might be expected in males because they had 31% greater cardiac iron than females ($p<0.0001$). However, DMT1 was positively correlated with cardiac iron in males ($R2=0.25$, $p<0.05$); this was not observed in females. Expression of the iron exporter, ferroportin, was positively correlated with cardiac iron in both males and females, as expected, but the relationship was shifted and steeper in females (Figure). Discriminant analysis showed a highly significant separation between male and female ferroportin response to cardiac iron ($p<0.0001$). Overall, ferroportin expression did not vary by sex or by steroid treatment among females. In males, ferroportin expression did depend on steroid treatment, with estrogen-treated animals having the highest ferroportin expression ($p<0.05$). Estrogentreated males also had the most cardiac iron ($p<0.05$), consistent with upregulation of ferroportin expression via the IRE/IRP system in response to cardiac iron.
Conclusion: Thus, sex differences in the "set point" for ferroportin upregulation could be responsible for the greater cardiac iron loading observed in males compared to females. In conclusion, we present evidence of a functioning IRE/IRP system in the mouse heart. Earlier upregulation of ferroportin in females may cause the lower cardiac iron seen in our iron overload model. The role of the human IRE/IRP system in cardiac iron overload should be explored.



Poster #29

CELLULAR IRON HOMEOSTASIS IN LEUKEMIA: EXPERIMENTAL AND MODELING APPROACHES

Emmanuel Pourcelot, PhD student[1]; Nicolas Mobilia[2]; Alexandre Donzé[3]; Fiona Louis[4]; Oded Maler[5], Pascal Mossuz[1], Eric Fanchon[2] and Jean Marc Moulis[4]
[1]Laboratoire d'Hématologie, Institut de Biologie et Pathologie, CHU Grenoble, France; [2]TIMC-Imag, Grenoble, France; [3]EECS Department, University of California Berkeley, USA; [4]CEA-iRTSV, Grenoble, France; [5]VERIMAG, UJF Gières, France
(Presented By: Emmanuel Pourcelot)

Introduction: Use of iron resources and variations of the redox balance are processes involved in cell proliferation and differentiation. They participate to normal hematopoiesis and their disturbance may have an oncogenic role. Hematological neoplasia, such as acute myeloid leukemia (AML), provide clinical evidence of the link between iron regulation, handling of redox active components, and malignancy. Yet, the properties favoring proliferation of leukemic clones detrimental to differentiation remain unclear. Iron and redox homeostasis are grounded in a series of molecular events belonging to several metabolic and regulatory networks. Our project aims at describing the functional differences between normal and pathological hematopoiesis by analyzing experimental data from leukemic cells with innovative modeling tools.
Methods and Materials: The core network of cellular iron regulation in mammals, organized around the Iron Regulatory Proteins (IRP), has been represented in a simplified generic theoretical model with five species (IRP, transferrin receptor, ferritin, ferroportin, and available 'labile' iron). The evolution over time of these species has been described by ordinary differential equations and the behavior of the biological system in hematopoietic precursors has been formalized in the Signal Temporal Logic using the software Breach [1]. Sets of consistent parameters were first estimated for the iron-replete situation corresponding to proliferating cells. The dynamic response to iron deprivation, which was previously shown to contribute to differentiation in some types of AML, was then monitored, and the relevant parameter region was determined.
Results: The salient features and new properties of hematopoietic precursors were evidenced, as well as identification of critical parameters [2]. Some of these important parameters, such as the iron threshold above which the IRP regulators loose function, have been experimentally measured in leukemic cell lines (KG1 and K562).
Conclusion: These data bear clinical relevance for patients supplemented with iron, or those under chelation therapy. They are also used to further implement the theoretical model of cellular iron homeostasis in order to improve its explicative and predictive power, and to unravel the details of its dynamic interactions with other redox-dependent regulatory networks.

[1] Donzé, A., Fanchon, E., Gattepaille, L. M., Maler, O. and Tracqui, P. (2011) PLoS One 6, e24246
[2] Mobilia, N., Donzé, A., Moulis, J.-M. and Fanchon, E. (2012) Electronic Proceedings in Theoretical Computer Science 92, 42-57. http://rvg.web.cse.unsw.edu.au/eptcs/paper.cgi?HSB2012.4

Poster #30

HYPERFERRITINEMIA-CATARACT SYNDROME: GENOTYPE/PHENOTYPE CORRELATIONS

Silvia Majore, Francesca Clementina Radio, Ilaria Cosentino, Gianfranco Biolcati, Caterina Aurizi, Francesco Brancati, Alfredo Dragani, Francesca Spina, Marco Castori, Rosanna Rinaldi, Carmelilia De Bernardo and Paola Grammatico
Medical Genetics, Molecular Medicine Department, Sapienza University of Rome, San Camillo-Forlanini Hospital, Rome, Italy
(Presented By: Silvia Majore)

Introduction: Hyperferritinemia-cataract syndrome (HCS; OMIM #600886) is an autosomal dominant disorder characterized by early-onset bilateral cataract and high ferritin levels in the absence of true iron overload. HCS results from different point mutations or deletions in the untranslated iron responsive element of the L-ferritin gene (FTL-IRE). The canonical IRE secondary structure consists of a six-nucleotide apical loop over a stem of five paired nucleotides, a small asymmetrical bulge and an additional lower stem of variable length. While the molecular mechanism leading to increased ferritin serum levels in HCS has been elucidated, the pathogenesis of cataract remains unclear.
Methods and Materials: We studied ten familial and five apparently sporadic cases with HCS, in addition to a sporadic 58-year-old woman with apparently isolated hyperferritinemia. Direct sequencing of the FTL-IRE region revealed six previously characterized mutations in individuals with complete HCS, while the novel 55C>G (c.-145C>G) mutation localized in the lowest stem region was identified in the patient with isolated hyperferritinemia.
Results: Ferritin average levels in our patients supported the hypothesis of a genotype/phenotype correlation between biochemical alterations and mutation site. In particular, ferritin levels seem higher in individuals with mutations in the apical zone compared to patients with mutations located in the stem region. In addition, we found the highest ferritin levels in patients with mutations located in the asymmetrical bulge region. Furthermore, cataract development seems influenced by mutation site in relation to the secondary structure of the FLT-IRE. Accordingly, its age at onset appears inversely related to the location of the affected nucleotide, with the most basal mutation associated with apparently complete lack of ocular manifestations.
Finally, the sporadic patient without ocular involvement pointed out the opportunity to consider HCS also in presence of isolated, apparently benign hyperferritinemia.

Poster #32
DEFECTIVE GLUCOSE METABOLISM IN IRP1-/- MICE
Nicole Wilkinson and Kostas Pantopoulos, PhD
Lady Davis Institute for Medical Research and McGill University
(Presented By: Nicole Wilkinson)

Introduction: Iron Regulatory Protein, IRP1, binds to several Iron Responsive Element (IRE)-containing mRNAs and controls their translation or stability. In ironreplete cells it acquires an iron-sulfur cluster converting it to a cytosolic aconitase at the expense of its RNA-binding activity. Aconitases catalyze the conversion of citrate to iso-citrate, which are metabolites involved in several pathways. IRP1-/- mice have not been reported to manifest any overt phenotypic abnormalities, suggesting redundancy in the genetic and enzymatic activities of IRP1. We hypothesized that IRP1-/- mice may exhibit metabolic defects and analyzed their responses to glucose challenges.
Introduction: IRP1-/- mice, IRP1+/- heterozygotes and wild type IRP1+/+ isogenic littermates (in C57B6 background) were first subjected to an oral glucose tolerance test.
Results: Compared to wild type controls and IRP1+/- heterozygotes, IRP1-/- mice presented significantly lower fasting glucose levels in the serum, as well as serum glucose levels at various time-points following gavage with a glucose bolus. No differences were observed in serum insulin concentration among the three genotypes, indicating that the hypoglycemia seen in IRP1-/- mice was not a result of insulin upregulation. When placed on a high fat diet IRP1-/- mice did not gain significantly more or less weight than IRP+/+ counterparts. Analysis by qPCR showed a ~2-fold increase of Glut1 mRNA in the liver ($p<0.05$) and kidneys ($p<0.01$) of IRP1-/- vs IRP1+/+ mice. Furthermore, mouse embryonic fibroblasts (MEFs) derived from IRP1-/- mice displayed a 2-fold increase in the uptake of fluorescently labeled glucose ($p<0.01$) as measured by fluorescence-activated cell sorting (FACS). Metabolite analysis of liver samples from IRP1-/- and IRP1+/+ mice demonstrate distinct profiles.
Conclusion: These data uncover previously unnoticed metabolic defects in IRP1-/- mice, which are apparently triggered by the absence of IRP1. We are currently investigating the molecular mechanisms underlying these responses.

Poster #34
MITOCHONDRIAL PROTEIN, FRATAXIN, IS DOWNREGULATED IN HEMODIALYSIS PATIENTS
Takeshi Nakanishi, MD, PhD; Yukiko Hasuike, MD,PhD; Soshi Yorifuji, MD; Ayako Matsumoto, MD; Mana Yahiro, MD, PhD; Aritoshi Kida, MD; Yasuyuki Nagasawa, MD, PhD; Takahiro Kuragano, MD, PhD
Hyogo College of Medicine
(Presented By: Takeshi Nakanishi)

Background: The mitochondrial protein frataxin regulates iron metabolism for heme and iron sulfur cluster synthesis in the mitochondria and could be associated with the regulation of oxidative stress. To clarify the expression of frataxin and its association with uremia, we evaluated the mRNA and protein levels of frataxin in the polymorphonuclear leukocytes of patients on hemodialysis.
Methods: Uremic patients on hemodialysis (n=18) and healthy control subjects (n=18) were investigated. Polymorphonuclear leukocytes were isolated by differential centrifugation. The mRNA levels of frataxin in isolated leukocytes were quantified by TaqMan real-time polymerase chain reaction. Frataxin protein expression in the cell lysate was evaluated using SDS-polyacrylamide gel electrophoresis and Western blotting. Serum tumor necrosis factor-□ (TNF-α) and interleukin-6 (IL-6) levels were measured with ELISA kits. Malondialdehyde (MDA) was measured as a marker of lipid peroxidation using the method developed by Yagi.
Results: The frataxin/GAPDH mRNA ratio in polymorphonuclear leukocytes from uremic patients was significantly lower than that in control subjects. Frataxin protein expression in uremic patients was also significantly lower than that in controls. Multiple regression analysis showed that frataxin mRNA levels were independently associated with the serum levels of both the oxidative stress marker, MDA and the proinflammatory marker, TNF-α. Single HD session did not affect the levels of frataxin.
Conclusion: The down-regulation of frataxin seems to be linked with uremic status, which is usually associated with chronic inflammation and the acceleration of oxidative stress. Mitochondrial iron regulation may play a role in several co-morbidities and poor prognosis in uremic patients. Further investigation is needed to elucidate whether reduced frataxin levels are linked to the pathological status of uremic patients and whether uremic substances affect frataxin expression.

Poster #35
FRATAXIN IS A REGULATOR OF THE FE-S BIOGENESIS
Salvatore Adinolfi, biologist
NIMR
(Presented By: Salvatore Adinolfi)

Introduction: Friedreich's ataxia is the most common inherited recessive ataxia. It is associated with reduced levels of frataxin, a small mitochondrial protein of still unclear function. Independent reports have linked frataxin to iron-sulphur cluster assembly through interactions with the two central components of this machinery, the desulphurase Nfs1/IscS and the scaffold protein Isu/IscU.
Objective: Our goal is the determination of the primary function of frataxin asthe prerequisite to understand pathogenesis.

Methods: We approached the problem by studying the structural bases ofthe interactions of frataxin with the components of the iron-sulpohur cluster biogenesis machinery. To simplify the problem we use the highly conserved bacterial orthologues.Our tools are a combination of biophysical and biochemical techniques that include NMR, CD, SAX, UV-VIS spectroscopy and enzymology.
Result: We have characterized the interaction of CyaY (the bacterial orthologue of frataxin) with the IscS/IscU complex and studied the effect on the enzymatic kinetics of cluster formation on the scaffold protein IscU. A single molecule of CyaY binds IscS in a pocket between the active siteand the IscS dimer interface through electrostatic interactionsof complementary charged residues. Cyay binding leads to a regulation of the desulfurase activity of IscS.
Conclusion: We propose that frataxins is a sensors that act as the regulator of iron-sulfur cluster formation to fine-tune the quantity of cluster formed to the concentration of the available acceptors.

Poster #37
IRON-DEPENDENT BINDING OF BOVINE MILK ALPHA-CASEIN WITH HOLO-LACTOFERRITN, BUT NOT HOLO-TRANSFERRITN
Koichi Orino, DVM, PhD; Kiyoataka Watanabe, DVM, PhD
Kitasato University
(Presented By: Koichi Orino)

Introduction: Bovine milk alpha-casein has been identified as ferritin-binding protein. Analysis of binding mechanism of alpha-casein with ferritin demonstrated that alpha-casein is iron- and heme-binding protein. However, the physiological role of its iron- or heme-binding remains to be elucidated in more detail. The purpose of this study is to clarify the physiological role of alpha-casein in bovine milk.
Methods: Alpha-casein was immobilized on CNBr-activated Sepharose 4B beads, and the binding of alpha-casein agarose beads ferrous ammonium sulfate ($Fe2^{+}$) and hemin was examined. Iron and hemin were measured with Ferrozine and 3,3',5,5'-tetramethylbiphenyl-4,4'-diamine reagents, respectively. The binding of alpha-casein beads with holo-lactoferrin (Lf) or holo-transferritin (Tf) was examine by measuring absorbance at wavelength of 280 nm.
Results and Discussion: The alpha-casein agarose beads efficiently bound hemin as well as Fe^{2+} as compared with control beads. Alpha-casein-beads bound bovine holo-Lf, but not holo-Tf. Following binding of alpha-casein beads to Fe^{2+}, holo-Lf caused the release of Fe^{2+} which had bound to the α- casein-agarose beads beforehand. These results suggest that bovine alpha-casein iron-dependently binds holo-bovine Lf more strongly than Fe^{2+}, and that strong binding between them may play a physiological role in regulating iron homeostasis in the bovine mammary gland.

Poster #38
NOVEL PRO-CHELATORS OF IRON AND THEIR ROLE IN PROTECTION AGAINST OXIDATIVE STRESS-INDUCED CARDIOMYOCYTE DAMAGE
Hana Jansova, Miloslav Machacek, Pavlina Haskova, Eliska Mackova, Tomas Simunek
Charles University in Prague, Faculty of Pharmacy in Hradec Kralove
(Presented By: Hana Jansova)

Introduction: Iron imbalance plays an important role in oxidative stress associated with numerous cardiovascular diseases. Therefore, iron chelation may be an effective therapeutic approach. However, the potential favorable effects of chelators against oxidative injury may be in conditions with no systemic iron overload hindered by own chelators' toxicity due to iron depletion and the ability to generate redox-active iron complexes. Therefore we studied novel "masked" iron pro-chelators that have little or no affinity for iron until the mask is selectively removed under the conditions of oxidative stress. Aromatic hydroxyl group, which is essential for chelataion of iron, is protected by formation of a boronic ester. This ester is converted to chelating active substances by the action of ROS such as H2O2 (Charkoudian et al. J Inorg Biochem. 2008; 102:2130-5).
Methods and Materials: H9c2 rat embryonal cardiomyoblast-derived cell line was used in our cardioprotection experiments. Neutral Red Uptake Assay and xCELLigence System were used for assessments of cellular viability. Calcein Assay was used to determine efficiency of prochelators' activation by H2O2 to effective chelators in buffered solution expressed as fluorescence intensity change. Calcein-AM Assay was used for determination of chelation efficiency inside the cells. Epifluorescence microscopy was used for photodocumentation of cellular morphology and damage, together with fluorescent staining for mitochondrial inner membrane potential by JC-1 probe. IC50 and EC50 were calculated using the CalcuSyn software. Software SigmaStat for Windows 3.5 (SPSS, USA) was used for statistical processing.
Results: All the tested pro-chelators and reference parent chelators showed some dose-dependent decrease of cellular viability, with exception of BSIH that did not display any own toxicity when incubated with the cells for up to 72 hours and concentrations up to its solubility limit of 600 μM. Fe chelators were able to significantly protect cells against injury caused by H2O2 (SIH at a concentrations ≥ 10 μM, HAPI and ICL670A ≥30 μM). Pro-chelators derived from ICL670A did not show significant protective effect. BHAPI, which is converted to HAPI (methyl ketone derivative of known chelator SIH), protected nearly 50 % of cells compared to control group at the concentrations 60 μM and 100 μM. Pro-chelators derived from SIH displayed the best protective properties. BSIH-PD and particularly the BSIH significantly protected H9c2 cells at a concentrations ≥ 60 μM their protective effects were comparable to parent chelators' efficiency. Our in vitro experiments

demonstrated conversion of pro-chelators in the presence of H2O2 to effective Fe chelators in solution as well as in cells, except for the pro-chelators derived from ICL670A, which did not show good chelating properties.
Conclusion: Our results demonstrate ability of pro-chelators BSIH, BSIH-PD and BHAPI derived from well known aroylhydrazone SIH and its novel derivative HAPI to protect cardiac cells against oxidative injury caused by H2O2. While retaining the useful antioxidant properties of parent chelators, they display very low potential to induce own toxicity. Therefore pro-chelators could offer solution of typical adverse effect of Fe chelators related to Fe deficiency.
This study was supported by the Charles University grant No. 367911.

Poster #39
MITOCHONDRIA-TARGETED IRON CHELATORS FOR THE MONITORING AND ADJUSTMENT OF CELLULAR LABILE IRON POOLS
Vincenzo Abbate, PhD[1]; Robert Hider, PhD[1]; Charareh Pourzand, PhD[2]; Olivier Reelfs, PhD[2]
[1]King's College London; [2]University of Bath
(Presented By: Vincenzo Abbate)

Introduction: Mitochondria play an important role in oxidative stress, acting as the main source of Reactive Oxygen Species (ROS) generation [1]. Recent studies of a range of human disorders suggested that mitochondrial redox active and chelatable labile iron (LI) plays a key role in conferring cellular sensitivity to oxidative damage and the resulting pathologies and cell death. A novel class of water−soluble small tetrapeptides (SS peptides) was recently developed, with the view to protect cells against ROS−mediated oxidative stress [2].These peptides consist of alternating aromatic and basic residues and are able to selectively target the inner membrane of mitochondria; furthermore, such small−peptide antioxidants are believed to penetrate cells in an energy−independent and non−saturable manner without a need for peptide transporters [3]. It is thus envisaged that the incorporation of iron−chelating moieties in the backbone of the SS peptides would result in a novel class of therapeutic agents for the treatment of disorders characterized by mitochondria iron overload, such as in Friedreich's ataxia (FRDA).
Methods and Materials: Peptides were synthesized using orthogonal Fmoc−solid phase peptide synthesis, fluorescently−labelled on the resin, cleaved from the solid support, purified by means of preparative RP−HPLC and finally characterized by ESI−MS. Confocal microscopy studies were conducted to study selective co−localization with mitochnodria using a mitochondria−specific marker (Mitotracker). Iron chelation ability, as well as drug cytotoxicity are currently under investigation. A series of fluorescently−labeled small tetrapeptides with or without iron−chelating groups (Fig. 1) were successfully prepared and tested for their ability to co−localize with mitochondria. The effect of different dyes on their biological behavior has been investigated.
Results: It was found that the size of the fluorescent probe strongly affected the ability of the compound to efficiently co−localize with mitochondria. Dansylated peptides were selected as models for the subsequent incorporation of 3−hydroxy pyridinones (as iron−chelating moieties) into the backbone sequence. Furthermore, a novel intrinsically fluorescent iron−chelator was designed, prepared and incorporated into the SS peptide−like sequence to avoid the presence of a fluorescent probe at the C−terminus, thus providing a 3+ net charge to the compound as in the parent SS peptide. Such novel mitochondria−targeted iron chelators will be screened to select a lead−sensor capable of monitoring the LI−pool in mitochondrial compartments and possibly remove excess LI.
Conclusion: These peptides may find use in the treatment of certain diseases such as Friedreich's ataxia which are caused by high (and therefore toxic) levels of iron in mitochondria.
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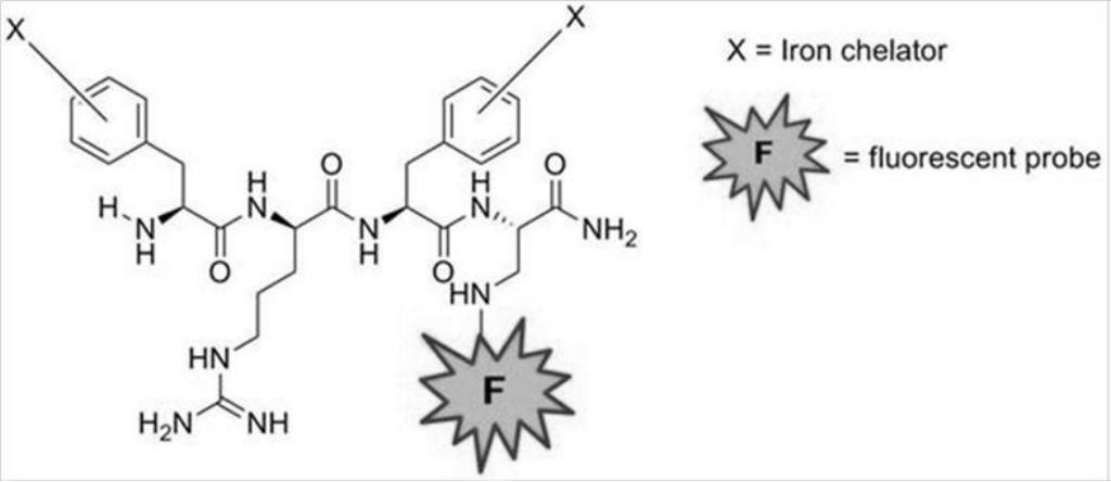
Fig. 1: Schematic representation of the new class of mitochondria−targeted iron chelators



Poster #40

NEW TRANSGENIC RICE TOLERANT TO LOW IRON AVAILABILITY IN A CALCAREOUS SOIL

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(Presented By: Naoko Nishizawa)

Introduction: One of the widest ranging abiotic stresses in world agriculture arises from low iron (Fe) availability due to high soil pH, with 30% of arable land too alkaline for optimal crop production. Rice, a staple food consumed by half the world population every day, is very susceptible to low Fe availability because of a low capacity to produce phytosiderophore, which is a Fe (III) chelator secreted by graminaceous plants. In addition to phytosiderophorebased system, by OsIRT1 transporter, rice also takes up Fe(II) which are abundant under the submerged condition. However, rice does not posses Fe (III) reduction activity. As a result, rice is very susceptible to low Fe availability because of absence of Fe(III) reduction activity and low phytosiderophore production.
Methods and Materials: Previously, we produced transgenic rice expressing the mutational reconstructed yeast ferric reductase, refre1/372 under the control of OsIRT1 promoter. This transgenic rice showed higher Fe(III) reductase activity and tolerance to low Fe availability [1]. We also produced transgenic rice over-expressing Fe-deficiency inducible basic helix-loop-helix transcription factor, OsIRO2, which regulates various genes participated in phytosiderophore-based Fe uptake system. This transgenic rice showed an enhanced phytosiderophore secretion and tolerance to low Fe availability in a calcareous soil [2].
Results: In the present study, we produced new transgenic rice with both Fe(III) reduction activity and enhanced phytosiderophore secretion. This new transgenic rice line exhibited enhanced tolerance to low Fe availability in a calcareous soil compared to non-transgenic line and the previous transgenic rice with single introduction of Fe(III) reduction activity or enhanced phytosiderophore-based Fe uptake system.
Conclusion: Our results show that an enhancement of both Fe(II) uptake system and phytosiderophore-based Fe(III) uptake in rice effectively contribute to further tolerance to low Fe availability in calcareous soil.
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[2] Ogo et al. (2011) Plant Mol. Biol. 75:593-605.

Poster #41

COMPUTATIONAL PREDICTION FOR CRITICAL RESIDUAL PAIRS IN COMPLEXATION BETWEEN DIFFERIC TF AND TFR2

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(Presented By: Tetsuya Sakajiri)

Introduction: Iron is an essential trace element for almost all living organisms. However, free Fe(II) is toxic and Fe(III) is insoluble in the aqueous environment of the blood. Therefore, in vertebrate circulation, the iron is bound in the form of Fe(III) by a serum transferrin (Tf). Mammals have 2 kinds of Tf receptors. Tf receptor1 (TfR1) and Tf receptor2 (TfR2), each of which is a homodimeric type II transmembrane protein. Both receptors share similar structures but possess different functions, TfR1 is ubiquitously expressed in all iron-requiring cells and mediates cellular iron uptake via the endocytosis of its ligand, diferric Tf (Fe_2Tf). TfR2 is predominantly expressed in hepatocytes and is involved in the production of the hepatic peptide hepcidin, which regulates systemic iron homeostasis. Recently, the computational structure model of the Fe_2Tf-TfR1 complex, and the crystal structure of a monoferric N-lobe Tf-TfR1 complex, have been reported. However, there is no report concerning the Fe_2Tf-TfR2 complex structure.
Methods: In this study we created a 3D structure model of the human Fe_2Tf-TfR2 complex by computational rigid body refinement. The structure of TfR2 was constructed based on the crystal structure of TfR1 by using a homology modeling technique. We carried out a molecular dynamics simulation to estimate bond energy of residual pairs between the Fe_2Tf and the TfR2. The residual pairs were confirmed by flow cytometry (FCM) analysis. Recombinant human TfR2 were expressed in HEK293 cells. A biotinylated Fe_2Tf which was bound to TfR2 on the cell surface and the Fe2Tf binding cells were labeled with fluorescent dyes (streptavidin-phycoerythrin) by binding to biotin. The number of the fluorescently-labeled cells was counted by FCM.
Results: The modeled structure of the Fe_2Tf-TfR2 complex has several typical bonds, including some ionic bonds and hydrogen bonds, between Fe_2Tf and TfR2, similar to the Fe2Tf-TfR1 complex. However, the Fe_2Tf-TfR2 complexation requires the cutting of one ionic bond within the TfR2 molecule, which is not needed upon the Fe_2Tf-TfR1 complexation. The energy for the cutting of the bond is supplied from the binding energy between Fe_2Tf and TfR2. This energy loss brings about a 30-50 times lower affinity of Fe_2Tf to TfR2 than to TfR1, resulting in facilitating the TfR2 in to becoming a sensor of the body's iron status. The FCM analysis using TfR2 mutants supported importance of the residual binding pairs which
were predicted in the modeled structure of Fe_2Tf-TfR2 complex.
Conclusion: Our computational structure model demonstrates the residual pairs which are decisive of formation of the Fe_2Tf-TfR2 complex.

Poster #42

ROLE OF FREE IRON IONS IN CATECHOLAMINE CARDIOTOXICITY AND THEIR CHELATION AS A POSSIBILITY OF PHARMACOLOGICAL CARDIOPROTECTION

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(Presented By: Pavlina Haskova)

Introduction: Pathogenesis of catecholamine cardiotoxicity is still incompletely understood. Although it has been traditionally attributed to β-adrenergic overstimulation, there is a hypothesis of involvement of catecholamine oxidation products, which may arise due to their spontaneous oxidation. This oxidation running under aerobic conditions leads to formation of reactive intermediates, including aminochromes, which are subject to redox cycling generating reactive oxygen species (ROS). Moreover, the autooxidation may be catalyzed by transition metals, which suggests free intracellular iron ions can promote not only the Haber-Weiss reaction of ROS, but also catalyze conversion of catecholamines to aminochromes and aggravate the cardiomyocyte damage. Iron chelation may therefore offer the possibility of cardiac cells protection against catecholamine toxicity.

Methods and Materials: The objective of this study was to explore the role of free iron ions in catecholamine cardiotoxicity and to evaluate the possibility of its protection by iron chelation. Protective potentials of different chelators (experimental and clinically used drugs) were studied. We evaluated spontaneous oxidation of catecholamines in buffered solution by spectrophotometry measurements and HPLC analysis, redox activity and oxidative effects of parent and oxidized catecholamines and their complexes with iron by determination of oxidized ascorbate, ROS production in the rat cardiomyoblast cell line H9c2 by determination of dichlorofluorescein, toxicity of catecholamines and their oxidation products to cells by neutral red vital staining and by fluorescent-stained nuclei microscopy.

Results: Spontaneous catecholamine (adrenaline, isoprenaline) concentration decrease was observed in buffered solution during the 24h incubation together with a gradual oxidation of catecholamines to unspecified products. This oxidation was significantly aggravated by the iron ions presence, which may form complexes with catecholamines and their oxidation products. Complexes of iron with the oxidized catecholamines had a pronounced redox activity towards ascorbate. Cellular experiments revealed higher toxicity of oxidation products than of parent catecholamines and confirmed the formation intracellular ROS after exposing of cells to oxidized catecholamines. Iron chelation significantly reduced spontaneous catecholamine oxidation and oxidation products formation, as well as redox activities of their complexes with iron. It was also able to provide significant protection of cardiomyoblasts, preserve their viability and suppress the ROS formation inside the cells. Small lipophilic chelators that are able to penetrate into cells in sufficiently-effective concentrations, eg. experimental salicylaldehyde isonicotinoyl hydrazone (SIH) and clinically-used deferiprone and deferasirox, have shown considerable protective potential. Furthermore, prochelator boronic ester of SIH was shown as very promising, as it was minimally toxic in comparison to the classic chelators.

Conclusion: Our results support the assumption that oxidative damage plays a significant role in the process of catecholamine-induced cardiotoxicity owning to catecholamines ability to undergo iron-promoted oxidation. That results in formation of unstable toxic oxidation products capable of redox cycling and ROS production. Both oxidation products and ROS are responsible for the cardiotoxicity of catecholamines. Free iron ions are significantly involved in all redox reactions leading to catecholamines oxidation and ROS formation. Iron chelation proved to be a suitable tool of cardioprotection, which has considerable potential to protect heart cells against catecholamine cardiotoxicity by the inhibition of catecholamines conversion and thus protects cardiac cells against oxidative damage.

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Poster #43

EFFICIENT AND FACILE OXIDATION OF CYCLIC MOUSE HEPCIDIN ANALOG TO BIOLOGICALLY ACTIVE CONFORMATION WITH THE HELP OF FE(III)

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Introduction: Hepcidin , which is a 25 amino acid residue cystine rich antimicrobial peptide hormone plays a crutial role in the iron homeostasis. Hepcidin binds to ferroportin causing its internalisation and degradation, and prevents the release of iron into the blood circulation. There are 8 cysteine residues that form four sets of disulphide bridges (1) (Cys7-Cys 23, Cys 10-Cys 13, Cys11-Cys 19, and Cys14-Cys22.) and are highly conserved across several species including mouse, rat, pig, dog and fish. It has been shown in earlier studies that Hepcidin is biologically active in the folded conformation irrespective of the nature of the disulphide bridge connectivity(2,3).The N terminal end of the peptide plays an important role in the function of the peptide and the interaction between hepcidin and ferroportin results in a covalent binding likely involving disulfide exchange between one of the disulfide bonds of hepcidin and Cys326 of Ferroportin, even though multiple disulfide bonds of hepcidin might be capable of forming a contact with Ferroportin (4).

Methods and Materials: We wanted to ask the question if Hepcidin could bind Fe(III) apart from playing a vital role in the iron

homeostasis and if the bound Fe(III) could assist the folding of Hepcidin to its native conformation by the oxidation of the thiols. A reduced cyclic mouse Hepcidin (because mouse hecpidin does not present the metal binding ATCUN center) analog NH2-DTNFPIc-(KIFCAKCCNNSQAGICD)KT-COOH has been designed to specifically aid the binding of Fe(III). Reduced cyclic mouse Hepcidin analog was metallated with Fe(III) under basic conditions and the complex was characterized by UV, EPR and EXAFS at low temperature. The oxidation of the reduced cyclic mouse Hepcidin analog has been studied both by the Ellman's test and by MALDI-MS spectrometry.
Results: It has been shown that in the presence of Fe(III), the reduced cyclic mouse Hepcidin analog is oxidized at a much faster rate than in its absence, both under Ar or when exposed to air. To study whether Fe(III) brings about folding of the peptide to native conformation, biological activity of the reduced cyclic mouse Hepcidin analog oxidized by Fe(III) was carried out and compared to that of synthetic cyclic peptides bearing 0 to 2 disulfide bridges. Finally the same strategy was succesfully applied to a fully reduced synthetic mouse hepicidin.
Conclusion: The results obtained indicate that the reduced linear mouse hepcidine and the cyclic mouse hepcidine analog underwent facile and prompt oxidation in the presence of Fe(III) and gave rise to the biologically active native form.
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Poster #44
WITHDRAWN

Poster #45
BOTH HUMAN FERREDOXINS EQUALLY EFFICIENTLY RESCUE FERREDOXIN DEFICIENCY IN TRYPANOSOMA BRUCEI
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(Presented By: Piya Changmai)

Introduction: Ferredoxins are highly conserved proteins that function universally as electron transporters. They not only require Fe-S clusters for their own activity, but are also involved in Fe-S formation itself.
Methods and Materials: We identified two homologues of ferredoxin in the genome of the parasitic protist *Trypanosoma brucei* and named them TbFdx and TbFdxL. TbFdx protein, which is homologous to other eukaryotic mitochondrial ferredoxins, is essential in both the procyclic (= insect-transmitted) and bloodstream (mammalian) stage, but is more abundant in the active mitochondrion of the former stage.
Results: Depletion of TbFdx caused disruption of Fe-S cluster biogenesis and haem synthesis. However, TbFdxL, which is present exclusively within kinetoplastid flagellates, was non-essential for the procyclic stage, and double knock-down with TbFdx showed this was not due to functional redundancy between the two homologues. Heterologous rescues with human orthologues HsFdx1 and HsFdx2 fully rescued the growth and Fe-S-dependent enzymatic activities of TbFdx knock-down.
Conclusion: In both cases, the genuine human import signals allowed efficient import into the T. brucei mitochondrion. Given the huge evolutionary distance between trypanosomes and humans, ferredoxins clearly have ancestral and highly conserved function in eukaryotes and both human orthologues have retained the capacity to participate in Fe-S cluster assembly.

Poster #46
IDENTIFICATION OF HEMOJUVELIN RESIDUES AS PUTATIVE TARGETS OF TMPRSS6
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(Presented By: Marco Rausa)

Introduction: The GPI-anchor protein Hemojuvelin (HJV) has a central role in hepcidin regulation and acts as a Bmp-coreceptor upregulating hepcidin transcription. The serine-protease TMPRSS6 inhibits hepcidin expression by cleaving membrane HJV in multiple soluble fragments in vitro. One cleavage site has been identified at Arginine (R) 288. The aim of this study was to investigate other potential TMPRSS6 cleavage sites on HJV.
Methods and Materials: To address this aim we mutagenized 23 HJV Arg residues to alanine (A) to generate potential TMPRSS6 resistant mutants. HJV variants were screened by western blot analysis for their correct translation and for the pattern of soluble fragments released in the conditioned medium of HeLa cells cotransfected with TMPRSS6, using an antibody raised against HJV C-terminal peptide (AA 226-402). Abnormal fragment patterns were identified for mutants at position 121, 176, 218, 288 and 326. As compared to HJV^{WT}, HJV^{R121A} lacked the 30 kDa fragment, HJV^{R218A} and HJV^{R326A} released only the low molecular weight (about 20 kDa) fragment, HJV^{R176A} showed a completely altered pattern of soluble fragments and HJV^{R288A} did not show any fragments in the culture medium. Maturation processing and cell surface localization of the HJV variants showed loss of the autoproteolytic cleavage (except for HJV^{R121A}) and a reduced cell surface localization for HJV^{R176A} and HJV^{R288A}. The absence of soluble fragments in the culture medium was not due to impaired interaction between the HJV variants and TMPRSS6, as assessed by co-immunoprecipitation. However, coexpression of each variant with TMPRSS6 decreased the amount of membrane HJV, suggesting that TMPRSS6 cleaves HJV at multiple sites. The

impaired autoproteolysis during HJV maturation led either to a decrease (HJV^{R218A} and HJV^{R326A}) or to a complete loss (HJV^{R176A} and HJV^{R288A}) of the BMP-coreceptor activity. HJV^{R121A}, which undergoes a correct autoproteolytic cleavage, behaved as HJVWT.
Results: Based on the soluble fragment patterns observed we hypothesize that HJV^{R121A}, HJV^{R218A} and HJV^{R288A} are resistant to the effect of TMPRSS6 and that the corresponding positions are putative TMPRSS6 cleavage sites. Since all mutants except HJVR121A do not undergo autoproteolysis and are unable to activate hepcidin in a luciferase-based assay we also hypothesize that R substitutions cause HJV misfolding, thus masking (loss of soluble fragments) or exposing (reduced membrane HJV) cryptic potential TMPRSS6 cleavage sites.
Conclusion: From our analysis HJV is probably cleaved at multiple sites and the cleavage is not only mediated by consensus sequences but requires a correct HJV folding, that is essential also for the coreceptor activity, as previously shown for HJV hemochromatosis-associated mutants. Further studies are needed to verify potential sites, not explored by the available antibody, in the N-terminal part of the protein.

Poster #47
THE HUMAN P-LOOP NTPASE CFD1 (NUBP2) IS A CYTOSOLIC IRON-SULFUR PROTEIN ASSEMBLY (CIA) FACTOR AND ESSENTIAL FOR IRON HOMEOSTASIS
Oliver Stehling, Brigitte Niggemeyer, Ralf Rösser, Roland Lill
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(Presented By: Oliver Stehling)

Introduction: Formation of iron-sulfur (Fe-S) clusters (ISCs) is one of the major iron-consuming processes in mammalian non-erythropoetic cells and tightly connected to cellular iron homeostasis via the bifunctional Fe-S cluster-containing iron regulatory protein 1 (IRP1). Maturation of Fe-S proteins including IRP1 is mediated by three complex assembly systems: The ISC assembly and the ISC export machinery are both residing within mitochondria, while the CIA machinery belongs to the cytosolic-nuclear compartment.
Methods and Materials: Studies performed in yeast as a model system revealed that the CIA system is acting in two consecutive steps: In an early reaction, an Fe S cluster is assembled in a transient fashion on a heterodimeric scaffolding complex consisting of the two related P-loop NTPases Cfd1 and Nbp35. In a consecutive reaction, the labile cluster is transferred to cytosolic and nuclear target proteins, a step assisted by dedicated late-acting CIA targeting complexes conferring target protein-specific maturation. In human cells, only one of the early acting scaffold components, huNBP35 (initially annotated as nucleotide binding protein 1, NUBP1), has been shown to be essential for cytosolic Fe-S protein maturation. In the present study we show the interaction of huNBP35 with huCFD1 (NUBP2) in HeLa cells. Further, RNAi was used to deplete huCFD1 and the cellular phenotype associated with huCFD1 deficiency was examined.
Results: Immunoblot analyses and enzyme activity measurements revealed an impaired maturation of the cytosolic Fe-S enzymes dihydropyrimidine dehydrogenase (DPYD) and glutamine phosphoribosylpyrophosphate amidotransferase (GPAT) as well as of the nuclear Fe-S protein POLD1, a subunit of DNA polymerase delta. Particularly, depletion of huCFD1 impaired maturation of IRP1 as indicated by a severe drop of cytosolic aconitase activity and lowered IRP1 protein levels. The improper assembly of Fe-S clusters on IRP1 had profound consequences for cellular iron acquisition and resulted in an elevated cellular transferrin binding activity, although responsiveness to iron deficiency and iron supply was maintained.
Conclusion: In summary, huCFD1 is required for the maturation of virtually all cytosolic-nuclear Fe/S proteins, and thus can be viewed as general CIA factor acting early in the pathway. Moreover, the protein is essential for proper cellular iron homeostasis re-emphasizing the interdependency of the latter process and Fe/S protein biogenesis.

Poster #48
DUODENAL CYTOCHROME B AND HEPHAESTIN EXPRESSION IS REGULATED BY THE SOLUBLE HFE ISOFORM
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(Presented By: Bruno Silva)

Introduction: Hereditary Hemochromatosis is an autosomal recessive disorder characterized by excessive intestinal iron absorption and iron deposition in organs such as liver, heart and pancreas, potentially leading to cirrhosis, hepatocelular carcinoma, diabetes, cardiac failure and arthritis. This disorder is mainly due to mutations in *HFE* gene.
HFE protein associates with beta-2 microglobulin (B2M) for trafficking to the cell surface. However, the HFE's role on iron homeostasis is not completely cleared. It may regulate hepcidin expression in the liver and iron trafficking in the duodenum. Several *HFE* alternative splicing transcripts have been reported, but their structural and functional characterization have been poorly studied.
Materials And Methods: Aiming to investigate the putative biological role of an alternative *HFE* transcript originated by the intron 4 inclusion, we measured its expression level in several human tissues by quantitative Real-Time PCR. Also, we produced the corresponding GFPtagged HFE variant. HepG2 cells were transfected with this construct and protein cellular location analyzed by immunofluorescence, using B2M, TfR1 and calnexin antibodies. In parallel, immunoprecipitation was performed. Finally the intron 4 inclusion variant was over-expressed in a human duodenum adenocarcinoma cell line (Hutu-80) under normal and iron overload conditions and the expression of several iron metabolism genes (*TFR1*, *DMT1*, *DCYTB*, *SLC40A1* and *HEPH*) evaluated by quantitative Real-Time PCR.

Results: We have found that the intron 4 inclusion transcript has an ubiquitous expression in the analyzed tissues, being its relative expression higher in duodenum and lower in the liver. Also, we found that this variant gives rise to a truncated protein (sHFE) that is secreted by the cells and is able to maintain its interaction with B2M. Its overexpression in HuTu-80 cells showed that sHFE down-regulates the duodenal cytochrome b (*CYBRD1*) expression in about 20% independently of cellular iron status, as it happens with the HFE_full length protein. Also, sHFE seems to be involved in the down-regulation of hephaestin (*HEPH*) expression, being its effect higher in the presence of iron overload (reduction of ~40 and ~50%, respectively).
Conclusions: Through this study we might have unveiled the contribution of the *HFE*'s intron 4 inclusion splice variant to the maintenance of iron homeostasis. sHFE may be secreted into the bloodstream and act in remote tissues such as the duodenum, down-regulating the expression of some of the iron metabolism related genes, as *CYBRD1* and *HEPH*, and consequently reducing dietary iron absorption. Also we are currently exploring the hypothesis of a possible effect of sHFE in the expression of other iron metabolism related genes in hepatic cells and macrophages.
Partially funded by FCT: PTDC/SAU-GMG/64494/2006; Pest-OE/SAU/UI0009/2011; SFRH/BD/21340/2005 and SFRH/BD/60718/2009

Poster #49
A MUTATION IN THE IRON-SULFUR CLUSTER BIOGENESIS FACTOR IBA57 CAUSES A FATAL MITOCHONDRIAL DISEASE WITH RESPIRATORY INSUFFICIENCY, AND HYPERGLYCINEMIA
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(Presented By: Claudia Wilbrecht)

Introduction: Iron-sulfur (Fe/S) clusters are inorganic co-factors present in proteins of all kingdoms of life. The most abundant forms are [2Fe-2S] and [4Fe-4S] clusters. Fe/S proteins are involved in various cellular processes including enzyme catalysis, electron transfer and sensing of environmental conditions. In mitochondria, their assembly is mediated by the iron-sulfur cluster (ISC) assembly machinery which comprises 18 conserved proteins some of which are essential for cell viability and which are required for biogenesis of all cellular Fe/S proteins. Besides the core ISC assembly machinery several specific maturation factors have been identified recently, all of which are crucial for maturation of distinct subgroups of target Fe/S proteins. Among these assembly factors are the so-called A-type ISC proteins, designated ISCA1 and ISCA2 in humans. We could show that these proteins act in conjunction with a third protein, IBA57, and are specifically involved in the maturation of mitochondrial [4Fe-4S] proteins such as respiratory complexes I and II, lipoate synthase, and aconitase. In contrast, maturation of mitochondrial [2Fe-2S] proteins and cytosolic Fe/S proteins occurs independently of these ISC assembly members. Over the past few years several severe diseases have been linked to genetic defects in various ISC components. These include more specialized maturation factors acting later in the ISC biogenesis pathway. For instance, mutations in NFU1 required for assembly of respiratory complexes I and II as well as lipoate synthase lead to juvenile encephalopathy with hyperglycinemia. A defect in the P-loop NTPase IND1 involved in complex I maturation is causative of a mitochondrial encephalomyopathy.
Methods and Results: Here, we report a mutation in the folate-binding protein IBA57 which leads to low levels of this protein and causes fatal symptoms such as hypotonia, respiratory insufficiency, and hyperglycinemia.
Conclusion: Ongoing biochemical analyses in human cell culture are aimed at describing the molecular consequences of this fatal mutation in order to fully understand the pathophysiology of affected individuals.

Poster #50
WITHDRAWN

Poster #51
SYNTHESIS OF DESFERRIOXAMINE-PEPTIDE CONJUGATES FOR MITOCHONDRIAL LABILE IRON CHELATION
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(Presented By: Roxana Pastrana)

Introduction: Iron is the most important transition metal in biological systems. However, metal overloads that are potentially harmful to the body by favoring oxidative stress. Some diseases are characterized by an overload pressure (thalassemia, hereditary hemochromatosis) or locally (eg. Mitochondria, characterized Friedrich's ataxia) of metal. Mitochondria are particularly sensitive to oxidative stress induced by iron, leading to lipid peroxidation and membrane permeabilization of this organelle. Efficient for iron chelators are therefore interesting to deplete certain overhead compartments. The desferrioxamine (DFO) is a bacterial siderophore high affinity for iron, but low cellular penetration.
Methods and Materials: In this project, we intend to produce conjugated synthetic peptide sequences with dfo (Mitochondria Penetrating Peptides) can promote the accumulation of chelating mitochondria. The characterization of the conjugates as well as its iron-binding characteristics and performance to suppress intracellular labile iron reservoirs will also be investigated, in

order to contribute to the treatment of neurodegenerative diseases such as Friedrich's ataxia. Therefore, a series of MPPs, natural or synthetic, have been developed as a means of carrying active mitochondria and specifically with a molecule of interest [39].
Results: Noteworthy among these are those derived from TAT (peptide constituent of the virus HIV) developed by the group of ShanaKelley ([40], [41]) and the SS-tetrapeptide (which have the added advantage of being antioxidant; [39]; [42]). The sequences of these MPPs are:
DLC CARGO-PPh3 (DLC= (Formylmethyl)triphenylphosphonium chloride)
TAT49-57 CARGO-R-K-K-R-R-Q-R-R-R , were cargo= DFO
1A CARGO-Fx-r-Fx-K-Fx-r-Fx-K
SS02 CARGO-Dmt-r-F-K
Conclusion: Therefore, this design is intended derivatization of desferrioxamine (DFO) with MPPsde order to increase the mitochondrial localization of siderophore and thus use it in the treatment of iron overload mitochondria.

Poster #52
LACTOFERRIN-CISPLATIN-EMBEDDED BIODEGRADABLE POLYMERIC THIN FILMS WITH ANTITUMORAL ACTIVITY IN VITRO
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(Presented By: Robert Evans)

Introduction: Combination therapy, the use of two or more drugs together for an enhanced biological effect, is of great interest in biomedical applications. Within this context, two compounds with anti-tumoral properties, namely lactoferrin [recombinant iron-free (Apo-rLf)] and cisplatin (Cis), were embedded in a biodegradable polycaprolactone (PCL) polymer matrix in order to obtain a new and more efficient chemotherapeutic agent.
Methods and Materials: PCL was proven to be more suitable for cell adherence when compared to a polyethyleneimine or polyethyleneoxide matrix on polystyrene. A modified approach of a laserbased technique, namely Matrix Assisted Pulsed Laser Evaporation (MAPLE), was used in biological compound thin film processing on glass slides. MAPLE has great potential as a facile and versatile deposition technique for the preparation of well-defined biomedical platforms.
Results: Structural, optical, and morphological properties of the biodegradable polymer cell platform containing the anti-tumor active factors, alone or in combination, were investigated by Fourier transform infra-red spectroscopy , Scanning Electron Microscopy, and atomic force microscopy. The *in vitro* effect of the thin films on the cell viability, adhesion, morphology and proliferation of B16-F10 murine melanoma cells was also investigated. Physico-chemical experiments demonstrated that when the anti-tumoral compounds were embedded within a PCL film there was no significant chemical modification. Biological assays showed decreased viability and proliferation, lower adherence, and morphological modifications in the case
of cells cultured on both Apo-rLf and Cis thin films. The effect was enhanced by deposition of Apo-rLf with Cis within the same film. The antitumoral effect was due solely to the biological compounds and not to the chemical composition of the substrate as shown by biological experiments in the presence of titanium-covered thin films.
Conclusion: Our results demonstrate the unique capability of the new approach based on MAPLE to embed antitumoral active factors within a biodegradable matrix in order to generate a novel biodegradable platform with increased antitumoral efficiency.
Acknowledgements: V.D. acknowledges financial support of a grant from the Romanian National Authority for Scientific Research, CNCSUEFISCDI, project number PN-II-RU-TE-2011-3-0289. L.S. acknowledges financial support from the POSDRU/89/1.5/S/60746 project. P.E.F. and A.R. acknowledge financial support from project nr. 3 of the Romanian Academy.

Poster #53
IDENTIFICATION OF TRANSCIPTION FACTORS THAT REGULATE MITOCHONDRIAL FERRITIN EXPRESSION
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(Presented By: Paolo Santambrogio)

Introduction: Mitochondrial ferritin (FtMt) is an iron-sequestering protein expressed in cell types characterized by high metabolic activity and oxygen consumption, suggesting a role in protecting mitochondria from iron-dependent oxidative damage. The human FTMT gene is encoded by an intronless sequence on chromosome 5q23.1. It is known that its 220bp segment upstream the ATG are densely methylated in normal tissues, however, no published data on
the characterization of its promoter are presently available.
Methods and Materials: To analyze the regulatory mechanisms controlling FtMt expression, we characterized the 5' ATG-flanking region by cloning a 1893bp segment in front of a luciferase reporter sequence. Its activity was examined in transient transfections in HeLa cells in basal condition or after treatment with 5_M 5-Aza-2'-deoxycytidine (DAC), a demethylating agent. The fragment induced a ~6 fold increase in basal condition and a ~9 fold increase after treatment with DAC compared

to mock luciferase activity. The DAC dependent FtMt upregulation was also confirmed in K562 cells by RT-PCR. To map the promoter, we prepared a series of 5' or 3' deletions and assayed their effect on activity.
Results: As expected, construct with deletion of region densely methylated didn't respond to treatment with DAC, but its activity was not null. This indicate that the promoter is not only regulated by methylation, but there are positive regulatory elements that we have identified between -1128 -874 and -464 -217nt regions and negative regulatory elements between -874 -464nt region. Analyses of these regions by Chromatin Immuno Precipitation (ChIP) resulted in the identification of four transcription factors involved in FtMt regulation. Three of them, CREB, SP1 and YY1 bound to positive regulatory elements, while one, FOXA1, bound to negative regulatory elements. CREB plays a role in the regulation of ROS detoxification, being a direct regulator of antioxidant gene expression. SP1 regulates the expression of genes involved in differentiation, apoptosis and mitochondrial biogenesis. FOXA1 positively regulates the activity of trascription factors, acting by opening the chromatin for other proteins.
Conclusion: The results indicate that the1893bp fragment contains regulatory elements for FtMt expression that bind specific transcription factors and that DAC transcriptionally activate the FtMt promoter. Thus the expression of FtMt appears regulated by a complex mechanism involving epigenetic events and an interplay between different transcription factors.

Poster #54

NON-TRANSFERRIN-BOUND IRON (NTBI) AND APOTRANSFERRIN STIMULATE THE EXPRESSION OF L-TYPE VOLTAGE-GATED CALCIUM CHANNELS OF CARDIOMYOCYTES

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(Presented By: Edith Wiener)

Introduction: In iron overload, the iron-binding capacity of transferrin is near saturation: NTBI species appear (0.4-10 μM) while apotransferrin is grossly reduced or absent. NTBI is taken up by the heart, liver and endocrine tissues inflicting damage to these organs. In a mouse model of iron-overload, the uptake of ferrous iron by the myocardium was related to L-type voltage-gated calcium channels (1) while recently, Kumfu et al showed that T-type calcium channels are involved in the cardiac pathology of β-thalassaemic mice (2). Additionally, iron loading of HuH7 liver cells causes modulation of membrane T- and L-type calcium channels (3). We have investigated the effect of NTBI and apotransferrin on the expression of L- and T-type calcium channels of HL-1 cardiomyocytes.
Methods: Cardiomyocytes expressing both L- and T-type calcium channels (4) were cultured (5) and plated out on chamber slides (4 chambers/slide, 2x105/chamber). Next day they were re-incubated in Claycomb medium containing different concentrations of ferric ammonium citrate (FAC) or apotransferrin. After 24h they were washed, fixed in paraformaldehyde, permeabilised in triton X-100, incubated overnight with rabbit antibodies against α 1 subunit D (L-type) or α 1 subunit H (T-type) and subsequently stained with an FITC-labelled anti-rabbit Ig antibody and submitted to quantitative confocal microscopy. Fluorescence analysis was performed using the Volocity program and the results expressed as object measurements (total cellular α 1 subunit) and line profiles (α 1 subunit intracellular distribution).
Results: Treatment of HL-1 for 24h with 2uM FAC consistently caused an almost two-fold rise in α1 subunit D ($p<0.05$) while with the higher FAC concentrations (20, 200 μM), stimulation was less pronounced. Line profiles revealed no change in the intracellular distribution. With the subnormal apotransferrin concentration of 10μM there was an almost two fold increase in α 1 subunit D. Line profiles revealed redistribution of this subunit from the cell centre to the periphery. By contrast, under any of these conditions, the expression of α1 subunit H remained unchanged.
Discussion: The stimulated expression by HL-1 cells of α1 subunit D at low concentrations of FAC and apotransferrin suggests that in iron overload, these factors bring about a rise in cardiac L-type calcium channels that mediate influx of iron into the heart. The angiotensin II-induced cardiac expression of Ltype α1C subunit is mediated via a protein kinase C-, reactive oxygen-mediated species- and CREB-dependent pathway (6). A similar mechanism might underlie the induction of the L-type α 1D subunit by NTBI/apotransferrin in the present system.
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Poster #55

NEET IS A NOVEL CLASS OF 2FE-2S PROTEINS WITH A UNIQUE NEET-FOLD AND A LABILE CLUSTER – LESSONS FROM STRUCTURE

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(Presented By: Rachel Nechushtai)

Introduction: MitoNEET (mNT:CISD1) and Miner1 (Naf-1:CISD2) are the major members of NEET, a newly discovered class of 2Fe-2S proteins involved in a diverse array of biological processes including autophagy, apoptosis, aging, diabetes, cancer and iron and reactive oxygen homeostasis. The proteins are endowed with a 'NEET fold' and a [2Fe-2S] cluster (ISCs) composed of one His and three Cys residues as Fe coordinating groups that render the cluster with uniquely predictable lability.

Methods and Materials: Those were demonstrated experimentally as ISC transfer from native NEETs to apo-acceptor proteins in solution or to mitochondria in permeabilized cells (1). Abrogation of the labile features is attained by mutating the His (87 in mNT and 114 in Naf-1) to Cys as well as by pretreatment with pioglitazone. Recently, structural and functional characterization (in knockdown and RNAi plant line) of Arabidopsis NEET revealed an ancient role for NEET proteins in iron metabolism (2). Moreover, the availability of the three NEET structures enabled the finding that despite their high structural similarity, the electrostatic surface and hydrophobic regions are different for each protein, predicting different protein-interacting partners and associated functions. Accordingly, we recently found NEET proteins to interact with key players in apoptosis and mitochondrial metabolism; while Naf-1 interacts with Bcl-2/BCl-XL and Bid, mNT interacts with VDAC.

Results: These interactions alter the stability of the NEET Fe-S cluster and its ability to be transferred to acceptor protein(s) or mitochondria.

Conclusion: The structural and functional characteristics of these interactions will be described and their implications for key aspects of metabolism in health and disease.

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Poster #56

MITOFERRIN IS ESSENTIAL FOR NORMAL DEVELOPMENT IN DROSOPHILA

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(Presented By: Maria Lind)

Introduction: Mitochondria are crucial for iron metabolism, being the unique site for heme synthesis and the major site for iron-sulfur (Fe-S) cluster biosynthesis. Iron is presumably transported into mitochondria by the mitochondrial carrier protein mitoferrin, which is located in the inner membrane of mitochondria. By studying different dmfrn *Drosophila* mutants we have previously found that dmfrn is essential for male fertility (Metzendorf & Lind, 2010). During this study, we also used two fly strains with deletions in the dmfrn gene. One deletion is small (*dmfrn*^{Df13}, removing a third of the 5´ untranslated region of dmfrn) the other is large (*Df(3R)ED6277*, removing genes *dmfrn* and *CG5514* and half of the 5´ untranslated regions of genes *Mes-4* and *Gp93*). Both of these deletions of dmfrn (homozygous *dmfrn*^{Df13}, transheterozygous *dmfrn*^{Df13}*/Df(3R)ED6277* or homozygous *Df (3R)ED6277*) cause lethality at larval stage.

Methods and Materials: In the current study we analyzed the developmental phenotype in further detail. As fertility of the hypomorph dmfrn P element mutant strains is depended on the level of dietary iron (Metzendorf & Lind, 2010), we were interested if the deletion mutants might show a similar iron sensitivity. Using a third chromosome balancer that allows distinguishing between heterozygous and transheterozygous flies at the larval, pupal and adult stage, allowed us to quantify the genotypes at different developmental stages.

Results: We found that transheterozygous *(dmfrn*^{Df13}*/Df(3R)ED6277*) flies, when on low iron food, developed to third instar larvae but very rarely started puparation (<0.25%). On normal food, transheterozygous flies developed to the pupal stage (~17%), but very few flies eclose as adults (~6%). Knocking down dmfrn expression by Gal4/UAS driven RNA interference (RNAi) with a ubiquitous driver, resulted in a phenotype similar to that of the dmfrn deletion strains. Introduction of the genomic construct *dmfrn*^{venusB32} (a fluorescently (venus) tagged dmfrn) rescued development of dmfrn deletion flies to pupal stage on low iron food, and increased the fraction of eclosed adults with the dmfrn deletion alleles on normal and high iron food. The presence of third instar larvae, homozygous for the deletion, indicates that dmfrn deletion might not cause larval lethality, but may rather slow down development or cause developmental arrest. We are currently investigating whichever is the case.

Conclusion: We conclude that mitoferrin is essential for normal development and since the developmental phenotype of mitoferrin deletion mutants can be rescued to some degree by addition of dietary iron, we propose that this phenotype is due to mitochondrial iron deficiency.
Metzendorf , C. and Lind M.I. (2010) *Drosophila* mitoferrin is essential for male fertility: evidence for a role of mitochondrial iron metabolism during spermatogenesis. BMC Developmental Biology, 10, 68.

Poster #57
DIVALENT METAL TRANSPORTER 1 (DMT1) IS FOUND IN MITOCHONDRIA
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(Presented By: Frank Thévenod)

Introduction: A large fraction of iron in mammalian cells is present in the mitochondria and a major portion of iron flux enters this organelle. Iron there is critical for heme and iron sulfur cluster formation but too much iron in that oxygen-rich environment is dangerous; thus careful management is necessary. Nevertheless, how iron enters mitochondria is not yet clear. We report here that a portion of intact divalent metal transporter (DMT1) is found in mitochondria of a pneumocyte model after centrifugal fractionation. DMT1 has 4 main isoforms: 1A/+IRE, 1A/-IRE, 1B/+IRE and 1B/-IRE based on variation at N- and C-termini. Moreover, split-ubiquitin yeast 2 hybrid experiments show that two mitochondrial proteins (cytochrome C oxidase subunit II (COXII) and translocase of the outer mitochondrial membrane (OMM) subunit 6 (Tom6) interact with human 1A/+IRE DMT1 as bait. Overexpressed in HEK293 cells and CHO cells, 1A/+IRE hDMT1-FLAG partially co-localized with endogenous COXII and Tom6.
Methods and Materials: While such data show that DMT1 could be in mitochondria, co-immunoprecipitation confirms that it interacts with COXII. Isolating mitochondria from rat renal cortex homogenates and subsequently separating mitoplasts and OMM indicate the presence of DMT1 in OMM by immunoblotting, while marker proteins confirm the identity of the fractions studied.
Results: Permanently transfected HEK293 cells that express either 1A/+IRE DMT1 or 1B/-IRE DMT1 in a doxycycline regulated fashion exhibit the specific isoforms in mitochondria and specifically OMM after fractionation then immunoblotting. Again marker proteins verify the identity of the fractions studied and probing the blots for early endosomal antigen (EEA1) and Lamp2 suggests that neither early nor late endosomes nor lysosomes are contaminants of the mitochondrial fraction. Immunogold labeling indicated co-localization of DMT1 and voltage-dependent anion-selective channel protein (VDAC1) in the OMM of rat renal tissue. DMT1 is found in mitochondria in the OMM in addition to its well-established locations in endosomal/lysosomal vesicles and some plasma membranes.
Conclusion: DMT1, encoded by a nuclear gene and translated on cytosolic ribosomes, represents a novel addition to the mitochondrial proteome. In mitochondria, it is likely that DMT1 may participate in the entry of iron and other metal ions for proper function of mitochondria and, possibly when dysregulated, contributing to disorders that originate in this organelle.
Funding: DFG TH345/11-1 (FT)

Poster #58
FURTHER EVIDENCE FOR A ROLE OF IRON IN DROSOPHILA SPERMATOGENESIS
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(Presented By: Tharse Pathmanathan)

Introduction: Mutations in the sole *Drosophila mitoferrin* gene revealed mitochondrial defects in spermatids, which failed to individualize into mature sperm (Metzendorf and Lind, BMC Developmental Biology, 10:68).
Methods and Materials: Male sterility in flies with hypomorph mitoferrin alleles was suppressed by iron supplementation of the food. From a genetic screen aimed to uncover new genes regulating ferritin expression (Mehta et al., Biochemical Society Transactions, 36:1313-6) we isolated a mutated chromosome, which when homozygous in flies resulted in male-specific reductions in fertility and fecundity; this phenotype was observed only in the absence of one functional copy of the ferritin genes.
Results: We have mapped the relevant mutation within the genomic region removed in Df(2L)BSC292. This deficiency deletes 16 transcribed genes, of which 7 are expressed specifically in the testis and none has a previously known function in iron homeostasis.
Conclusion: On the basis of these findings, we suggest that the major *Drosophila* ferritin (the secreted form) may have a special function in spermatogenesis as has been proposed for its mitochondrial ferritin, which is also a testis specific gene (Missirlis et al., PNAS, 103:5893-8).

Poster #60
WITHDRAWN

Poster #61

IDENTIFICATION OF A SOLUBLE FORM OF TRANSFERRIN RECEPTOR 2 (TFR2)

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(Presented By: Laura Silvestri)

Introduction: TFR2 is a type II transmembrane protein, highly expressed in hepatocytes and erythroid cells, homologous to transferrin receptor 1 (TFR1) with a proposed function of sensor of diferric transferrin (holo-TF). Holo-TF binding to TFR2 causes cell surface stabilization of the receptor; in vivo this mechanism has been proposed to activate hepcidin. In analogy with what is known for soluble TFR1 we search for a soluble counterpart of TFR2.
Methods and Materials: HeLa, Hep3B, HuH7 and CHO-Trvb0 cell lines were transiently transfected with wild type and mutated TFR2 expressing vectors, subjected to different manipulation and the culture supernatants processed and analyzed with western blot for the presence of released forms of TFR2. sTFR2 is released in the culture media of transfected cells as a 75 kDa protein. The shedding is inhibited in a dose dependent manner by holo-TF, suggesting that the ligand binding interferes with the proteolytic cleavage, likely inducing changes in TFR2 folding.
Results: Indeed TFR2 G679A variant, with a mutation in the holoTF RGD binding motif, releases equal amounts of sTFR2 in the presence of holoTF. sTFR2 shedding occurs at the membrane level: when endocytosis is blocked by co-transfection with the mutant form of dynamin (K44A) the release of sTFR2 increases, while brefeldin-A, an inhibitor of the secretory pathway, reduces sTFR2 release. Among several protease inhibitors tested, the furin inhibitor chloromethyl ketone (CMK) inhibits TFR2 shedding. However, overexpression of furin did not modify the amount of sTFR2 suggesting that CMK-sensitive proteases other than furin are involved in this process. By mass spectrometry analysis we identified as possible cleavage targets two sites highly conserved among species, compatible with the molecular weight of the soluble protein (about 75 kDa). To study the role of sTFR2 in the modulation of hepcidin, we used the hepcidin promoter luciferase assay in HuH7 and Hep3B cells transfected with TFR2/HFE. However, in our hands the study of the effect of sTFR2 on hepcidin modulation is hampered by the inability of membrane TFR2 to activate hepcidin in the promoter assay.
Conclusion: Additional studies are needed to clarify a potential functional role of sTFR2.

Poster #62

DOWN REGULATION OF RESPIRATORY ENZYMES IN FRATAXIN DEFICIENT YEAST IS MEDIATED BY THE METABOLIC REGULATORS ADR1 AND CTH2

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(Presented By: David Alsina)

Introduction: Friedreich ataxia is an hereditary disease caused by deficient expression of Frataxin. This mitochondrial protein may play a role in ironsulfur cluster biogenesis, iron storage or iron sensing. Budding yeast has been widely used to study Frataxin function, as it contains a frataxin ortholog of yeast Frataxin depletion on several metabolic pathways.
Methods: We used a conditional yeast strain in which the YFH1 gene is under the control of tet promoter regulated by Doxycycline. We have performed a 2-D based proteomic analysis and a transcriptomic analysis to identify genes and proteins altered after Frataxin depletion. The transcription factor Adr1p was tagged with HA epitope to study its levels by Western Blot, and with GFP to study its localization using fluorescent microscopy. Quantitative RT-PCR was also used to measure mRNA levels from several genes. Aconitase enzymatic activity was measured spectrophotometrically.
Results and Discussion: The proteomic and transcriptomic analysis revealed that the content of many glucose regulated proteins was decreased in Yfh1 deficient cells. Interestingly, most of these proteins are regulated by ADR1, a key transcription factor required for respiratory growth. We observed that after Yfh1 depletion Adr1p was exported from the nucleus, while their protein and mRNA levels were not altered. Moreover, when cells were treated with hydrogen peroxide this transcription factor had a similar response. This regulation mechanism had not been previously described and is different from those observed in response to the presence of glucose. The signaling pathway which triggers Adr1p export from the nucleus is under investigation. We also identified CTH2 as a highly induced gene upon Yfh1 depletion. This gene, usually induced under iron deprivation, is involved in the degradation of mRNAs from several iron-containing enzymes such as Aconitase. We found that in the double mutant (tetO7-YFH1 cth2) Aconitase activity and levels were not altered in comparison with the single tetO7-YFH1 mutant.
Conclusions: From these results we can conclude that Frataxin depletion, in addition to altered iron homeostasis, it also involves drastic metabolic rearrangements governed by ADR1 and CTH2 that finally lead to downregulation of genes involved in respiration. These results also indicate that the deficiency in iron-sulfur enzymes observed in Yfh1p deficient cells is due to down-regulation of these proteins by Cth2p.

Poster #63

IN VITRO STUDIES ON THE TRANSFER OF IRON BETWEEN CERULOPLASMIN AND TRANSFERRINS

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(Presented By: Robert Evans)

Introduction: *In vitro* binding and molecular modelling studies indicate that lactoferrin and transferrin can bind to the serum ferroxidase ceruloplasmin (White et al 2012) such that iron, in its ferric form following oxidation of ferrous iron, can be transferred directly from ceruloplasmin to iron-free transferrin or lactoferrin (White *et al* 2012). Such a mechanism would prevent both the utilisation of iron by pathogenic bacteria and the generation of potentially toxic hydroxyl radicals. However, a direct transfer of iron between the ferroxidase and members of the transferrin family of iron-binding proteins, without its release into the immediate environment, has not been demonstrated experimentally. One approach to address this issue is to investigate the rate of the ceruloplasmin-mediated uptake of ferric iron by transferrins in the presence of an iron chelator. Such a chelator would, however, have to be kinetically faster at sequestering ferric iron than the iron-free protein. Although extensive studies have been reported on the ability of iron chelators to mobilise transferrin-bound iron (Evans *et al* 2012), data on the direct competition between iron-free transferrins and iron chelators is limited. A systematic study of a number of different iron chelators in such competitive studies is therefore required prior to monitoring the effects of chelators on the ceruloplasmin-mediated uptake of iron by transferrins.
Methods and Results: Desferioxamine, a therapeutically useful iron chelator, has a log affinity constant for Fe(III) at least four orders of magnitude higher than transferrin. However, it was found that when desferioxamine and iron-free transferrin are present at an equimolar iron-binding capacity, the chelator is inefficient at competing with the protein for iron in the ferric form, irrespective of the nature of the ferric complex.
Conclusion: Further studies are now warranted on a range of different classes of iron chelators with varying affinities for Fe(III) in order to identify chelators that are kinetically more efficient than members of the transferrin of iron-binding proteins at sequestering ferric iron.
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Poster #64

DEFINING THE ARCHITECTURE OF THE MITOCHODRIAL IRON-SULFUR CLUSTER ASSEMBLY MACHINERY

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(Presented By: Belinda Galeano)

Introduction: Iron-sulfur clusters (ISC) are essential enzyme co-factors that participate in a large number of cellular reactions and processes. In eukaryotic cells, ISC are primarily synthesized in the mitochondria although ISC assembly also occurs in other cellular compartments. The mitochondrial proteins known to participate in ISC synthesis are highly conserved from yeast to humans, and many were inherited from bacteria. They include early components that provide the building blocks to assemble new [2Fe-2S] and [4Fe-4S] clusters, and late components that transfer these clusters to mitochondrial apo-proteins.
Methods and Results: The general roles played by individual components have been assessed through genetic and biochemical studies in prokaryotes and S. cerevisiae and confirmed for the corresponding human components. According to current models, synthesis of [2Fe-2S] clusters in the mitochondrial matrix is initiated on a scaffold protein (yeast Isu1/mammalian ISCU) and involves (i) a cysteine desulfurase (yeast Nfs1/mammalian NFS1, stabilized by a small binding partner, Isd11/ISD11) that serves as the sulfur donor; (ii) frataxin (yeast Yfh1/mammalian FXN) that serves as the iron donor; and (iii) the electron donor chain formed by ferredoxin reductase and ferredoxin (yeast Arh1-Yah1/mammalian FDXR-FDX2). Subsequently, release of ISC from Isu1/ISCU and their transfer to apo-enzymes are assisted by various chaperone and co-chaperones, proteins needed to reduce cysteine residues on apo-enzymes, and ISC carrier proteins. In these models, the ISC scaffold protein, Isu1/ISCU, is thought to cycle between the early and the late components. However, recent studies indicate that mitochondrial ISC synthesis occurs on stable complexes built-up of at least four components: Nfs1, Isd11, Isu1 and Yfh1 in yeast; NFS1, ISD11, ISCU and FXN in humans. These complexes have been reconstituted in vitro with purified proteins (Gakh et al., 2010; Li et al., 2009; Schmucker et al., 2011; Tsai and Barondeau, 2010) and have also been reconstituted with native proteins from yeast or human cells (Gakh et al., 2010; Gerber et al., 2003; Li et al., 2009; Schmucker et al., 2011).
Conclusion: Together, these findings point to the existence of complex macromolecular machineries that eukaryotic cells have developed to perform ISC synthesis inside mitochondria. Given the vital functions controlled by these machineries, it is important to elucidate their structural architecture and the catalytic mechanisms. We are focusing on the yeast core components and their sub-complexes using single-particle reconstruction and complementary techniques. We have obtained three-dimensional models of the iron-loaded yeast frataxin, Yfh1, oligomer bound to the ISC scaffold, Isu1. The models consistently indicate that Isu1 binds to the 24-subunit Yfh1 oligomer near the channel at the 4-fold axis of the oligomer, a region that contains amino acids known to be critical for the safe handling of iron by frataxin.

Poster #65

THE ROLE OF TMPRSS6 CAUSING IRIDA DURING FETAL AND NEONATAL LIFE

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(Presented By: Luigia De Falco)

Introduction: Mutations in TMPRSS6 (matriptase-2), a transmembrane serine protease expressed by the liver, result in the clinical phenotype of iron refractory iron deficiency anemia (IRIDA). TMPRSS6 mutations in patients with IRIDA were firstly described by Finberg et al (Finberg, et al., 2008). Until now, 32 IRIDA families reporting 49 patients of different geographic and ethnic origin have been described (Pellegrino, R M, et al. 2012, Guillem, F, et al. 2012, Choi, H S, et al 2012, Cuijpers, M L, et al. 2010, Beutler, E, et al. 2010, Altamura, S, et al. 2010, De Falco et al., 2010, Tchou, et al., 2009, Edison, et al., 2009; Silvestri, et al., 2009, Ramsay, A J, et al. 2009, Melis, et al., 2008; Guillem, et al., 2008; Finberg, et al., 2008). The role of the proteolytic enzyme matriptase-2 in iron metabolism was first demonstrated in murine models. Mask homozygotes (Du, et al., 2008) and Tmprss6 null mice (Folgueras, et al., 2008) are slightly smaller than their normal littermates and show microcytic anemia with low plasma iron levels and depleted iron stores. Previously we described 12 IRIDA patients belonging to 7 unrelated families and identify 10 (9 novel) TMPRSS6 mutations spread along the gene sequence: 5 missense, 1 non sense and 4 frameshift (De Falco et al., 2010).

Methods and Materials: In this work we reported a Turkish family with a novel homozygous deletion in exon15 which produces a truncated protein like in Mask mouse. Furthermore this Turkish family have three affected children one of which had a molecular diagnosis before she developed clinical phenotype at the age of 3 months. In 2010 at the ASH congress Finberg K. et al reported that Tmprss6 regulates hepcidin expression in the fetal and neonatal periods in mice. However, Tmprss6 deficiency does not appear to be associated with systemic iron deficiency at these stages of development, and fetal Tmprss6 expression does not have a significant effect on maternal iron homeostasis in late gestation.

Results: From all the cases reported in the literature, and from the personal experience of the authors, it is clear that anaemia is not detected at birth and the clinical phenotype develops only after the neonatal period, suggesting that MT-2 is probably dispensable during fetal life. This is compatible with the idea that the regulation of iron metabolism by hepcidin becomes effective only after birth, when the oral diet starts. Also we hypothesized a switch mechanism for TMPRSS gene family from the fetal/neonatal period to the first 3 months of life as well as in haemoglobin switch.

Conclusion: These results may have implications for understanding the maintenance of iron homeostasis in early development, and may provide insight into the evolution of IRIDA as well as other disorders of iron homeostasis.

Poster #66

MOLECULAR ANALYSIS OF GROWTH DIFFERENTIATION FACTOR 15 IN ANEMIA OF PREGNANCY

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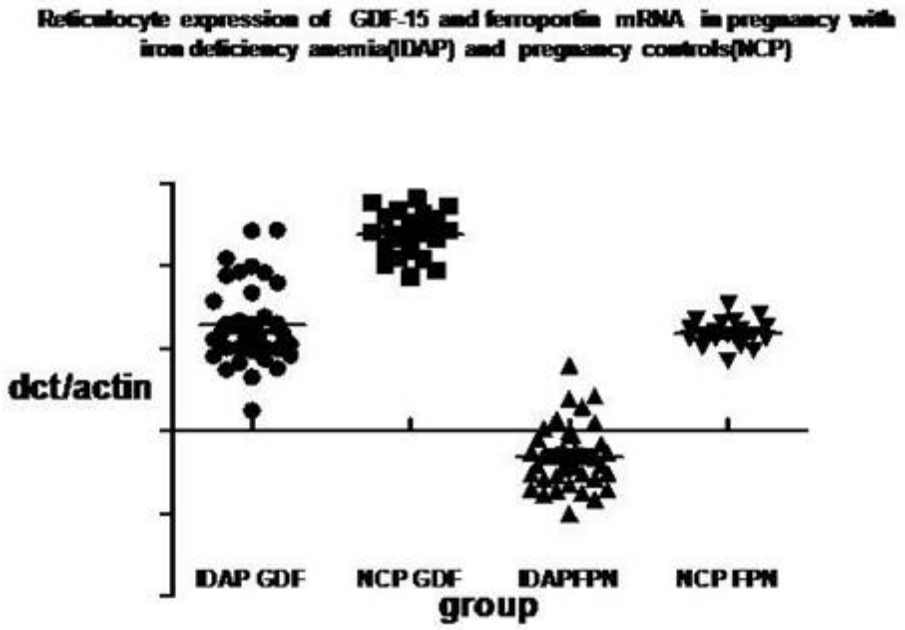
(Presented By: Rekha Athiyarath)

Introduction: Anemia of pregnancy is mostly attributable to iron deficiency associated with increased iron requirements due to expansion of the maternal erythrocyte mass and high iron demand of the growing foetus. Maternal anaemia is associated with poor intrauterine growth and increased risk of preterm births and low birth weight rates.Cytokines in the transforming growth factor beta (TGF-β) super family such as growth differentiation factor 15 (GDF-15) are thought to have multiple functions during pregnancy.GDF-15,secreted mainly by the placenta, is present in high levels in the sera of pregnant woman and rises substantially with gestational age. The role of GDF-15 is mainly ascribed to promote foetal survival by suppressing the production of maternally derived pro-inflammatory cytokines within the uterus. In contrast GDF-15 has been associated with increased iron absorption in patients with β-thalassemia, by negatively regulating hepcidin. It has also been shown that iron depletion will increase GDF-15 expression.

Methods and Materials: Thus we sought to investigate the expression of GDF-15 and its role in iron deficiency anemia of pregnancy. We included fifty pregnant women with anemia (IDAP) [Hb≤11g/dl MCV<78fl] between 10-18 weeks of gestation and twenty healthy pregnant controls (NCP) in the same gestational age. Serum GDF15 levels were measured using ELISA (Ray Biotech, Inc, Georgia) and ferritin by chemiluminescent immunoassay. Reticulocytes from patients and controls were isolated from peripheral blood using cellulose columns. RNA was extracted by Trizol method and cDNA was synthesised. The relative expression of GDF15 and FPN mRNA was analyzed using actin as the control gene.

Results: The median Hb in IDAP group was 9.9g/dl (6.2-11) and MCV was 71.3fl (55.7-79.3).The median ferritin and transferrin saturation in IDAP group was 7ng/ml (0.2-42.70) and 6.42% (2.3-34.7) respectively. The median level of GDF-15 in IDAP was 12395pg/ml (6461-15694) and 11502pg/ml (8118-12377) in NCP. Serum GDF-15 levels were significantly higher in IDAP compared to NCP ($p=0.01$).The reticulocyte expression of GDF-15 and FPN mRNA was significantly higher in IDAP compared to NCP group ($p<0.0001$) (Fig.1). A significant correlation was observed between GDF15 and FPN mRNA in

IDAP (r=0.645, p=0.000) which was not seen in NCP. In IDAP we observed a significant correlation between GDF15 mRNA and ferritin level (r=0.38, p=0.008).
Conclusion: These findings indicate a possible role of GDF-15 in regulating maternal iron homeostasis in pregnancy in addition to its anti-inflammatory function. We hypothesise that high level of GDF-15 in IDAP helps to enhance iron absorption in pregnancy by up regulating FPN.



Poster #67
ASSOCIATION OF SINGLE NUCLEOTIDE POLYMORPHISMS WITH TIBC AND ERYTHROCYTE COUNT IN IRON DEFICIENCY ANEMIA OF PREGNANCY IN INDIAN POPULATION
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(Presented By: Rekha Athiyarath)

Introduction: Iron deficiency is one of the major public health problems in India. It affects 80% of pregnant women in India and anemia during pregnancy remains a problem despite of routine iron supplementation. The etiology of iron deficiency anemia has been mainly attributed to poor socioeconomic status and poor dietary intake. In spite of addressing these etiologic factors, there is still a high frequency of patients in whom the cause for IDA could not be identified. Recently, genome wide association studies have shown a possible association of iron regulatory gene polymorphisms with various haematological and biochemical parameters that determines the iron status of an individual.
Methods and Materials: We have selected two SNP's of high impact based on previous GWAS studies and literature.TMPRSS6 rs855791 SNP was strongly associated with Hb, iron status and erythrocyte volume in multiethnic population. Blanco et al has shown that SNP in HFE gene rs1799945 (H63D) is associated with low transferrin and high ferritin.H63D could have a protective effect against the development of anaemia in menstruating women. Hence, we undertook this study to analyse the possible contribution of TMPRSS6 (rs855791) and HFE (rs1799945) polymorphisms to iron status and response to iron supplementation in pregnancy. One hundred and fifty (N=150) pregnant women with anemia between 10-18 weeks of gestation were enrolled in this analysis. All were supplemented with elemental iron of 100mg.The haematological and biochemical parameters were reassessed after 8 weeks of supplementation (follow up).DNA was extracted, amplified and rs855791 and rs1799945 were screened using RFLP.
Results: Statistical analysis was performed using SPSS software and a p value of <0.05 was termed significant. The mean Hb at diagnosis was 9.4g/dl and MCV 71.5fl.The mean Hb and MCV in follow up samples were 10.8g/dl and 80.53fl respectively. The mean compliance was found to be 87% .The T allele frequency of rs855791 was 52.3% and C was 47.6% in our cohort. The wild type allele frequency of rs179995 was 90.3% and minor allele showed a frequency of 9.6%. In contrast to previous studies we couldn't find any association with Hb and ferritin between groups with these polymorphisms. But TIBC was significantly higher in follow up samples with rs855791 variant and H63D was significantly associated with low RBC count at diagnosis.

Conclusion: These findings suggests that there are other genetic factors apart from these polymorphisms associated with predisposing one to iron deficiency anemia but it also may influence the response to iron supplementation.

Table.1. Mean±SD of haematological and biochemical values and its association with SNP's

SNP	Hb (g/dl)		MCV (fl)		RBC (*10⁶/µl)		TIBC (µg/dl)	
	D(149)	F(124)	D(149)	F(123)	D(149)	F(123)	D(97)	F(97)
rs855791								
TT	9.4±1.14	10.5±1.11	72±6.8	80.4±5.9	4.15±0.37	4.04±0.39	386±61.7	359±46.7
TC	9.4±1.27	11.0±1.30	71±6.6	81.0±5.8	4.23±0.29	4.20±0.45	388±114	362±58.1
CC	9.5±1.43	10.7±1.42	71±8.1	80.0±6.0	4.26±0.46	4.15±0.49	385±59.5	400.6±69.9
p value	0.96	0.155	0.76	0.98	0.71	0.74	0.99	0.01
rs1799945								
CC	9.5±1.1	10.7±1.2	71.0±6.9	80.2±5.6	4.24±0.36	4.14±0.45	392±85.7	375±70.8
CG+GG	9.07±1.6	11.1±1.4	71.4±8.2	81.6±7.0	4.05±0.35	4.12±0.39	360±54.9	351.5±39.1
p value	0.36	0.34	0.88	0.28	0.82	0.79	0.11	0.36

Poster #68

IRON PROPHYLAXIS IN PREGNANCY: FERROUS BISGLYCINATE 25 MG IRON MATCHES FERROUS SULPHATE 50 MG IRON

Nils Milman, MD[1], Lisbeth Jønsson, MD[2], Pernille Dyre, Midwife[2] and Palle Pedersen, MSci[3]
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(Presented By: Nils Milman)

Objective: To compare the effects of oral ferrous bisglycinate 25 mg Fe2+/day vs. ferrous sulphate 50 mg Fe2+/day in order to assess the lowest dose of iron needed to prevent iron deficiency (ID) and iron deficiency anemia (IDA) in pregnant women.
Methods: A non-inferiority, randomised, double-blind, intention-to-treat study comprising 80 healthy Danish pregnant women allocated into two groups taking either ferrous bisglycinate in a dose of 25 mg elemental iron (n=40) or ferrous sulphate in a dose of 50 mg elemental iron (n=40) from 15-19 weeks gestation to delivery. Hematological status (hemoglobin, red blood cell indices) and iron status markers (plasma iron, plasma transferrin, plasma transferrin saturation, plasma ferritin) were measured at 15-19 weeks (baseline) 27-28 weeks and 36-37 weeks gestation. ID was defined as ferritin <12 µg/L and IDA was defined as ferritin <12 µg/L and hemoglobin <110 g/L. Gastrointestinal symptoms during iron supplementation were recorded.
Results: At inclusion, there were no significant differences between the two iron supplementation groups concerning hematological status and iron status. Body weight was higher (p=0.02) in the bisglycinate group than in the sulphate group. At 27-28 weeks gestation, there were no significant differences between the two groups concerning hematological status or iron status. At 36-37 weeks gestation, hematological status was similar in the two groups. Concerning iron status, the bisglycinate group had slightly lower transferrin saturation (18 ± 9% vs 24 ± 9%, p=0.02) and log10 ferritin (1.31 ± 0.291 vs 1.48 ± 0.291, p=0.02) than the sulphate group. There were no significant differences between the bisglycinate vs the sulphate group concerning the frequency of ID (4/27 vs 1/29 women, p=0.4) and IDA (0/27 vs 1/29 women, p=0.9). The frequencies of various gastrointestinal symptoms were not significantly different in the two groups, except that black stools was less frequently observed in the bisglycinate group (p=0.003). Newborns' birth weight was higher in the bisglycinate group vs. the sulphate group (3601 ± 517 vs 3360 ± 435, p=0.05).
Conclusions: In the prevention of ID and IDA in pregnant Danish women, a supplement of 25 mg ferrous iron/day as bisglycinate was not inferior to 50 mg ferrous iron/day as sulphate, probably because ferrous bisglycinate displays a relatively higher absorption than ferrous sulphate. Ferrous bisglycinate in a low dose of 25 mg iron/day appears to be adequate to prevent IDA in more than 95% of the women during normal pregnancy.

Poster #69

ROLE OF TRANSFERRIN IN RECOVERY FROM PHLEBOTOMY IN IRON REPLETE AND IRON DEFICIENT MICE

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(Presented By: Yelena Ginzburg)

Introduction: The most common reason for deferral of blood donation is low hemoglobin concentration and anemia. Furthermore, repeated donations, especially in young females, may lead to iron deficiency, drop of hemoglobin, and

consequent iron deficiency anemia. Recent data suggests that iron deficiency, in the absence of anemia, may be associated with symptoms in otherwise healthy individuals. Our previous findings suggested that additional transferrin ameliorates anemia in mouse models of β-thalassemia; this effect appears driven by an increase in the number of circulating red blood cell (RBC) by extending RBC survival (Li Nat Med 2010).

Methods and Materials: We hypothesize that exogenous transferrin may increase RBC count and enable a more rapid recovery after phlebotomy. To test this hypothesis, C57 mice were fed an iron replete (35ppm iron) and iron deficient (2.5ppm iron) diet for 3 months to simulate the human condition. Mice were phlebotomized an average of 220ul of blood weekly for four weeks. Additionally, all mice received intraperitoneal (IP) injection of either of 10mg of human apo-transferrin (apo-Tf) (200 ul volume) or 200ul PBS daily Monday – Friday for 4 weeks (in total 20 days of injection). Mice (n=4/group) were sacrificed and samples analyzed at the end of the 4 weeks. RBC survival was evaluated using an in vivo biotin avidin assay previously described (Li Nat Med 2010).

Results: We analyzed all RBC indices using ADVIA 120 (Bayer). MCV decreased in mice on 2.5ppm compared to 35ppm diet ($p=0.012$) and after apo-Tf injection ($p<0.05$ in 2.5ppm diet, and $p<0.001$ in 35ppm diet). Average of 50% higher reticulocytes counts was found in apo-Tf treated compared to PBS treated mice on 2.5ppm diet ($p=0.006$); no effect was observed in mice on 35ppm diet ($p=0.23$). However, apo-Tf resulted in an increase in RBC counts in phlebotomized mice on 35ppm diet ($p=0.056$). Furthermore, using antibodies to CD44 and TER119 in flow cytometry (Chen PNAS 2010), we assessed the proportion of erythroid precursors at all stages of terminal erythroid differentiation. Apo-Tf injections result in more erythroid precursors in spleen and bone marrow ($p<0.05$ in early stage precursors on 2.5ppm diet and $p<0.05$ for all erythroid precursors on 35ppm diet) without changes in the degree of apoptosis or quantity of reactive oxygen species. Although no differences in spleen size or spleen / body weight is observed in apo-Tf treated mice on 35ppm diet, 2.5ppm diet resulted in mice with smaller spleens relative to 35ppm diet (0.002 vs. 0.004 spleen / body weight, $p=0.05$) that increased as a consequence of apo-Tf injection (0.003 vs. 0.002 spleen / body weight, $p=0.02$). Immunohistochemistry of spleen samples using anti-TER119 antibodies revealed more extramedullary erythropoiesis in all apo-Tf treated mice. Lastly, no difference in RBC survival was observed in mice as a consequence of diet ($p=0.36$) in linear mixed effects model.

Conclusion: Taken together, our data suggest that exogenous apo-Tf in recurrently phlebotomized mice on iron deficient diet stimulates erythropoiesis and a relative reticulocytosis. Because all stages of erythroid precursors in mice on 35ppm diet are also increased in apo-Tf treated mice without an effect on reticulocytosis but an increase in circulating RBCs, the relative role of iron availability and iron delivery on different stages of erythroid differentiation requires further analysis.

Poster #70

CLINICALLY IDENTIFIED TMPRSS6 MUTATIONS RESULT IN EITHER TRAFFICKING DEFECTS OR INABILITY TO CLEAVE HEMOJUVELIN

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Queensland Institute of Medical Research
(Presented By: Nigel C. Bennett)

Introduction: Mutations in the *TMPRSS6* gene are associated with severe Iron Refractory Iron Deficiency Anaemia, resulting from an over−expression of hepcidin leading to a blockade of iron absorption and recycling. The TMPRSS6 protein is thought to act to prevent over−expression of hepcidin by cleaving hemojuvelin (HJV, also known as HFE2), a co−receptor in the BMP6−hepcidin signalling pathway. *TMPRSS6* loss−of−function mutations result in no cleavage of HJV and consequently the pathway of hepcidin up−regulation remains constitutively active.

Methods: Site−directed mutagenesis was used to create FLAG−tagged expression constructs of six clinically identified *TMPRSS6* variants. The Y141C and I212T mutations occur with the SEA domain, R271Q and G442R within the CUB domains, and C510S and D521N within the LDLR domains of the TMPRSS6 protein. The HepG2−C3A cell line was transiently transfected with the *TMPRSS6* constructs with either low (200ng / 9.6 cm2 well) or standard DNA (1 µg / 9.6 cm2 well) concentrations. Cellular localisation of the expressed proteins were assessed by whole−cell or cell−surface immunofluorescence (IF). A clonal HepG2−C3A cell line stably expressing HJV was also transfected with the *TMPRSS6* constructs to assess their ability to cleave HJV.

Results: The concentration of transfected DNA had a significant influence on the cellular localisation of the TMPRSS6 mutants. In wild type, R271Q and G442R expressing cells, the number of cells showing surface localisation of the TMPRSS6 protein was equal to the number of cells detectable as transfected in whole−cell IF at both DNA concentrations. However, in cells expressing the Y141C, I212T, C510S and D521N mutants the proportion of cells with surface expression was approximately 10% with low DNA concentrations, but increased with higher DNA concentrations to around 50%. In cells with clonal stable expression of HJV, co−expression of wild type TMPRSS6 led to cleavage of HJV as evidenced by the lack of detectable HJV on the surface of only those cells expressing TMPRSS6. In cells transfected with standard concentrations of DNA where cell surface localization of the Y141C, I212T, C510S and D521N mutants was detected, HJV cleavage was also apparent. The R271Q and G442R appeared unable to cleave HJV.

Conclusion: These results suggest that the use of over expression systems in TMPRSS6 studies should be approached with caution. It is apparent that at lower levels of expression, the Y141C, I212T, C510S and D521N mutants are retained in the endoplasmic reticulum (ER) and are not trafficked to the cell surface. Thus in spite of their retained capacity to cleave HJV, they should be classified as loss−of−function mutations. However, at higher levels of expression, an ER retention threshold appears to be reached in some cells, resulting in protein release from the ER and trafficking to the cell surface where it retains

the ability to cleave HJV. In this instance, they may be incorrectly assigned as retention−of−function mutations. These results demonstrate that the SEA and LDLR domains of TMPRSS6 are important for protein structure and passage through the ER, but are not required for cleavage of HJV. Conversely, CUB domain mutations do not significantly affect trafficking, but do result in a loss of the HJV cleavage function.

Poster #71

STUDIES ON IN VITRO IRON AVAILABILITY FROM LIGAND-MODIFIED NANO FE(III) OXO-HYDROXIDE COMPOUNDS IN CULTURED HUMAN CELLS

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(Presented By: Yemisi Latunde-Dada)

Introduction: Anaemia is a major nutritional problem affecting all population groups globally. Strategies for preventing iron deficiency include iron supplementation and iron fortification of foods. However, some iron salts that are used for these purposes may cause undesirable gastrointestinal side effects arising from their free radical-generating potentials.
Methods and Materials: Iron uptake from nano iron compounds was evaluated in HuTu cells using the ferritin synthesis ELISA approach. In these cells, ferritin synthesis from nanoparticulate iron poly oxo-hydroxide decreased when pH was increased from 5 to 7 and uptake of iron was dose dependent.
Results: Although silencing the DMT1 gene inhibited iron uptake from FeSO4, there was no effect on iron uptake from the nano compounds. Similarly, uptake from these nano compounds was unaffected by the ferrous iron chelator ferrozine which inhibited uptake from FeSO4 and Fe-NTA. Furthermore, while anti Dcytb antibody did not influence iron uptake from the nano compounds, iron uptake from Fe-NTA was significantly inhibited.
Conclusion: The ligand-modified nano Fe(III) oxo-hydroxide compounds appear to be absorbed through a pathway that is independent of DMT1.

Poster #72

RESPONSIVENESS TO ORAL IRON THERAPY OF TWO IRIDA PATIENTS

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(Presented By: Luigia De Falco)

Introduction: Iron Refractory Iron Deficiency Anemia (IRIDA, OMIM #206200, ORPHA209981) is an autosomal recessive genetic disorder caused by mutations in TMPRSS6 gene mapping to chromosome 22q12-q13. TMPRSS6 is involved in iron metabolism through the regulation of the expression of hepcidin, which is the master regulator of iron hemostasis. Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. (Nemeth et al 2004). TMPRSS6 mutations lead to failure of negative control of hepcidin expression resulting in high levels of hepcidin with consequent IRIDA phenotype. Clinically, IRIDA subjects are characterized by hypochromic (low MCH), microcytic (low MCV) anaemia, and very low serum iron levels and transferrin saturation. However, serum ferritin may be normal, or even slightly elevated in patients following intravenous iron treatment. IRIDA patients fail to respond to oral ferrous sulfate therapy and partially respond to parenteral iron administration (Melis et al, 2008, Guillem et al, 2008, De Falco et al, 2010, Sato et al, 2011, Tchou et al, 2009, Beutler et al, 2010). So far there are only few reports on the efficacy of the iron treatment and on the follow up of these patients and only one case on the efficacy of the oral iron therapy when supplemented with ascorbic acid (Cau etal , 2012).
Methods and Materials: Here we report two siblings homozygous for a loss of function mutation in TMPRSS6 gene (R599X), who responded to oral iron therapy. The patients were referred to our department at the age of 10 and 7 years, respectively, however, their anemia was detected first when they were 2 and 5 years old, respectively. The parents informed us that in the past (before admitting to us), significantly higher Hb values were reached with p.o. iron treatment, however, after the cessation of therapy, Hb declined rapidly again. They have been using p.o. iron for more than one year (since October 2011).
Results: We began p.o. iron (ferro glycine sulphate) at a dose of 6 mg/kg/d. The children put on weight significantly after p.o.iron. In their evaluation about 3 months ago, the dose was decreased to 2-3 mg/kg/d. The haematological and iron parameters results are reported in Table1.

	PATIENT 1 (11) 21.10.2011	PATIENT 1 (8) 21.10.2011	PATIENT 1 (11) 5.12.2012	PATIENT 1 (8) 5.12.2012
Hb (g/dl)	8.96	7.93	12.68	12.12
Hct (%)	29.3	26.04	39.46	37.48
MCV (fL)	57.79	57.12	70.91	78.51
MCH (pg)	17.68	17.4	22.78	25.38
MCHC (g/dl)	30.59	30.47	32.13	32.33
RDW	na	na	16.46	15.14
Plt	536900	536600	393800	380000
WBC	5490	7850	7270	7350
Reticulocyte (%)	0.809	0.858	1.378	1.375
RBC	5.07	4558	5565	4775
Ferritin (ng/ml)	na	56.1	166	262
Serum Fe (µg/dl)	7	6	na	na
Transferrin saturation (%)	2	1.9	7.8	15.3
Ceruloplasmin (mg/dl)	na	na	na	na
Haptoglobin (mg/dl)	na	na	na	na
Bilirubin (T and Indirect)	0.64/0.37	0.75/0.45	na	na

Poster #73

IS IRON DEFICIENCY THE MOST PROGNOSTICALLY ADVERSE CAUSE OF ANAEMIA IN PATIENTS UNDERGOING CORONARY ARTERY BYPASS SURGERY?

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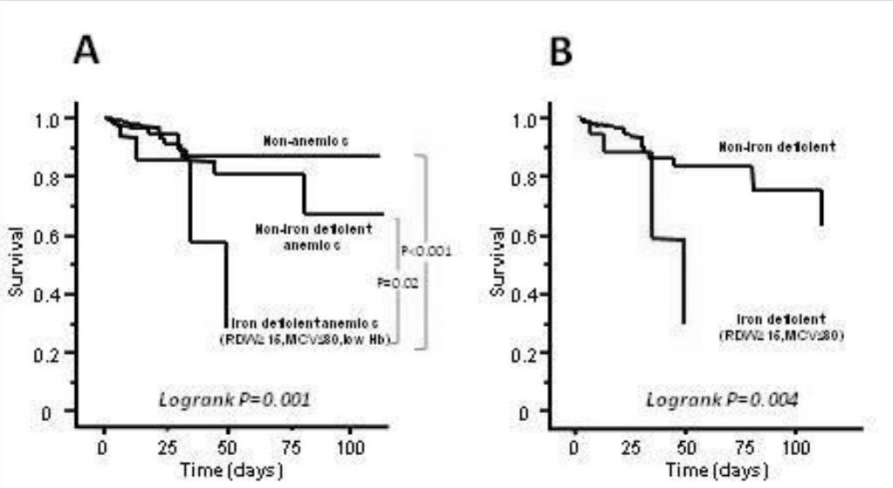
Imperial College London
(Presented By: Nelson Amaral)

Introduction: Whilst pre-operative anaemia increases the risk of morbidity and mortality after coronary artery bypass grafting (CABG), it is unclear whether different causes of anaemia confer different magnitudes of risk. Because iron is quantitatively the most important biocatalyst in human physiology, we hypothesized that iron deficiency (ID) anaemia (IDA) might confer the greatest hazard. Additionally, we supposed that ID even in the absence of anaemia might be detrimental.
Methods and Materials: We analysed hospital outcomes in 1910 CABG patients (mean [±SD] age 67±10 years, EuroSCORE 4±3%, 79% male, 36% Canadian Cardiovascular Score [CCS] >2). The combination of a red cell distribution width≥15% and a mean cell volume ≤80fL was utilised to identify ID as it has a ≥90% sensitivity and specificity for this diagnosis. A haemoglobin <13g/dL in males and <12g/dl in females was used to define anaemia.
Results: On admission, 70%, 27%, and 3% of patients had no anaemia, non-iron deficiency anaemia (NIDA), and IDA, respectively. Patients with IDA were more likely to be female (38% vs 21%), diabetic (53% vs 25%), with poorer left ventricular ejection fractions (51% vs 33% with EF<50%), more congestive symptoms (15% vs 5%), and higher CCS class (2.5±1.6 vs 2.1±1.2), EuroSCOREs (5±3 vs 4±3) and post-op renal (15% vs 8%) and neurological (16% vs 5%) complications (all P<0.05). Over a median (±IQR) hospital stay of 10±12 days, 46 (2.4%) patients died. IDA was associated with increased inpatient mortality (Hazard ratio 3.9, 95% confidence interval 1.6-9.3, P=0.002) independently of all covariates including the EuroSCORE, and conferred a greater risk than NIDA (Fig A). Interestingly, ID per se also predicted an escalated risk of death (unadjusted hazard ratio 3.3, 95% confidence interval 1.4-7.8, P=0.007, Fig B) and did so independently of the presence of anaemia (ID adjusted hazard ratio 2.6, 95% confidence interval 1.1-6.5, P=0.03).
Conclusion: Pre-operative iron deficiency appears to be the most ominous cause of anaemia in CABG patients and confers adversity even in the absence of anaemia. Optimizing iron status in anaemic and non-anaemic subjects might improve outcomes.



Poster #74

ORIGINS OF ANAEMIA VARY WITH CHRONIC HEART FAILURE SEVERITY AND AN IMPROVED IRON STATUS RELATES TO IMPROVED SURVIVAL

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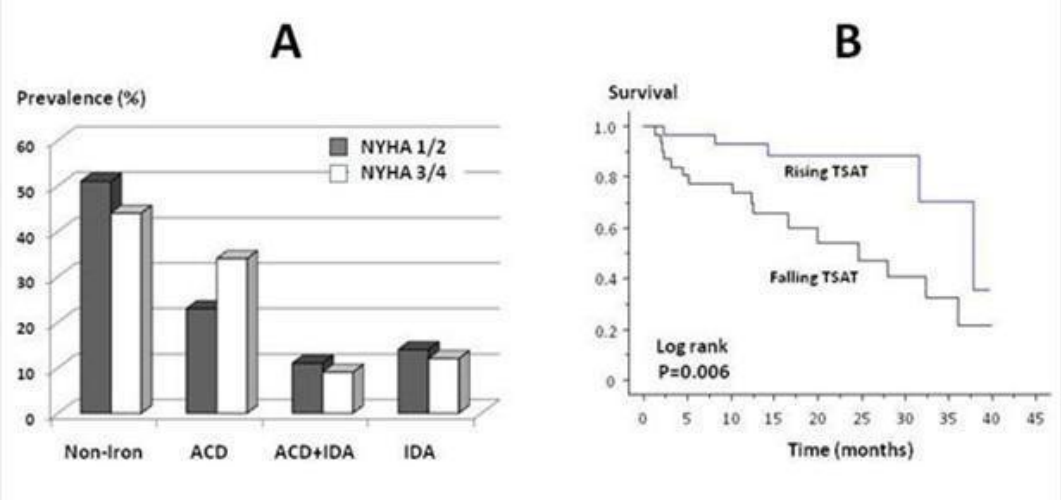
(Presented By: Mohamad Barakat)

Introduction: Iron deficient erythropoiesis due to defectively mobilised (anaemia of chronic disease [ACD]) and/or depleted (iron deficiency anaemia [IDA]) stores is the commonest cause of anaemia globally. However, despite the dire implications of anaemia in chronic heart failure (CHF), few studies have utilised simple iron indices to discriminate IDA from ACD, and to identify IDA in the midst of ACD. Consequently, it is unclear whether the causes of anaemia vary with disease severity or whether improved iron status over time confers a survival advantage.

Methods and Materials: We identified 134 CHF patients who had anaemia (haemoglobin <13 g/dL in men, <12 g/dL women) and available iron indices. IDA (transferrin saturation <20%, ferrtin ≤30 µg/L), ACD (transferrin saturation <20%, ferritin >30 µg/L, total iron binding capacity<65 µmol/L) and ACD+IDA (transferrin saturation <20%, ferritin >30 µg/L, total iron binding capacity ≥65 µmol/L) were diagnosed.

Results: At baseline ACD, ACD+IDA, IDA and a non-iron deficient anaemia (NIDA) were evident in 52%, 9%, 9% and 29% of hospitalised, and 28%, 10%, 13%, and 48% of non-hospitalised patients. ACD increased whilst NIDA decreased in prevalence with increased CHF severity as defined by increasing New York Heart Association class (Fig A). Over a median (± interquartile range) follow-up of 14±18 months, 61 patients had repeat iron studies and 22 (36%) died. Increasing levels of transferring saturation over time related to better survival (hazard ratio 0.95, 95% confidence interval 0.92-0.98, P=0.002) independently of New York Heart Association class, left ventricular ejection fraction and baseline transferrin saturations (Fig B).

Conclusion: The origins of anaemia in CHF vary with disease severity and an improved iron status confers prognostic benefits. Large scale iron repletion trials are needed to corroborate these findings.



Poster #75

SINGLE NUCLEOTIDE POLYMORPHISMS IN THE TMPRSS6 GENE AND IRON STATUS INDICATORS IN KENYAN SCHOOL CHILDREN

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(Presented By: Alida Melse-Boonstra)

Introduction: Iron deficiency is commonly present in Africa, and it has been speculated that this may partly be due to natural selection for protection against infectious diseases such as malaria. Various single nucleotide polymorphisms (SNPs) in the TMPRSS6 gene have been associated with decreased iron status and anaemia in GWAS studies. However, this comprises almost exclusively Caucasian populations and currently there are no data on SNPs in the TMPRSS6 gene from African populations.
Methods and Materials: Therefore, we have assessed six SNPs (rs855791, rs2413450, rs4820268, rs228918, rs228919, and rs228921) in the TMPRSS6 gene in an African population and determined differences in iron status between genotypes. Venous blood samples were taken from schoolchildren (n=360) in Eastern Kenya, and 500 µL of whole blood was immediately added to a similar volume of buffer (Qiagen). DNA was isolated from the blood samples and isolates were stored at -80 °C until SNPs analysis by Taqman assay. Blood serum was analysed for ferritin concentration by ELISA assay and for transferrin receptor by turbidimetric assay.
Results: Haemoglobin concentrations were determined directly in the field by Hemocue device. In three of the SNPs, the frequency of the mutant allele was higher than that of the wild-type allele (rs228918, 59%; rs228919, 57%; rs228921, 55%). All children were heterozygous for rs855791, the SNP which has most frequently been associated with decreased iron status in the existing literature so far. After adjustment for age and gender, we found a significantly higher concentration of serum ferritin (15.5 ng/mL for GG vs. 20.9 ng/mL for TT; P=0.04) and a lower concentration of transferrin receptor (7.17 µg/mL for GG vs. 6.11 µg/mL for TT; P=0.03) in the mutant genotype for rs228919. Moreover, we found a lower concentration of transferrin receptor in the mutant genotype for rs4820268 (6.62 for AA vs. 4.62 for CC, P=0.01).
Conclusion: Significant differences in haemoglobin were not encountered for any of the SNPs. Genotype distributions of SNPs in the TMPRSS6 gene in these African children generally deviated from those published in the literature on Caucasian populations. The mutant T allele in rs228919 with an allele frequency of 57% appeared to be associated with higher iron status in our study population.

Poster #76

DIETARY IRON SHIFTS GUT MICROBIAL METABOLISM TOWARDS A MORE TOXIC PROFILE

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(Presented By: Guus Kortman)

Introduction and Objectives: Iron deficiency is the most prevalent nutrition related disorder worldwide, displaying major health consequences and should therefore be treated. However, safety of oral iron supplementation and fortification in African children has recently been questioned as it was associated with a shift to a more pathogenic gut microbiota profile, increased gut inflammation and an increased incidence of infectious disease. In this study, the effects of different iron preparations on growth and metabolism of the gut microbiota has been investigated.
Materials and Methods: TNO's *in vitro* model of the large intestine (TIM-2) simulates the average conditions in the lumen of the human proximal colon. To assess the effect of iron, TIM-2 was inoculated with a human microbiota that was at first diminished of iron. Next, fermentation was continued for 72 hours with or without addition of 50/250 µM ferrous sulfate, 50/250 µM ferric citrate or 50 µM hemin. Samples were taken regularly and analyzed on the iron content, profiled by 1H-NMR and specifically analyzed for microbial metabolites Short Chain Fatty Acids (SCFA), Branched Chain Fatty Acids (BCFA) and ammonia. Shifts in the microbiota were analyzed by micro-array analyses using a phylogenetic micro-array, the Intestinal (I)-Chip. Intestinal epithelial Caco-2 cells were used for assessment of the fecal water cytotoxicity.
Results and Discussion: Our studies showed that both the composition and metabolism of the microbiota had clearly changed in the iron rich conditions compared to the "low iron" controls. The most prominent shift in microbiota composition concerned an increase of *Prevotella* spp. under iron rich conditions, while Bacteroidaceae content remained similar. The metabolic analyses remarkably showed that microbial protein fermentation pathways were stimulated by iron. More specifically, the production of potentially toxic BCFA and ammonia increased significantly, in particular with ferrous sulfate, while the production of health-promoting SCFA remained similar and ethanol content decreased. Furthermore, under all iron conditions we found a strong decrease in lactate and formate content, which are generally seen as metabolites that are beneficial to intestinal health. Cell viability tests confirmed an increased cytotoxicity of the fecal water originating from iron rich conditions.
Conclusion: Together, these findings indicate that iron stimulates microbial protein fermentation pathways, resulting in increased production of toxic putrefactive metabolites. This could be either a direct effect of iron on bacterial metabolism

and/or indirect via early depletion of carbon sources that resulted in a shift to protein breakdown and/or via growth advantages of certain species. However, we found no increase in protein fermentation when *Prevotella* monocultures were grown with iron. Current metagenomic sequencing efforts will allow mapping of the observed metabolic changes to the microbial metabolic potential at the population level. Ultimately this research will aid in the design of safe iron supplementation programmes in the tropics.

Poster #77

DEVELOPMENT OF A NOVEL ORAL COMPLEX FOR IRON DELIVERY: ENCAPSULATION OF HEMIN IN POLYMERIC MICELLES AND ITS IN-VITRO ABSORPTION

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(Presented By: Kimberley Span)

Purpose: The aim is to develop a novel micellar complex for oral iron delivery. By encapsulating an analogue of heme iron into micelles composed of a biodegradable and thermosensitive diblock copolymer, we want to achieve increased bioavailability over current iron supplements.
Methods. Hemin which is an iron containing heme analogue, was encapsulated into polymeric micelles consisting of biodegradable thermosensitive diblock copolymer poly(ethylene glycol)-b-poly[N-(2-hydroxypropyl) methacrylamide-dilactate]; (mPEG-b-p(HPMAm-Lac2)) using different hemin : polymer ratio and concentrations. The hemin-loaded micelles were characterized in terms of loading capacity, encapsulation efficiency and size. The micelles with the highest loading capacity were chosen for further in vitro absorption testing. In vitro absorption of iron was measured after incubation of the hemin-loaded micelles on Caco-2 cells for 24 hours. Iron sulfate, a commercial oral supplement, iron sulfate in combination with ascorbic acid, free hemin as also dissolved in sodium hydroxide were taken along as controls by incubating the same concentrations as the heminloaded micelles on the cells. Lysed cells were tested for human ferritin (ELISA), which is the iron storage protein and thus a marker for intracellular iron levels and iron absorption. The ferritin levels were normalized against total protein content of the cells.
Results. Hemin was most efficient encapsulated in the micelles with a concentration of 120 µg/ml of hemin in 1,8 mg/ml polymer. This resulted in an encapsulation efficiency of 70% and a loading capacity of 4,5%. The average particle diameter of the hemin-loaded mPEG-b-p(HPMAm-Lac2) micelles was determined by dynamic light scattering and ranged from 80 to 130 nm for concentrations of 40 – 200 µg/ml hemin added to the polymer solution. In vitro testing showed that iron sulfate induced maximum ferritin levels of 150 ng/mg cellular protein. The effect of iron sulfate on ferritin reached a plateau at 50 µM. In contrast, hemin-loaded micelles strongly enhanced ferritin expression up to 2700 ng/mg cellular protein, without reaching a plateau at the highest concentration tested (150 µM). Therefore the in vitro absorption of Hemin-loaded micelles is 18 times higher than that of iron sulfate.
Conclusion. Hemin-loaded micelles with a loading capacity 70% and an encapsulation efficiency of 4,5% were successfully developed. In vitro these micelles drastically increased absorption of iron, and therefore ferritin levels, compared to the most often used commercial iron product.
Acknowledgment. This research was financially supported by Vifor Pharma.

Poster #78

PREPARATION AND CHARACTERISATION OF SOLID LIPID PARTICLES FOR ORAL IRON DELIVERY

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(Presented By: Mohammed Gulrez Zariwala)

Introduction: Iron deficiency which often leads to iron deficiency anaemia (IDA) is one of the most prevalent nutritional disorders worldwide, and is generally treated by supplementation with oral iron preparations. The iron salt ferrous sulphate (FeSO4) is the most commonly used iron supplement. Ferrous sulphate however has a high propensity to cause gastrointestinal side effects by irritation of the gastric mucosa, generally leading to low patient compliance. This has led to the application of microencapsulation technology for oral iron delivery, wherein iron is entrapped and protected within a lipid shell. Lipids have the advantage of high cell membrane permeability, and several liposome based iron delivery products have been developed over the past two decades. The main limitation of liposome formulations is the large particle size which impedes cellular absorption, has relatively low encapsulation efficiency and poor inherent stability due to the high lipid content. Solid lipid particles (SLP's) offer an attractive alternative to liposome's, as they retain the advantages of traditional lipid based systems while overcoming their major limitations.
Methods and Materials: We aimed to formulate iron loaded SLP's and evaluate iron absorption from these *in vitro* using the well characterised human intestinal cell line Caco -2, with intracellular ferritin formation as a marker of iron absorption. Four batches of solid lipid nanoparticles (SLP-Blank, SLP-Fe, SLP-Chi-Blank, and SLP-Chi-Fe) were prepared using stearic acid by hot homogenisation-emulsification process with polyethylene glycol (PEG) as an emulsifier. The mucoadhesive polysaccharide chitosan (Chi) was added to the aqueous phase during emulsification to prepare chitosan coated SLP's. SLP's demonstrated stable physiochemical characterstics as assessed by particle size, zeta potential and morphological analysis.

Results: Particle size analysis reveals a sub-micron size range (232 ± 8.1 nm to 336 ± 13.4 nm), with SLP-Chi-Fe particles having the largest dimensions. As expected, chitosan conferred a net positive charge on particle surface. The encapsulation efficiency of SLP-Fe and SLP-Chi-Fe was 74.9 ± 1.8 % and 81.1 ± 2.2 % respectively, as determined by quantifying iron concentrations in SLP's before and after ultrafiltration. Iron loading did not significantly alter size or morphological features of the SLP's, suggesting good entrapment characteristics. FeSO4 was used as a reference standard in iron-uptake experiments. An equal dose of iron (20 μM) from each preparation was added to each well of Caco-2 cells cultured in six-well plates (n = 5 per sample). Trials were standardized at 2 hours, the estimated physiological transit time through the duodenum, and cells harvested after 24 hours. Intracellular ferritin concentration was determined by ELISA. Caco-2 iron absorption from SLP-Fe (583.98 ± 40.83 ng/mg cell protein) and SLP-Chi-Fe (642.77 ±29.37 ng/mg) was 13.42 % and 24.9 % higher than that from FeSO4 respectively (514.66 ± 20.43 ng/mg). Iron absorption from SLP preparations was therefore significantly greater than that from free ferrous sulphate control ($P \leq 0.05$).
Conclusion: In this study, we describe for the first time the preparation and characterisation of iron-SLP *in vitro*, and demonstrate their superior iron-absorption properties suggesting their potential as a novel alternative to conventional iron delivery.

Poster #79
EXPLORING THE EFFECTS OF IRON DEFICIENCY ON SKELETAL MUSCLE METABOLISM AND FUNCTION
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(Presented By: M.K. Curtis)

Introduction: Physical work or exercise capacity has been widely reported as being impaired by iron deficiency. Previous, separate, studies using diet-induced iron deficient rats have shown decreased work rate capacity, diminished contractile force generation and an impairment of energetics during exercise, when compared to rats on normal chow. The aim of this project was to develop a technique that allows for the simultaneous assessment of force production, fatigue resistance and metabolic function of murine gastrocnemius muscle during exercise. To address this, a method of *in vivo* gastrocnemius muscle stimulation was established. A bespoke Perspex cradle was designed and manufactured for the hindlimb preparation.
Methods and Materials: In this technique, under gaseous anaesthesia, the ischiatic nerve is isolated surgically and electrodes are placed distal to the tibial nerve branch. The knee and ankle joints are immobilised, the calcaneal tendon is attached to a force transducer, via a suture thread, before a 31P saddle-shaped coil is placed over the muscle. The cradle is then placed into the 7 Tesla magnet and images of cross-sectional area (CSA) of the gastrocnemius muscle are obtained using the 1H volume coil. The measurement of CSA of the muscle means the forces produced between muscles of different sizes can be compared. A stimulation protocol consisting of a train of eight pulses of 100 μs at 30 Hz, followed by a rest period of 1.25 seconds, is repeated over 10 minutes. During this exercise period, and the subsequent 20 minute recovery period, 31P spectrum are acquired every 85 seconds.
Results: On average there is a 50% reduction in contractile force produced over the 10 minute exercise period, and an increase in inorganic phosphate and a decrease in phosphocreatine by 31P MRS.
Conclusion: Now that the technique has been established, it will be used to assess muscle function and metabolism, including fatigue resistance, of diet-induced or genetic mouse models of iron deficiency in the future.

Poster #80
CONTINUOUS ERYTHROPOIETIN RECEPTOR ACTIVATOR (C.E.R.A.) PROMOTES IRON UTILIZATION FOR ERYTHROPOIESIS THROUGH INTENSIVE SUPPRESSION OF HEPCIDIN LEVELS IN MICE
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(Presented By: Yusuke Sasaki)

Introduction: Continuous Erythropoietin Receptor Activator (C.E.R.A.) is a novel long-acting erythropoiesis stimulating agent, proven to maintain hemoglobin (Hb) levels effectively with once monthly administration. We investigated iron dynamics associated with erythropoiesis after C.E.R.A. injection in mice.
Methods: C57BL/6N (B6) mice were intravenously injected with 2, 10 μg/kg of C.E.R.A. or vehicle. Iron loaded or overloaded mice were prepared by intraperitoneal injection of 0.5 or 2.5 mg/mouse of iron dextran into B6 mice. Five days after iron loading, 10 μg/kg of C.E.R.A. was injected intravenously. Hematological and iron parameters including serum hepcidin levels were analyzed. Splenic ferroportin (FPN) expression and iron deposition were also evaluated by immunohistochemistory and Berlin Blue stain.
Results: C.E.R.A.-treated mice showed significantly higher Hb levels than in control mice for 14 days after injection, while serum hepcidin levels were intensively suppressed until 8 days after C.E.R.A. injection and nevertheless serum iron was markedly decreased at day 5. There were no difference in increase of Hb levels by C.E.R.A. injection in control and iron loaded mice, but the increase was suppressed in iron overloaded mice. Serum hepcidin levels and splenic iron deposition were decreased at day 5 after C.E.R.A. injection in iron loaded mice and there were no difference in splenic FPN expression levels in control and iron loaded mice. On the other hand, C.E.R.A. injection in iron overloaded mice could not decrease

serum hepcidin to the level of retaining splenic FPN expression. There was no change of splenic iron deposition after C.E.R.A. injection in iron overloaded mice.
Conclusions: These results indicate that C.E.R.A. stimulates effective erythropoiesis with marked iron consumption and leads to effective iron recruitment into erythroblasts from splenic iron storage through intensive hepcidin suppression. It is evident that expansion of iron storage than normal status might not be effective to enhance the C.E.R.A. efficacy, because iron storage expansion failed to up-regulate FPN expression, the rate limiting transporter of iron efflux from storage. It is also revealed that excess iron storage caused low efficiency of iron metabolism and erythropoiesis with functional iron deficiency resulting from high hepcidin levels. These results suggest that there is a need to regulate iron status adequately in order to maximize the effects of C.E.R.A. on enhancement of erythropoiesis and iron metabolism.

Poster #81
IRON SUPPLEMENTATION IN SUCKLING PIGLETS: HOW TO CORRECT IRON DEFICIENCY ANEMIA WITHOUT INCREASING PLASMA HEPCIDIN LEVELS
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(Presented By: Rafal R. Starzynski)

Introduction: Among mammalian species neonatal iron deficiency anemia is particularly frequent and severe pathology in pig, regardless of the breed and the system of piglet rearing. Therefore, intramuscular administration of large amounts of iron dextran to newborn piglets, which aims at rectifying their hematological status is current practice in the swine industry. However, high amounts of iron dextran routinely given to piglets can generate a negative feedback loop that reduces duodenal iron uptake and the recycling of supplemental iron deposited in the reticulo-endothelial system. Hepcidin, a peptide induced by iron and secreted mainly by the liver, acts as a negative regulator of iron absorption and reutilization.
Methods and Materials: The aim of the study was to establish a protocol of iron dextran administration to pig neonates, which could meet their iron requirements for erythropoiesis without increasing plasma hepcidin levels. Here, we compare the development of red blood cell indices, plasma iron parameters and plasma hepcidin levels during the 28-day period after birth (till the weaning), after intramuscular administration of iron dextran to Polish Landrace x Polish Large White suckling piglets according to 3 different protocols involving injections of: A) 150 and 40 mg Fe/kg b.w. on days 3 and 21 postpartum, respectively; B) 37.5 mg Fe/kg b.w. on days 3 and 14 postpartum; C) 37.5 mg Fe/kg b.w. on day 3 postpartum only. Piglet plasma hepcidin-25 measurements were performed by a combination of weak cation exchange chromatography and time-of-flight mass spectrometry, as described previously for human plasma samples [Kroot et al., 2010; Swinkels et al., 2008].
Results: The routine split supplementation, involving injection of high amount of iron on day 3 and 21 postpartum (protocol A) resulted in the recovery of piglets from iron deficiency but induced high plasma hepcidin concentration. Although a single injection on day 3 after birth (protocol C) did not increase plasma hepcidin level, it provided iron for erythropoiesis only till day 14 postpartum. Our split protocol involving injection of reduced amounts of iron on day 3 and 14 after birth (protocol B) improved hematological status of piglets while maintaining plasma hepcidin at very low levels. Considering that iron up-regulates hepcidin synthesis, this result strongly implies that supplemental iron was directed towards erythropoietic sites while passing by hepatocytes, main site of hepcidin synthesis in the body.
Conclusion: Our results pave the way for planning mixt supplementation of piglets starting with intramuscular injection of iron dextran on day 3 after birth and continuing with dietary supplementation, which could be largely efficient under condition of very low plasma hepcidin level.

Poster #82
MACROPHAGES HAVE AN IRON-INDEPENDENT ROLE IN THE REGULATION OF STRESS AND INEFFECTIVE ERYTHROPOIESIS AND CONTRIBUTE TO HEPCIDIN REGULATION
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(Presented By: Bart J. Crielaard)

Introduction: In erythropoiesis, macrophages have been thought to mainly fulfill a function in iron recycling and disposal of nuclei expelled by the developing erythroblasts surrounding them. We hypothesized a more direct involvement of macrophages in the regulation of erythropoiesis and iron absorption.
Methods and Materials: Therefore, we investigated the role of macrophages in different physiological and pathological conditions of enhanced erythropoietic activity, and whether their contribution is independent of their function in iron recycling.
Four mouse models of enhanced erythropoiesis were utilized: phlebotomy-induced stress erythropoiesis (SE) and increased erythropoiesis by erythropoietin (Epo) administration, representing physiological stress erythropoiesis; Polycythemia Vera (Jak2V617F/+ or PV), and beta-thalassemia intermedia (Hbbth3/+ or BTI), as models of chronic stress erythropoiesis (CSE), defined as a pathological expansion of erythroid progenitors, altered erythroid cell cycle and splenomegaly. Macrophages

were chemically depleted by administration of liposomal clodronate. Furthermore, we utilized iron-overloaded mice using dietary or parenteral iron or animals affected by hemochromatosis (*Hfe-/-* and *Hamp-/-*).
Results: While the chronic (3 months) depletion of macrophages in healthy mice showed little effect on steady state erythropoiesis, it significantly impaired their recovery from anemia following phlebotomy, strongly restrained the increase in hematocrit (Htc) upon treatment with Epo, and significantly decreased the number of erythroid progenitors observed in PV and BTI. This was associated by lack of hepcidin suppression and accelerated differentiation of the erythroid progenitors (as discussed in the companion abstract by Crielaard *et al.*). Chronic depletion of macrophages in PV mice completely reversed splenomegaly and Htc. Additionally, macrophage depletion in BTI mice ameliorated the ineffective erythropoiesis and reduced splenomegaly. Moreover, macrophage-depleted BTI mice exhibited a significant increase (~2 g/dL) of hemoglobin, which was not mediated by an impaired phagocytosis of RBCs, but rather a decreased hemichrome formation, improved RBC morphology and red cell distribution width. This resulted in a significant extension of the RBC lifespan, assessed by the transfusion of RBCs from macrophage-depleted BTI mice into normal mice with an intact macrophage reservoir. We then investigated whether these effects were primarily mediated by reduced serum iron levels, considering the essential role of macrophages in iron homeostasis. Macrophage depletion did not affect the elevated serum iron and transferrin saturation levels in iron-overloaded healthy mice and animals affected by BTI and hemochromatosis. Consistent with non-overloaded animals, recovery from phlebotomy-induced anemia by ironoverloaded animals upon macrophage depletion was impaired. However, macrophage depletion in iron-overloaded BTI mice resulted in improvements in both erythroid differentiation and splenomegaly, indicating an iron-independent function of macrophages in stimulating erythropoiesis. These and previous observations, in which overexpression of hepcidin or low iron diet suppressed stress and ineffective erythropoiesis (Ramos *et al.*, Blood; Gardenghi *et al.*, JCI), indicate that iron is necessary, but not sufficient to sustain SE and CSE in absence of macrophages.
Conclusion: This study demonstrates the role of macrophages in maintaining a proper erythroid response under conditions of increased erythropoiesis in healthy mice, while contributing to the pathological expansion and impaired maturation of erythroblasts in PV and BTI. Exploring the mechanism by which macrophages modulate SE/CSE may have significant therapeutic implications in the near future.

Poster #83
ACE-536 CORRECTS INEFFECTIVE ERYTHROPOIESIS, ANEMIA, IRON OVERLOAD AND OTHER CO-MORBIDITIES IN B-THALASSEMIA SYNDROME
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Acceleron Pharma
(Presented By: Ravi Kumar)

Introduction: Deficiency of beta globin chain expression in b-thalassemia results in precipitation of unpaired alpha globin protein in red cell membranes. In bthalassemia patients, severe anemia caused by hemolysis of damaged mature RBC and ineffective erythropoiesis leads to erythroid hyperplasia, high EPO levels, reduced hepcidin expression and iron overloading. In chronic transfusion dependent patients, blood transfusions are essential for survival, but further contribute to iron toxicity of various critical organs such as liver and heart. A therapeutic approach that can correct ineffective erythropoiesis and prevent erythroid hyperplasia, splenomegaly and severe iron overloading is warranted. Several TGF-beta superfamily ligands are involved in erythropoiesis. ACE-536 is a modified extracellular domain of the human activin receptor type IIB linked to the Fc region of human IgG1. The ligand binding profile of ACE-536 is significantly altered from the wild type ActRIIB receptor. ACE-536 modulates the availability of TGF-beta ligands during erythropoiesis. While EPO is responsible for proliferation of early stage erythroblasts, ACE -536 promotes maturation of late stage terminally differentiating erythroid precursors.
Methods and Materials: In this study, we investigated if RAP-536 (murine ortholog of ACE-536) corrects ineffective erythropoiesis and increases hemoglobin in a murine model of b-thalassemia. For this study, we used mouse model of b-thalassemia intermedia (Hbb th1/th1). Three months old mice were treated subcutaneously twice per week with TBS (vehicle) or RAP-536 (1mg/kg) for two months. At the end of treatment, cell blood counts, bilirubin and iron levels were measured. Bone marrow and splenic erythroid differentiation analysis was carried out using anti-CD71 and anti-Ter119 antibodies and flow-cytometry. Prussian blue staining was performed on liver, kidney and spleen sections to assess iron deposition.
Results: Prior to dosing, b-thalassemic mice were severely anemic with significantly decreased RBC (-31.6% $p<0.001$), hemoglobin (-35.0% $p<0.001$) and hematocrit (-34.8% $p<0.001$) compared to wild type littermates. Following two months of dosing with RAP-536, significant increases in RBC (+32.9%, $p<0.01$), hemoglobin (+17.4%, $p<0.01$), hematocrit (+11.0%, $p<0.01$) were observed compared to vehicle treated mice. Differentiation analysis of bone marrow and spleen cells revealed significant decreases in basophilic and increases in poly- and ortho-chromatophilic erythroblasts. In addition, EPO levels were significantly reduced in RAP-536 treated mice (639.7±111 vs. 1769.7± 517 pg/mL, $p<0.05$). Furthermore, RAP-536 treatment reduced splenomegaly (-38%, $p>0.05$). RAP-536 treatment also restored bone mineral density in b-thalassemic mice to levels observed in wild type mice. RAP-536 also improved iron homeostasis in b-thalassemic mice as observed with reduced serum iron, transferrin saturation and decreased iron staining of liver, kidney and spleen. Reduced hemolysis was evident in blood smears from RAP-536 treated b-thalassemic mice which was confirmed by reduced bilirubin and reactive oxygen species along with longer RBC life-span.

Conclusion: In conclusion, these data demonstrates that RAP-536 reduces ineffective erythropoiesis and improves anemia in b-thalassemic mice. Thus ACE-536 represents a novel potential therapy for patients with b-thalassemia or other conditions associated with ineffective erythropoiesis. Phase 2 clinical studies of ACE-536 in patients with b-thalassemia and MDS are ongoing.

Poster #84

TOXICITY STUDY OF A NOVEL ORAL IRON CHELATOR: 1-(N-ACETYL-6-AMINOHEXYL) 3-HYDROXYPYRIDIN-4-ONE (CM1) IN TRANSGENIC β-THALASSEMIA MICE

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(Presented By: Nittaya Chansiw)

Introduction: Deferiprone (DFP) (MW = 139 Da, Kpart = 0.11) is an effective iron chelator used for the treatment of iron overload in thalassemia patients, but the drug is not free from side effects. We have synthesized a novel oral bidentate iron chelator, 1-(N-acetyl-6-aminohexyl)-3-hydroxypyridin-4-one (CM1) (MW = 256 Da, Kpart = 0.53) which is an analogue of DFP. This compound is more lipophilic than DFP and can bind iron efficiently. Our current results have demonstrated that the CM1 reduced iron-induced redox damage and decreased levels of intracellular iron pool (LIP) in cultured hepatocytes effectively. However, the toxicity of the CM1 remains largely unknown.
Methods and Materials: The aim of this study was to therefore examine the toxicity of CM1 treatment in an animal model under normal and iron overload conditions. To induce iron overload, transgenic β-thalassemia (BKO) mice were fed with a 0.2% (w/w) ferrocene-supplemented diet (Fe diet) for 240 days. The mice received three doses of CM1 orally (50,100 and 200 mg/kg) every day for 180 days.
Results: Blood was collected from the tail vein every 45 days during treatment for measurement of hemoglobin (Hb) level, white blood cell (WBC) and platelet numbers. We also determined activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP), which are markers of liver damage. Treatment with CM1 at the assigned doses did not markedly alter the numbers of WBC and platelets, and Hb level in BKO mice fed with either N diet or Fe diet. Importantly, all the treatments slightly increased activities of plasma AST, ALT and ALP in BKO mice after 150 days. Nonetheless, hematoxylin and eosin staining result did not show abnormal morphological changes of their spleen, liver and heart tissues.
Conclusion: The results imply that CM1 may not be toxic to bone marrow cells and liver cell function in BKO mice under normal and iron overload conditions.

Poster #85

REDUCTION OF LABILE IRON AND REACTIVE OXYGEN SPECIES IN PRIMARY HEPATOCYTES BY A NOVEL SYNTHETIC IRON CHELATOR, 1-(N-ACETYL-6-AMINOHEXYL)-3-HYDROXYPYRIDIN-4-ONE (CM1)

Kanokwan Kulprachakarn, PhD Student, Kanjana Pangjit, PhD, Suthat Fucharoen, MD and Somdet Srichairatanakool, PhD
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(Presented By: Kanokwan Kulprachakarn)

Introduction: Iron overload associated with oxidative stress is a serious problem in transfusion-dependent patients with beta-thalassemia major. The increased iron overload in several organs may be caused by higher intestinal absorption along with less intensive chelation therapy. Liver iron overload could in turn facilitate the development or persistence of chronic progressive liver disease. Previous studies have shown that chelation with desferrioxamine (DFO) and deferiprone (DFP) substantially reduced body-iron scores in beta-thalassemia patients with transfusional iron overloads.
Methods and Materials: We have synthesized and characterized a new bidentate iron chelator, 1-(N-acetyl-6-aminohexyl)-3-hydroxypyridin-4-one(CM1). The compound has a molecular weight of 256 Da), pKa values of 3.520+/-0.003 and 9.800+/-0.002, partition coefficient (Kpart) value of 0.53, and is uncharged over the pH ranges of 6-8. The CM1 is more lipophilic than deferiprone (DFP) (Kpart = 0.11), and can efficiently bind the iron in the forms of ferrous ion, ferric ion and plasma nontransferrin bound iron (NTBI). In this study we studied the efficacy of the CM1 treatment in decreasing the levels of labile iron pool (LIP) and reactive oxygen species (ROS) in iron-loaded mouse hepatocyte culture. Liver was obtained from C57/BL6 mice and perfused with collagenase type IV solution. The isolated hepatocytes were cultured in the supplemented DMEM, and treated with DFO, DFP and CM1 at different concentrations.
Results: The treated cells were analyzed cytotoxicity using (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, intracellular LIP using calcein fluorescent technique and ROS level using dichlorofluorescein (DCF) fluorescent method. It was found that DFP, DFO and CM1 were dose dependently toxic to the cultured hepatocytes; however, treatment with low doses of CM1 enhanced the viability of the cells. During 12 and 24 hour incubation periods, CM1 at the doses of 25 and 50 μM effectively decreased the levels of intracellular LIP and hydrogen peroxide-induced ROS in the treated hepatocytes in a concentration-dependent manner.
Conclusion: Our findings support the evidence of iron-chelating and free radical-scavenging activities of CM1 in the livers with iron overload, which potentially can protect against oxidative liver inflammation and fibrosis. The efficacy of the CM1 treatment needs to be further investigated intensively in both animals and human patients with iron overload.

Poster #86

IN VITRO FERROPORTIN EXPRESSION IN NON TRASFUSION DEPENDENT THALASSEMIA DURING ERYTHROID DIFFERENTIATION

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(Presented By: Laura Sonzogni)

Introduction: Ferroportin (FPN) is the only known iron exporter protein. It is expressed in different cell types including duodenal enterocytes, hepatocytes, macrophages and erythroblast cells. Recently it has been reported the existence of multiple alternative transcripts of FPN with or without an iron−responsive element (IRE) in their promoter (FPN1A and FPN1B isoforms respectively). The expression of the different ferroportin isoforms as well as the mechanisms regulating their expression on erythroid cells in non transfusion−dependent beta thalassemia syndromes (NTDT) are not known.

Aim: To investigate the expression profile of ferroportin isoforms during erythroid differentiation in control and NTDT cell cultures and to elucidate the mechanisms regulating their expression.

Methods: An in vitro model of erythropoiesis derived from human peripheral CD34+ cells from healthy volunteers (control) and NTDT patients was used. The expression profiling of FPN isoforms (FPN1A and FPN1B) was evaluated at baseline (day 0) and at day 7 and 14 of culture (proerythroblasts and orthochromatic erythroblasts stage respectively) by real−time PCR (2^−dCt). The relative percentage of each isoform was calculated on the basis of total ferroportin expression (FPN1A+FPN1B). The intracellular iron concentration was analyzed using an Iron Assay Kit (Biovision). In independent experiments, control cultures were treated with iron (Ferric Ammonium Citrate [FAC] 100μM), heme (Hemin 10μM) or hydrogen peroxide (H_2O_2 0.1mM) to investigate a possible role of these compounds in ferroportin regulation; FPN expression was evaluated at day 14 in standard and treated conditions by real−time PCR (2^−ddCt; untreated cells used as calibrator).

Results: The ferroportin expression increased during erythroid differentiation, with the highest level at the end of erythroblasts stage (day 14 of cultures) both in control and NTDT cultures. (Table 1). The FPN1A was the more expressed isoform in both conditions. Its expression was higher at the initial and final steps of erythropoiesis (day 0 and 14), while FPN1B expression was higher at the intermediate erythroblast stages (day 7). Noteworthy, the FPN1B expression, although lower compared to FPN1A, was significantly higher in NTDT cultures than in control ones, particularly at day 14. The intracellular iron concentration decreased significantly during erythroid differentiation (from day 7 to day 14) both in control and NTDT cultures, however, at day 7 (early erythroblasts stage) the iron levels in NTDT cultures were notably lower than in controls. The addition of FAC and Hemin in control cultures did not modified the ferroportin expression compared to untreated cultures (FPN1A: untreated cells: 1; FAC: 0.9 ± 0.5; Hemin: 0.9 ± 0.01. FPN1B: untreated cells: 1; FAC: 0.9 ± 0.2; Hemin: 1 ± 0.01). H_2O_2 added to control cells increased the expression of both ferroportin isoforms (FPN1A: untreated cells: 1; H_2O_2: 1.73. FPN1B: untreated cells: 1; H_2O_2: 2.11).

Conclusions: The ferroportin expression increases during erythroid differentiation either in control than in NTDT cultures, suggesting its role in exporting the excess intracellular iron. In both conditions the FPN1A is the more expressed isoform. However the expression of the non−iron responsive FPN1B isoform, although lower compared to FPN1A, is significantly higher in NTDT than in control conditions. In control cultures, FPN expression, and particularly the FPN1B isoform, seems to be upregulated by H_2O_2 addition. These data suggest that the oxidative stress, notably higher in NTDT conditions, could be one of the major regulator of FPN1B expression, with a major iron export from NTDT erythroblast cells.

Table 1.

		Day 0	Day 7	Day 14
Control	Total FPN	0,16 ± 0.11	0,15 ± 0.08	2,3 ± 1*
	FPN1A %	95 ± 2	70 ± 4	97 ± 1.7*
	FPN1B %	5 ± 2	30 ± 4 •	3 ± 0.7
	Intracellular IRON (nM)	n.d.	28 ± 2,8	9,5 ± 4,5*
NTDT	Total FPN	0,40 ± 0.17	0,14 ± 0.04	2.9 ± 1.7*
	FPN1A %	93 ± 2.5	58 ± 8	95 ± 2.5*
	FPN1B %	7 ± 2.5	42 ± 8 •	5 ± 2.5°
	Intracellular IRON (nM)	n.d.	13,5 ± 3,6°	5,8 ± 3,4*

• day 7 vs day 0: p<0.05; * day 14 vs day 7: p<0.05; ° NTDT vs control: p<0.05

Poster #87

GROWTH DIFFERENTIATION FACTOR 15 EXPRESSION AND REGULATION DURING ERYTHROID DIFFERENTIATION IN NON TRANSFUSION DEPENDENT THALASSEMIA SYNDROMES

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(Presented By: Luisa Ronzoni)

Background: Growth differentiation factor 15 (GDF-15), a cytokine member of the TGF-beta superfamily that is released by mature erythroblasts, has been proposed to be an hepcidin inhibitor able to down-regulate the hepatic expression and release of hepcidin probably in response to ineffective erythropoiesis and increased iron demand from the bone marrow. However, the exact mechanisms regulating GDF-15 expression remain unknown. Moreover, correlation between GDF-15 levels and the degree of ineffective erythropoiesis has been never explored. NTDT represents an ideal model to study thalassemic erythropoiesis and its relationship with iron metabolism.
Aim: To determine the GDF-15 expression and regulation during normal and thalassemic erythroid differentiation in vitro. To evaluate if GDF-15 levels relate with ineffective erythropoiesis degree, estimated by transferrin receptor (TFR1) levels.
Methods. After informed consent, CD34+ cells were obtained from peripheral blood of healthy volunteers (control group) and NTDT patients and cultured for 14 days with a medium stimulating erythroid differentiation. GDF-15 expression was evaluated at day 0 (erythroid progenitors), 7 (proerythroblasts) and 14 (mature erythroblasts) of culture by real-time PCR (2^-dCt). GDF-15 and TFR1 levels in culture supernatants were evaluated by ELISA assay (R&D Systems, Minneapolis, MN). Intracellular iron concentrations were estimated by colorimetric assay (BioVision, Milpitas, CA).
Results: GDF-15 expression and secretion increased significantly during erythroid differentiation (from day 7 to day 14 of cultures) both in control and NTDT cultures. However, GDF-15 levels as well as TfR levels were significantly higher in NTDT cultures compared to controls (p=0,02). Noteworthy, GDF-15 levels correlated positively with TfR levels in NTDT cultures but not in control ones (NTDT: R=0,96 p<0,01; Control: R=0,1). Intracellular iron concentrations significantly decreased during erythropoiesis in both control and NTDT cultures; however, at day 7 iron levels were significantly lower in NTDT cultures compared with controls (Table 1).
Conclusions: GDF-15 expression and levels in erythroid cultures are related to the erythropoietic stage of differentiation, being higher in mature erythroblasts. However, GDF-15 expression and secretion in NTDT cultures is higher than controls and correlate positively with TfR levels, reflecting the higher erythropoietic activity of thalassemic cells. There is an association between GDF-15 levels and intracellular iron concentrations, with lower concentration being associated with higher GDF-15 production. All these findings suggest that GDF-15 can be considered a marker of erythropoietic activity and that intracellular iron concentration could be a major contributor to GDF-15 regulation, although other factors, such as oxidative stress,
can still be involved in this process.

Table 1.

	GDF-15 expression (2^-dCt)			GDF-15 levels (pg/mL)		TfR1 levels (µg/mL)	Iron concentrations (µM)	
	Day 0	Day 7	Day 14	Day 7	Day 14	Day 14	Day 7	Day 14
Control	0,01	0,02	5,93*	208	4708*	0,35	29,6	7,8*
NTDT	0,18	0,64	20,06*°	452	9064*°	0,84°	11,5°	3,2*

*day 14 vs day 7: p<0.05; ° NTDT vs control: p<0.05.
Data are expressed as median of ten indipendent experiments

Poster #88

EVALUATION OF A NEW ORAL IRON CHELATOR 1-(N-ACETYL-6-HEXYL)-3-HYDROXYPYRIDIN-4-ONE (CM1) FOR TREATMENT OF IRON

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(Presented By: Kanjana Pangjit)

Introduction: Desferrioxamine (DFO), deferiprone (DFP) and deferasirox (DFX) are promising effective iron chelators for the treatment of iron overload in ßthalassemia patients; nonetheless, their side effects have also been reported. 3-Hydorxypyridinone derivatives are being developed as a safer new chelator and in combined chelation therapy.
Methods and Materials: We evaluated the iron-chelating activity of 1-(N-acetyl-6-aminohexyl)-3-hydroxypyridin-4-one(CM1) in iron-loaded C57BL6 mice. The feeding of a ferrocene-supplemented diet (Fe diet) to mice resulted in iron overload, detectable plasma nontransferrinbound iron (NTBI) and labile plasma iron (LPI), and increases of red cell membrane iron, plasma malondialdehyde (MDA) and excessive tissue iron deposits. Like DFP, the CM1 lowered the levels of the membrane non-heme iron, the NTBI and LPI ($p < 0.05$) and the MDA after 3 months of treatment.

Results: Administration of the Fe diet and the Fe diet along with the chelators did not change the morphology of the liver and heart. Numerous iron accumulations were observed in the liver and spleen tissues of the Fe diet-fed mice, whereas the CM1 reduced such iron deposition.
Conclusion: Thus, 1-(N-acetyl -6-aminohexyl)-3-hydroxypyridin-4-one (CM1) can be considered a candidate bidentate oral iron chelator and is effective in the removal of toxic irons in blood compartment and tissues. The effectiveness and toxicity of the CM1 need to be investigated extensively in thalassemia mice and patients with iron overload.

Poster #89
INNATE IMMUNE CELL EXPRESSION OF PATTERN RECOGNITION RECEPTORS FROM TRANSFUSED THALASSEMIA MAJOR PATIENTS DURING INTENSIVE COMBINATION CHELATION THERAPY
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(Presented By: Patrick Walter)

Introduction: Despite improved chelation therapies, thalassemia major patients endure iron overload, chronic inflammation, organ failure, infection and oxidative stress, resulting in elevated levels of pro− and anti−inflammatory proteins and pro−apoptotic proteins. Infection is the second most common cause of death in thalassemia. The innate immune system provides the first line of defense against infection and its specificity depends on pattern recognition receptors (PRRs) specific to microbial pathogens. One class of PRR called the toll−like receptors (TLRs) interacts with CD14 on innate immune cells transducing the signal for bacterial Lipopolysaccharide (LPS), resulting in cytokine production. The role iron plays in thalassemia in determining expression level of PRRs is unknown. Thus, the goal in these studies is to investigate the relationship of iron overload and chelation to innate immune cell expression of PRRs in thalassemia.
Patients and Methods: Eighteen transfusion dependent thalassemia patients (11 – 29 yrs old) participating in the combination trial of deferasirox and deferoxamine (Novartis sponsored CICL670AUS24T) were enrolled in a substudy investigating innate immunology (Novartis sponsored CICL670AUS42T). Fasting blood samples were obtained i) at baseline after a 72 hr. washout of chelator, and ii) at 6 and 12 months on study. Fourteen healthy controls (10 − 35 yrs old) were also enrolled. Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples and then monocytes and granulocytes were purified using antibody−linked magnetic microbeads (Miltenyi Biotec Inc). Highly enriched populations of CD14+ monocytes and CD15+ granulocytes were verified by flow cytometry. CD15 is not a PRR, but a trisaccharide adhesion molecule, used by granulocytes to mediate phagocytosis and chemotaxis. The expression level of CD14 and CD15 in PBMCs and TLR4 in purified cells was determined and reported as the median fluorescent intensity (MFI). Liver iron concentration (LIC) was determined by biomagnetic susceptibility ("SQUID", Ferritometer®) in patients with thalassemia; healthy controls were shown to have normal ferritin.
Results: In PBMCs from thalassemia patients at baseline, the expression of monocyte CD14 and TLR4 were significantly increased 22% and 27% respectively compared to healthy controls ($p < 0.05$). Granulocytes from patients with thalassemia were also found to have a 50% and 23% higher expression of TLR4 and CD15 respectively at baseline compared to controls. In longitudinal analysis markers of iron burden and the expression of TLR4 on granulocytes all significantly decreased in the follow−up period in thalassemia patients receiving intensive combination chelator therapy ($p<0.05$).
Conclusions: These studies support the hypothesis that iron burden influences the innate immune response in thalassemia as demonstrated by the increased monocyte and granulocyte expression of TLR4 at baseline. Also increased at baseline were the expression of monocyte CD14 and granulocyte CD15. All of these changes are likely to contribute to the chronic inflammation in thalassemia and could also contribute to the commonly observed susceptibility to infection. After intensive chelation, the levels of TLR4 decreased, indicating that decreased iron overload with chelation may improve chronic inflammation in thalassemia. These changes in CD14 and TLR4 may be able to restore proper innate immune function in thalassemia patients.

Poster Abstracts

Poster #90

IRON DISTRIBUTION ASSESSED BY MRI IN SICKLE CELL DISEASE, THALASSEMIA AND DIAMOND BLACKFAN ANEMIA (MCSIO PILOT STUDY)

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(Presented By: Zhiyue J. Wang)

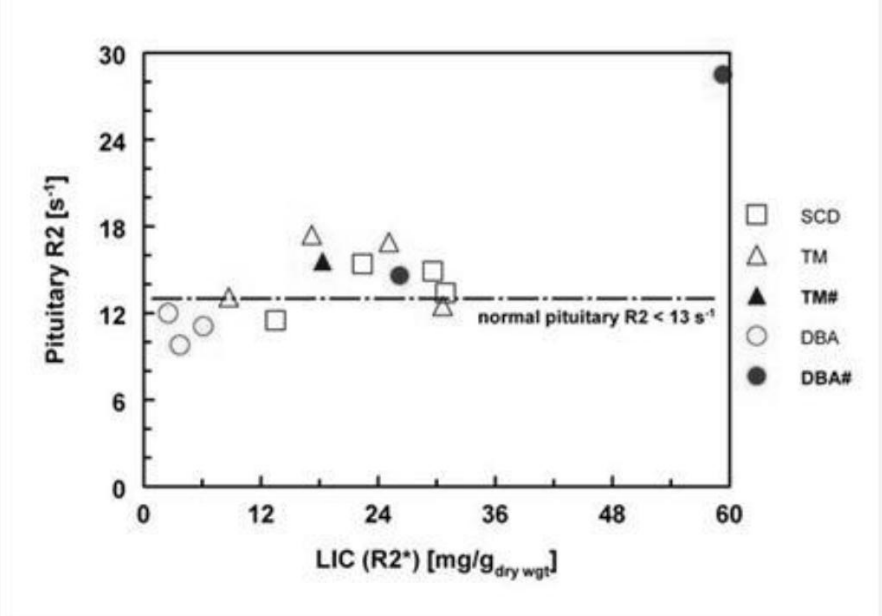
Background: Transfused patients with hemoglobinopathies (thalassemia major, TM; sickle cell disease, SCD; Diamond Blackfan Anemia, DBA) benefit from iron chelation treatment, avoiding morbidities and death, but may suffer longterm from actual or past iron load. A low rate of extrahepatic iron deposition has been reported in SCD patients despite heavy hepatic iron burden. In patients with DBA, little is known about iron deposition in organs other than the liver. Here we report liver, cardiac, and pituitary iron measurements by MRI-R2*/R2 as well as cardiac function assessments by MRI in TM, SCD, and DBA patients. This information is part of the MCSIO pilot study and will be related to parameters of inflammation, hepcidin synthesis, and erythropoiesis.

Methods: Iron-overloaded patients with TM, SCD, DBA (5 per group) with ferritin > 1500 µg/L or LIC > 7 mg/gd.w., age ≥16 y, and 10 to 20 years of transfusion exposure were enrolled from 3 sites at Oakland (SCD), London (TM), and Hamburg/Freiburg (DBA). Fasting, early morning blood samples were obtained from each patient at the mid-point and end of the transfusion cycle. Chelation and vitamin supplements were held for 72 hours prior to each sample. Equal iron measurement methods were adjusted to the technical requirements at the three MRI sites. Central analysis was performed for each organ by one of the authors.

Results: On average, DBA patients had the lowest median liver iron concentrations (Figure 1) independent of being determined by MRI-R2* or –R2 (nonlinear regression between R2 and R2*: r2 = 0.86), while cardiac R2* (1/T2*) rates were comparable with those in TM patients. Interoperator variability was low for liver R2 and R2* (r2 = 0.97 and 0.99, respectively), although different analysis methods were used (author JCW: pixel-wise vs. RF: global ROI). LV ejection fraction ranged from 48 to 69% with 6/15 patients revealing an EF < 56%. Age related pituitary R2 rates were elevated in 9/14 patients above the 95% range of normal values. The highest cardiac and pituitary iron levels were observed in a severely iron overloaded (LIC) 25year-old DBA patient.

Discussion and Conclusion: For pituitary iron (R2), a significant Spearman rank correlation could only be found with LIC (R2, R2*) (rS=0.68, p=0.01). Assuming that the actual LIC echoes the total iron burden from the past, pituitary R2 may indicate early extra-hepatic iron deposition.

Figure_1. Pituitary iron correlates significantly with liver iron (rS=0.68, p=0.01): solid symbols indicate patients with elevated cardiac R2* rates > 50 s^{-1} (TM#, DBA#), normal 95% pituitary R2 threshold from Noetzli et al (2012).



Poster #91

PHARMACOGENETIC STUDY OF UGT1A1 AND UGT1A6 POLYMORPHISMS AND HAPLOTYPES IN TRANSFUSION DEPENDENT THALASSAEMIA PATIENTS REVEALED A COUNTERBALANCE EFFECT BETWEEN TWO UGT ISOFORMS ON DETERMINING CLINICAL RESPONSE TO ONCE DAILY DOSE OF DEFERASIROX THERAPY

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(Presented By: Sarayuth Rodmai)

Introduction: Earlier studies suggested that deferasirox is mainly metabolised via glucuronidation to metabolites M3 (acyl glucuronide) and M6 (2-O-glucuronide) through fecal and renal excretion. Recently, we have showed that up to 44.2% in our 45 Thai thalassaemia patients were not achieve satisfactory iron balance at once daily dose of 30 mg/kg/day of deferasirox. These patients could be classified as “inadequate responder (IR)” and difference in bioavailability with a lower area under the curve (AUC) of each IR individual was the only main mechanism identified earlier. The majority of our patients (n=15/17) could achieve a better iron chelation efficacy by dividing dose administration of deferasirox into twice daily suggesting that these patients might have a different pharmacokinetics from the rest.
Methods and Materials: To further explore the possible pharmacogenetic role over rate of glucuronidation on deferasirox metabolise and related efficacy, we determine genotypes of two UGT isoforms; UGT1A1 and UGT1A6 in thirteen IR to compared with another twelve matched age-sex control Thai thalassaemia patients who adequately responded (AR) to once daily dose of deferasirox . These two UGT isoforms might be different on their affinity and capacity to metabolise on overlapping substrates.
Results: Four variants of UGT1A1; F83L(T>C), G71R (G>A), -3279(T>G) and TA7 repeats were determined using PCR based analyses. Three UGT1A6 SNPs; Ser7Ala(T>G), Thr181Ala(A>G) and Arg184Ser(A>C) were analysed to construct common haplotypes: *1/*2/*4. In addition, at least 150 unrelated Thai individuals were determine for all these genotypes and revealed that the distribution of all major and minor allele frequencies of each SNP was in Hardy-Weinberg equilibrium (data not shown). Interestingly, three patients in IR were heterozygotes for TA6/TA7 variants, but none in AR group. The strongest correlation was identified at -3279 allele with a mutant (G) allele was found significantly higher in AR group (p=0.021). In a further analysis, patients with > 1 mutant UGT1A1 alleles had a higher risk (RR = 3.33) to be found in the AR group. In contrary, compound heterozygous with minor UGT1A6 haplotypes; *1/*2 and *1/*4 were only found in 5 individuals in IR group while only a wild type; *1/*1 was found in AR patients. All minor alleles at each three SNP sites of the UGT1A6 were significantly different between two groups (p<0.05). These data suggest that mutant UGT1A1 variants in which have been associated with Gilbert syndrome that can decrease rate of glucuronidation of bilirubin and so deferasirox limiting its metabolites. On the other hand, the UGT1A6*2 allozyme that has been shown earlier to have high intrinsic clearance values with higher Vmax compared with UGT1A6*1 might result in a higher rate of deferasirox glucuronidation and clearance of the drug resulting in IR patients. Further analysis using individual data combining two UGT alleles, a significant association between AR patients with UGT1A1 variants was hindered by co-inheritance of UGT1A6*2 haplotype as five individuals with two independent UGT polymorphism were all in IR group (p=0.000).
Conclusion: In conclusion, a thorough genetic analysis of both loci might be useful for future prediction of clinical response to standard deferasirox dosing and tailoring iron chelation regimens.

Poster #92

DETERMINATION OF UGT1A1 AND UGT1A6 GENETIC POLYMORPHISMS ON CLINICAL RESPONSE TO DEFERIPRONE MONOTHERAPY FROM A 1-YEAR MULTI-CENTRE PROSPECTIVE, SINGLE ARM, OPEN LABEL, DOSE ESCALATING PHASE III PEDIATRIC STUDY (GPO-L-ONE; A001) FROM THAILAND

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(Presented By: Suchada Riolueang)

Introduction: In many developing countries in particular Asia-Pacific where thalassaemia and haemoglobin disorders are highly prevalent, accessibility to current standard iron chelators including deferoxamine and deferasirox is rather limited due to high cost and limited health resources. To provide an alternative, The Government Pharmaceutical Organization of Thailand (GPO) has produced a local deferiprone to provide an alternative.
Methods and Materials: Previously, we have shown in 73 pediatric patients with severe β thalassaemias, age range 3.2-19 yrs, from a 1-year multi-centre prospective, single arm, open label, dose escalating Phase III study of deferiprone that this drug had clinical efficacy and acceptable safety profile. Sixty-four patients (87.6%) completed the study with good compliance (>94%). Average deferiprone dose was 79.1 ± 4.3 mg/kg/day.
Results: Although, overall mean serum ferritin (SF) levels at 1 year were not significantly changed from baseline. However 45% of patients (response group) had SF reduced > 15% from baseline at 1 year with a median reduction of 1,065 ng/ml. A subgroup analysis by MRI-T2* confirmed that the response group had higher baseline liver iron and deferiprone could

significantly reduce liver iron overload and normalize levels of ALT at 1 year while the results from non-response group was quite the opposite. Baseline SF > 3500 ng/ml and transfusion iron intake (<0.25 mg/kg/d) were only two clinical factors distinctive between response and non-response group. To provide a possible genetic makers for predicting clinical response to deferiprone, we determined four polymorphic variants of UGT1A1; F83L(T>C), G71R(G>A), -3279(T>G) and TA7 repeats and three UGT1A6 polymorphic sites; Ser7Ala(T>G), Thr181Ala(A>G) and Arg184Ser(A>C) using PCR based analyses. In addition, a common variant in TMPRSS6; A736V previously shown to be associated with iron status was also evaluated. Furthermore, 150 unrelated Thai controls were studied to evaluate genetic epidemiology including allelic frequency of each polymorphic site and they were all in normal distribution. There was no statistically significant difference for all SNPs and variants of these three genes analysed between response and non-response groups using different analytical algorithms. Moreover, in a haplotype analysis of UGT1A6 gene, the presence of haplotype*2 associated with increase glucuronidation rate was evenly distributed between both groups.

Conclusion: This finding is consistent with previous study by Limenta et al. (BJCP, 2008) that genetic polymorphisms in UGT1A6 do not appear to exert statistical effects on the single-dose pharmacokinetics of deferiprone in healthy controls in the absence of iron loading and also in thalassaemia patients with iron overload as shown in this study. Further analysis in these groups of patients including genome wide association study (GWAS) and a more comprehensive pharmacogenomic approach are warranted in order to identify possible genetic basis underlying different clinical efficacy in patients who receive deferiprone therapy.

Poster #93

IRON CHELATION THERAPY IMPROVES OXIDATIVE DNA DAMAGE IN HEMATOPOIETIC CELLS DERIVED FROM TRANSFUSION-DEPENDENT MYELODYSPLASTIC SYNDROME

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(Presented By: Masayoshi Kobune)

Introduction: Myelodysplastic syndrome (MDS) is characterized by dysplastic and ineffective hematopoiesis, peripheral blood cytopenias and a risk of leukemic transformation. Most MDS patients eventually require red blood cell (RBC) transfusions for anemia and consequently develop iron overload. Excess free iron in cells catalyzes generation of reactive oxygen species that cause oxidative stress, including oxidative DNA damage. However, it is uncertain how iron-mediated oxidative stress affects the pathophysiology of MDS.

Methods and Materials: This study included MDS patients who visited our university hospital and affiliated hospitals (n=43). Among them, 13 patients received iron chelation therapy when their serum ferritin (SF) level was greater than 1000 ng/mL or they required more than 20 RBC transfusions (or 100 mL/kg of RBC). We prospectively analyzed 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels in peripheral blood mononuclear cells (PBMC) obtained from MDS patients before and after iron chelator, deferasirox administration.

Results: We showed that reactive oxygen species (ROS) in hematopoietic cells was higher in MDS patients with high SF level. Furthermore, the 8-OHdG levels in MDS patients were significantly higher than those in healthy volunteers and were positively correlated with SF and chromosomal abnormalities. Importantly, the 8-OHdG levels in PBMC including CD11b+, CD3+, CD19+ and CD34+ cells of MDS patients dramatically decreased 3 month after deferasirox administration although SF level were slightly reduced in this period, suggesting that iron chelation reduced oxidative DNA damage and 8-OHdG is a sensitive marker of cellular iron chelation therapy by deferasirox which readily passes through the plasma membrane. When patients were divided into the low SF group (<500 ng/ml) and high SF group (≥500 ng/mL), the survival time was significantly longer in the former group than the latter group (118.8 M vs. 10.2 M, P=0.002). Further, leukemia-free survival (LFS) was significantly longer in the low SF group than the high SF group (P=0.010).

Conclusion: Thus, excess iron could contribute to the pathophysiology of MDS and iron chelation therapy could improve the oxidative DNA damage in MDS patients.

Poster #94

A NOVEL, LONG ACTING, POLYMERIC IRON CHELATOR FOR THE TREATMENT OF SECONDARY IRON OVERLOAD

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(Presented By: J L. Hamilton)

Introduction: Secondary Iron Overload remains a significant cause of morbidity and mortality in transfusion dependent patients with severe hemoglobin disorders. Desferrioxamine (DFO) mesylate is effective in promoting iron excretion. However, its usefulness is limited by a short intravascular half-life (t ½) and toxicity. This necessitates continuous 8-12 hr subcutaneous infusion, often leading to limited patient compliance. Using hyperbranched polyglycerol (HPG), we have generated DFO conjugates (HPG-DFO) which have significantly prolonged the t ½, reduced toxicity and improved the iron excretion efficiency of DFO in mice.

Methods: HPG-DFO was synthesized by conjugating HPGs of various molecular weights (MW) to varying numbers of DFO molecules using Schiffbase chemistry. The influence of HPG MW and DFO density on iron binding of HPG-DFO was determined using Isothermal Titration Calorimetry (ITC) and UV-visible spectroscopy. The biocompatibility of HPG-DFO was evaluated by examining blood coagulation, platelet activation and complement activation. The effect of HPG-DFO on cell viability was evaluated in human umbilical vein endothelial cells (HUVECs). Tolerance was studied by injecting up to 1000 mg/kg of HPG-DFO in mice and monitoring body weight, lactate dehydrogenase levels and organs morphology 14 days post administration. Tritium labeled HPG-DFO was used to determine the circulation t ½ in mice. Iron excretion efficacy of HPG-DFO compared to DFO was tested in iron overloaded mice by treating the animals with 150 mg/kg DFO or DFO equivalent. The iron content of organs, urine and feces were analyzed to determine the amount of iron excreted. All major organs were stained with Prussian blue and examined under light microscopy.
Results: ITC and UV analyses demonstrated that the conjugation of DFO to HPGs did not alter the iron chelating properties of DFO. HPG-DFOs did not activate platelets, coagulation or the complement system. In vitro cell tolerance studies showed that cell toxicity of conjugates were reduced compared to DFO. Mice tolerance studies showed that the HPG-DFO conjugates were non toxic up to the maximum injected dose of 1000 mg/kg; maximum tolerated dose is not reached. The t ½ of HPG-DFO in normal mice was 16 h for the 50 kDa conjugate and 44 h for the 500 kDa conjugate. The iron excretion efficiency as measured by urinary iron of the 50kDa HPG-DFO in iron overloaded mice showed significant improvement compared to placebo ($p=0.0002$) and DFO treated mice ($p=0.0011$). There was a significant reduction in the iron content of all major organs (liver, heart, pancreas, spleen) in HPG-DFO treated mice compared to DFO treated mice.
Conclusions: HPG-DFO showed significant increase in t ½, which resulted in the maintenance of much higher plasma concentrations and more effective iron removal. This approach has the potential to significantly decrease the number and duration of injections required to offload significant amounts of organ and total body iron in transfusion dependent patients. Current studies are focused on refining this polymer therapeutic.

Poster #95
ASCORBATE STATUS MODULATES RETICULOENDOTHELIAL IRON STORES AND RESPONSE TO DEFERASIROX IRON CHELATION IN ASCORBATE-DEFICIENT RATS.
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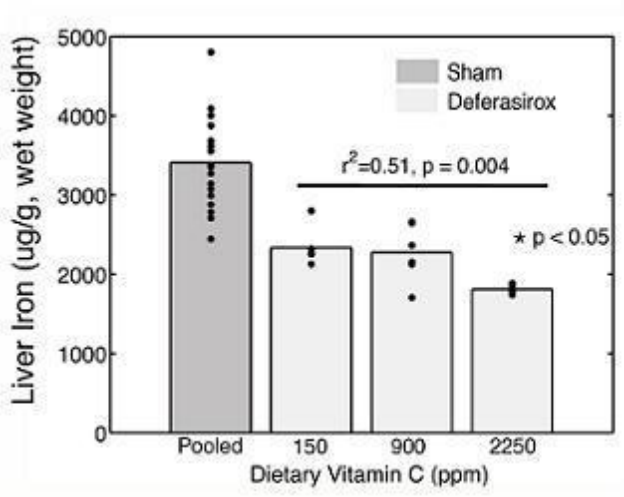
Children's Hospital Los Angeles
(Presented By: Casey Brewer)

Introduction: Iron chelation is essential to patients on chronic blood transfusions to prevent toxicity from iron overload and remove excess iron. Deferasirox (DFX) is the most commonly used iron chelator in the United States; however, some patients are relatively refractory to DFX therapy. Vitamin C is an important cofactor for a number of physiologic processes; its redox properties including its putative role as a pro-oxidant are most relevant in ironoverloaded patients. Ascorbate deficiency is common in iron overloaded subjects, and scorbutic patients appear to favor iron storage with iron deposition skewed towards reticuloendothelial cells. We postulated that vitamin C supplementation would improve the availability of transfusional iron to DFX treatment by promoting iron's redox cycling, increasing its soluble ferrous form and promoting its release from reticuloendothelial cells.
Methods and Materials: Osteogenic dystrophy rats (n = 54), which have a mutation to a key enzyme in L-ascorbic acid biosynthesis and are therefore ascorbate-deficient, were given iron dextran injections for 10 weeks. Ascorbate supplementation of 150 parts per million (ppm), 900 ppm, and 2250 ppm was used in the chow to mimic a broad range of ascorbate status; plasma ascorbate levels were 5.4 ± 1.9, 8.2 ± 1.4, 23.6 ± 9.8 micromolar, respectively ($p < 0.0001$). Cardiac and liver iron levels were measured after iron loading (n = 18), 12 weeks of sham chelation (n = 18), and 12 weeks of DFX chelation (n = 18) at 75 milligram/kilogram/day.
Results: The lowest ascorbate supplementation (150 ppm) produced reticuloendothelial retention, lowering total hepatic iron by 29% at the end of iron loading ($p < 0.05$). After 12 weeks of sham chelation, liver iron concentration declined 14.4%, or just under 5% per week, independent of ascorbate supplementation. In contrast, cardiac iron concentration varied considerably with ascorbate supplementation after sham chelation, with halving of the cardiac iron levels at maximal ascorbate supplementation ($p<0.05$). Most importantly, ascorbate supplementation at 2250 ppm improved DFX efficiency, allowing DFX to remove 21% more hepatic iron than ascorbate supplementation with 900 ppm or 150 ppm (Figure, $p < 0.05$). Histological evaluation of iron deposition mirrored the results predicted by tissue iron levels – rats on 150 ppm ascorbate showed almost no parenchymal iron staining in the liver, while rats on 2250 ppm ascorbate showed combined hepatocyte and reticuloendothelial cell iron loading.
Conclusion: We conclude that vitamin C facilitates the release of iron from the reticuloendothelial system and correlates positively with DFX chelation efficiency. Ascorbate deficiency is quite common in thalassemia major and could potentially create a phenotype of relative DFX refractoriness. Our findings suggest that ascorbate status should be probed in patients with unsatisfactory response to DFX or whose serum ferritin and transferrin saturation are not commensurate with their transfusional iron burden. Given the number of patients who are inadequately controlled by DFX monotherapy, even at maximal dosing, controlled studies of ascorbate replacement in humans are warranted. Ascorbate repletion must be done cautiously in any patient having cardiac iron stores because of the theoretical risk of increased labile myocyte iron; however, the long half-life of DFX may ameliorate the potential oxidative stress of iron mobilization.



Poster #96

IRON OVERLOAD AND INDUCTION OF APOPTOSIS IN CULTIVATED PANCREATIC BETA CELLS NES2Y

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(Presented By: Kamila Balusikova)

Introduction: Iron is an essential element for all organisms due to its participation in many biochemical processes. However, high iron levels in the cells, which may arise from various causes, can lead to the reduction of cellular functions. Iron accumulation in organism affects particularly cells of parenchymatic organs, which can lead to their damage or even cell death and serious organ injury. Excess iron is harmful especially due to its ability to produce reactive oxygen species (ROS) followed by oxidative stress and probably reduced antioxidant enzymes levels in the cells. Although different cell types have similar basic mechanism of the induction of apoptosis, current knowledge suggests that such endoplasmic reticulum stress (ER stress) caused by iron overload could also play a role in organ injury.
Methods and Materials: Therefore, we tested the effect of high doses of ferric citrate on the cellular injury and cell death induction in human pancreatic cell line NES2Y. Cells were maintained in a medium based on RPMI 1640, and supplemented with 10% fetal bovine serum. Iron overload conditions were simulated by the addition of ferric citrate to the medium. A suitable concentration of ferric citrate, i.e. 4 mM and 40 mM ferric citrate in medium, was determined using dose-response experiments. Changes in cell redox status, ER stress induction, activity of autophagy and induction of apoptosis was determined via changed expression of relevant molecules using western blot analysis.
Results: In pancreatic cells exposed to ferric citrate, high iron stores and oxidative stress occurred already after 12 h of treatment. Increased expression of ER stress markers such as BiP and CHOP was also observed. The level of LC3B used as a marker of autophagy was significantly increased as well. The phosphorilation of IRE1α as well as activation of JNK kinases is believed to cause apoptosis by activating downstream caspases. Detected changes in the expression of various caspases indicate their cleavage and thus their activation.
Conclusion: Taking together, high levels of iron in the medium result in increased iron intake into the cells and lead to the formation of reactive oxygen species and oxidative stress. Persistent oxidative stress subsequently results in ER stress and autophagy activation. Unless the high iron levels are sufficiently compensated, cellular damage becomes more severe and the cell undergoes apoptosis.
This work was supported by grant MSM 0021620814 from Ministry of Education and Youth of the Czech Republic, grant 264703/SVV/2012 and projects UNCE 204015 and PRVOUK P31 from Charles University in Prague, Czech Republic.

Poster #97

THE INFLUENCE OF STORAGE ON IRON STATUS, OXIDATIVE STRESS AND ANTIOXIDANT PROTECTION IN PAEDIATRIC PACKED RED BLOOD CELL UNITS

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(Presented By: Keith Collard)

Objectives: The receipt of blood transfusions in premature babies has been shown to be associated with the development of the serious consequences of prematurity. This could be mediated, in part, by transfusion related iron overload and associated oxidative stress. The preparation of packed cell units removes most of the plasma proteins capable of binding and sequestering iron, and also the extracellular antioxidants. In addition, the premature baby is poorly equipped to deal with excess iron or free radical load and is likely to be at risk of transfusion-related iron and oxidative overload. The aim of

this study is to examine the influence of storage on the iron and oxidative status of paediatric packed cell units.
Materials and Methods: Standard paediatric packed cell units (6 from a single donor) prepared at the NHS Blood and Transplant Centre Bristol were used in this study. Packs were stored alongside packs for clinical use at the Blood Bank at Derriford Hospital. One pack was removed on arrival (3 days post donation), another at 7 days post donation and then weekly until 35 days storage. The contents of the packs were removed and centrifuged at 1,500 x g for 10 minutes. The extracellular fluid was removed for measurement of the following: total iron, iron binding capacity, non-transferrrin bound iron [NTBI], haemoglobin, malondialdehyde and reduced and total ascorbate.
Results: Iron was present in the extracellular phase from day 3 (6.09 ± 1.42 μM) and rose throughout storage to maximum levels of 32.14 ± 4.29 μM on day 35. A high percentage was present as the potentially damaging NTBI (14.99 ± 2.87μM on day 35). As expected iron binding capacity was low (5-6 nmol/ml). Both haemoglobin and malondialdehyde rose slowly from day 3 to day 21 and then more rapidly to day 35. Ascorbate levels were low (6.0 ± 2.68 μM day 3; 1.47 ± 0.88 μM day 35) and a large proportion was present in the oxidised state (55% on day 3; 99% on day 35). There were significant correlations between malondialdehyde and NTBI, Malondialdehyde and extracellular haemoglobin and malondialdehyde and ascorbate.
Conclusions: The data suggests that initial damage occurring during preparation of the paediatric packs causes iron to appear in the extracellular phase. This is associated with oxidative damage (probably to the red blood cell membranes) leading to further loss of iron and haemoglobin from the stored red blood cells as storage time increases. Further iron-induced oxidative damage to red blood cell membranes and haemoglobin could set up a potential vicious cycle leading to further iron loss from the red blood cell etc. Depending on length of storage, the fluid surrounding erythrocytes is rich in iron, highly redox active with limited antioxidant protection and ironbinding capacity. This is then available for transfusion to subjects ill-equipped to deal with an increase in iron and oxidative load. The implications for the clinical outcome, current transfusion practice, and the preparation, storage and use of packed cell units in neonatal intensive care units will be discussed.

Poster #98
CHANGES OF THE EXPRESSIONS OF THE GENES INVOLVED IN IRON METABOLISM BY THE IRON CHELATION USING DEFERASIROX IN THE IRON OVERLOADED MOUSE MODEL
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(Presented By: Yasumichi Toki)

Introduction: Iron chelation is the most important therapy for iron overload. Some iron chelators have been applied for iron overload patients. Deferasirox (DSX), an oral iron chelator, has been thought to accelerate iron excretion from the body. However, the gene expressions involved in iron metabolism and the precise target cells of DSX during the treatment are not clear. Therefore, we investigated the changes of gene expression profiles involved in iron metabolism in DSX treatment to elucidate the responses of various organisms to DSX.
Methods: Iron overload mouse models were made by intraperitoneal injection of iron dextran (the iron-loaded group). DSX was given orally for the iron overload mice (the iron-loaded + DSX treated group). The iron chelation by DSX had continued for 4 weeks. Mice without any treatment were participated as the control group. After 4 weeks, mice were sacrificed, and then the liver and small intestine were collected. The tissue samples were processed for hematoxylin-eosin and Berlin-blue staining. Total RNA was extracted from the tissues and quantitative RT-PCR (qRT-PCR) was performed for genes involved in iron metabolism. The genes investigated in this study were hepcidin-1 (*Hamp1*), transferrin receptor 1 (*Tfr1*), transferrin receptor 2 (*Tfr2*), divalent metal transporter 1 (*Dmt1*), ferroportin 1 (*Fpn1*).
Result: Stainable iron was observed in both hepatocyte and Kupffer cell in iron-loaded group, while these were not observed in control group. In iron-loaded + DSX group, although the stainable iron was eliminated from hepatocytes, it was not eliminated from Kupffer cell. The result of qRT-PCR analysis, in the liver of iron-loaded + DSX group, down-regulation of *Hamp1* and up-regulation of *Tfr1*, *Tfr2* and *Dmt1* were observed compared to the ironloaded group. In the duodenum of the iron-overload + DSX group, higher level of *Dmt1* was observed than the iron-loaded group.
Discussion and Conclusion: In addition to removal of iron from iron-loaded liver, our data showed that iron chelation by DSX affected on genes expressions involved in iron metabolism. The DSX treatment caused iron excretion from hepatocyte effectively, which might secondary induced decrease of *Hamp1* and increase of *Tfr1*, *Tfr2* and *Dmt1*. On the other hand, increase of *Dmt1* was observed in the duodenum by DSX treatment. *Dmt1* is the gene which promotes iron uptake from diet in duodenum, so this result indicated that iron limited diet might be needed for iron overload patients during the DSX treatment.

Poster #99

CONTRASTING SYNERGIES OF PLASMA NON-TRANSFERRIN BOUND IRON (NTBI) REMOVAL BETWEEN DIFFERENT CHELATOR COMBINATIONS DEPEND ON KINETIC DIFFERENCES OF IRON ACCESS BY SHUTTLE MOLECULES

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(Presented By: Patricia Evans)

Introduction: Not all iron that is unbound to transferrin (NTBI), in the presence of iron overload, is rapidly available for iron chelation. In particular only a fraction of plasma NTBI is rapidly chelated by desferrioxamine (DFO). By simultaneously applying deferiprone (DFP) with desferrioxamine (DFO), we have previously demonstrated pronounced shuttling of NTBI onto DFO, with relatively modest concentrations of DFP (3-10μM) both in iron-overloaded serum and in NTBI mimetics, such as iron citrate and iron citrate albumin solutions1. With the increasing clinical use of other chelator combinations, we have now extended these studies to include the combinations DFO + deferasirox (DFX, exjade) and DFP/DFX.
Methods: Chelators were added either singly or in combination at clinically relevant concentrations to iron citrate and iron citrate albumin solutions (both containing 10 μM iron) and iron-overloaded sera containing NTBI. The combinations DFO + DFX and DFP + DFX were used. Samples of the reaction mixtures were extracted at timed intervals and assayed for either ferroxamine or DFX iron complex using established HPLC methods.
Results: DFX failed to shuttle NTBI onto DFO (10 μM) whatever the presentation of NTBI across a wide range of DFX concentrations (20-60 μM), including those observed in patient sera in contrast to DFP and other bidentate chelators such as salicylaldehyde isonicotinoyl hydrazone (SIH). In contrast, transfer of all these forms of NTBI onto DFX was markedly catalysed by even low concentrations of DFP (1 μM) at trough (20 μM) and maximal (80 μM) DFX levels. All physiologically observed concentrations of DFP (1-100 μM) promoted shuttling of iron from iron citrate onto DFX with a maximal effect when the chelators are present at equivalent iron-binding concentrations (20 μM DFX and 30 μM DFP).
Discussion and Conclusion: These studies show that DFP acts as an effective shuttle to either DFO or DFX, where these molecules act as an iron 'sink'. By contrast DFX does not shuttle NTBI species. The origin of these differences in shuttling between chelators lies in the relative rates of access of the respective chelators for NTBI species. NTBI species are heterogeneous consisting of citrate monomers, dimers, oligomers and polymeric species, as well as protein bound forms (2) and these are not equally accessible to chelators. Shuttling requires the presence of smaller, more dynamic chelators that are able to penetrate large plasma NTBI complexes and access the iron that is then passed to a more thermodynamically stable but slower acting chelator sink such as DFO or DFX. Thus combinations of chelators with similar kinetics of iron access are not liable to show synergy in NTBI removal but act additively whereas those combinations with pronounced differences in rate of chelator access to iron are likely to show synergy. As NTBI is considered to be the mechanism by which iron is distributed extrahepatically in iron overload disorders, these chelator combinations offer enhanced
NTBI removal over use of a single chelator regime or a regime where chelators are alternated.
References:
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Poster #100

SUCCESSFUL TOLERANCE OF DEFERASIROX FOLLOWING DESENSITIZATION FOR SIGNIFICANT SKIN RASH

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(Presented By: Hatoon Ezzat)

Introduction: Deferasirox is an oral iron chelator that has become available in recent years. The long term tolerability profile are still being evaluated however; a common side effect is skin rash, which may occur in up to 10%. Severe rash is rare however, may require interruption of therapy and reintroduction at a lower doses. Occasional cases of angioedema have been reported for which cessation of drug is recommended but re-introduction is difficult. We report four patients with significant hypersensitivity to deferasirox manifest as NCI CTC grade 2-3 skin rash, who were able to tolerate deferasirox following desensitization.
Methods: Two patients with β thalassaemia major (TM), one with congenital dyserythropiotic anemia (CDA) and one with Hemoglobin H disease. All patients had evidence of iron overload and experienced significant skin rash to deferasirox but received a desensitization protocol. Desensitization was given orally as shown:

STEP 1		*STEP 2*	
Solution 1 (Deferasirox 1.25 mg/ml)		Solution 2 (Deferasirox 12.5 mg/ml)	
Deferasirox (ml)	Days	Deferasirox (ml)	Days
1 ml	1-3	1 ml	10-12
2 ml	4-6	2 ml	13-15
3 ml	7-9	5 ml	16-18

The dose of deferasirox was subsequently increased by 125 mg/day every week until the desired dose was reached.

Results: *Case 1:* A 23 year-old female with βTM switched to deferasirox 20mg/kg/day. Nine days after starting deferasirox she experienced extensive erythematous maculopapular rash and facial edema. Deferasirox was discontinued. Two days later the rash had progressed and she was started on prednisone. While still tapering prednisone, deferasirox was re-started at 500 mg/day. The rash recurred within 2 days and deferasirox was discontinued. One year later the desensitization protocol was given and well tolerated.
Case 2: A 21 year-old female with βTM switched to deferasirox 20mg/kg/day. Ten days later after starting deferasirox she developed diffuse erythema multiforme. Deferasirox was stopped and prednisone started with marked improvement. Five days later, she was re-challenged with deferasirox 500 mg/day. The rash recurred and deferasirox was discontinued. One year later, the desensitization protocol was given successfully.
Case 3: A 23 year-old female with CDA switched to deferasirox 20mg/kg/day for ease of administration. Eight days after starting deferasirox she developed a maculopapular rash with pruritis confined to the palms of the hands and feet. Deferasirox was stopped and the patient declined prednisone. She was offered the deferasirox desensitization protocol. Desensitization was given successfully but required a three day extension of treatment with solution 1 (2ml) because of recurrent rash, which resolved over the following two days with the temporary addition of benadryl.
Case 4: A 43 year-old male with Hemoglobin H disease started on deferasirox 15mg/kg/day for non transfusional iron overload. Ten days after starting he developed a maculopapular rash on his trunk and progressed to his face. Medication stopped and he was started on prednisone and offered desensitization protocol. Deferasirox was well tolerated after desensitization at full dose.
Conclusion: Deferasirox is often preferred by patients given its ease of administration and its promising effects on the removal iron, however skin rash is a common side effect that may limit its use in some patients. To our knowledge, this is the first report of a protocol that may successfully desensitize patients, allowing them to tolerate deferasirox in therapeutic doses.

Poster #101
VITAMIN D DEFICIENCY AND LIVER IRON CONCENTRATION IN TRANSFUSION DEPENDENT HEMOGLOBINOPATHIES IN BRITISH COLUMBIA
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University of British Columbia
(Presented By: Hatoon Ezzat)

Introduction:
Patients with inherited chronically transfused heamoglobinopathy disorders are at increased risk of endocrinopathies and bone disease due to iron overload. An adequate vitamin D level plays a major role not only in bone health but also in preventing other diseases such as colon cancer and cardiovascular diseases. Vitamin D deficiency is a relatively common manifestation in these patients, the mechanism of which remains unclear. The first step in vitamin D metabolism, known as vitamin D hydroxylation, occurs in the liver. Some liver disease can affect this step however; it is not clear whether liver iron overload has a possible role in interfering with this step. This study addresses the association between the degree of liver iron overload and vitamin D levels in patients with transfusion dependent haemoglobinopathies.
Methods: Patients with transfusion dependent haemoglobinopathies including TM, haemoglobin Eβ TM (EβTM), and congenital dyserythropiotc anaemia (CDA) attending the Inherited Bleeding and Red Blood Cell Disorder Program at either the adult (St. Paul's Hospital) or the paediatric (BC Children's Hospital) programs in British Columbia, Canada were identified using the programs database. Patients included were those who had an assessment of liver iron concentration (LIC) done by MRI (T2* or R2*) and underwent endocrinology assessment including 25-hydroxyvitamin D3 (25 OH D3) levels between January 2009 and the 31st of December 2011.
Results: Thirty patients with transfusion dependent haemoglobinopathies (21 with β TM, 6 with Eβ TM and 3 with CDA) were identified. Thirty patients had both LIC and vitamin D levels available for assessment. Patients had the following characteristics: age range 16-51 years and 16 (53.3%) were female. Their ethnicities were as follows: 12 (40%) were Asian, 8 (26.7%) were Indian, 6 (20%) were of unclear ethnicity, 3 (10%) were from the Middle East, 1 (3.33%) was Caucasian. All patients were receiving iron chelation therapy: 18 (60%) were receiving deferasirox, 9 (30%) were on desferrioxamine, 3 (10%) were on a combination and none were on deferiprone alone. 26 (86.7%) patients were receiving vitamin D and calcium supplementation. The median serum ferritin level was 693.5 mcg/L while LIC median was 4.25 mg/g dry weight (DW). Vitamin D deficiency/insufficiency was common in our patients, occurring in 15 (57.7%). There was a significant association between LIC >5 mg/gDW and vitamin D level <60nmol/L (P= 0.027), with 11 (38.5%) patients having moderate iron overload (LIC ≥5 mg/gDW), 8 of whom had a vitamin D level <60 nmol/L, indicating vitamin D insufficiency.
Conclusion: Vitamin D is important in regulating bone health and preventing against cancer and cardiovascular diseases. This study addresses the association between vitamin D deficiency and the presence of liver iron overload in patients with transfusion dependent hemoglobinopathies in a single center. We hypothesized that vitamin D hydroxylation in the liver may be affected by liver iron overload. The results of this study show a significant association between LIC ≥5 mg/gDW and vitamin D levels <60 nmol/L, suggesting that liver iron overload may affect vitamin D metabolism in the liver. These results, if verified in prospective trials, may help to identify patients requiring intensification of vitamin D and/or intensification of iron chelation therapy to reduce the incidence of clinical consequences.

Poster #102

"TRANSFERRIN-IMMUNE COMPLEX DISEASE" IS AN UNDERDIAGNOSED ACQUIRED ALTERATION OF IRON METABOLISM REVEALED BY COESISTENCE OF M-GUS AND HYPERSIDEREMIA

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(Presented By: Valeria Pinto)

Background: Transferrin-immune complex disease is a disorder of the iron metabolism whose pathogenesis is attributed to the presence of a monoclonal autoantibody against the transferrin and characterized by a high level of serum iron. Only five cases are described in literature.
Methods and Materials: We recently described a new case and consequently we looked for other cases searching patients with levels of serum iron more than 300 µg/dl among those referring to the general lab of our Hospital. Excluded cases related to a recognized cause of iron metabolism alteration or acquired iron overload due to transfusion therapy we pointed out 2 cases with hypersideremia and concomitant MGUS. By affinity chromatography and by western blot we analyzed the sera of these two patients. Levels of Hepcidin and LIC were performed too.
Results: We found the presence of immunoglobulin that selectively recognised transferrin and we demonstrated that this complex is present because transferrin is co-elute by chromatography with the immunoglobulin. Interestingly we identify Ig type M for a patient and G for the others although different was the light chain type composition. The value of LIC was normal, on the contrary the patient that we have previously described had liver iron overload. We present the data of these new patients and a revue of the cases previously described in literature (tab).
Conclusions: Our data and observation suggest that transferring-immune complex disease is an under diagnosed acquired alteration of iron metabolism that could lead to iron overload.

Patient	Sex	Age	Serum Iron µg/dL	Transferrin mg/dL	% Tf Saturation	Ferritin ng/L	Monoclonal Ig type	Monoclonal Ig level g/dL	Hb gr/dL	Liver Iron Overload (liver Biopsy-MRI)
Acta Haemat., 1977	F	71	780	ND	ND	ND	IgG1-k	ND	ND	Present
Blood, 2007	F	55	669	540	89	55	IgG2-k	1,46	14,1	ND
Blood, 2007	M	53	809	638	91	200	IgG-k	0,84	15,5	ND
AJH, 2008	M	62	710	570	100	800	IgG	1,5	14,9	Present Moderate
1	F	46	400	500	61	76,7	IgG-k	0,5	13	Absent
2	M	73	385	600	48	109	IgM-k	1,46	14,9	Absent
Normal range	NA	NA	F 37-145 M 59-158	200-360	18-60	F 13- 150 M 30-400	NA	NA	F 12,3-15,3 M 14-17,5	Absent

Poster #103

PHYTATE AND DIISATIN-PROPYLENEDIIMINE: IRON BINDING AND ANTIOXIDANT ACTIVITY OF CANDIDATES TO DECREASE LABILE POOLS OF IRON

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(Presented By: Breno Esposito)

Introduction: The investigation of known chelators as candidates for the redistribution of iron has been a pursuit of our group in order to increase the therapeutic arsenal to control iron overload disorders. Isatin-derived Schiff bases are low toxic imine chelators able to ferry metal ions to human cells. Phytate (phyt) is a polyphosphate inositol chelator present in plant tissues which markedly decreases dietary iron absorption. In this study, diisatinpropylenediimine (isapn) was synthesized. The ability of isapn and phyt to remove iron from a ferric-calcein derivative in a timely manner was used as an indication of chelator performance in a physiological relevant situation.
Methods and Materials: The antioxidant activities of the chelators against iron(III)-mediated prooxidation of ascorbate were investigated by the alteration of the oxidation rate of the fluorescence probe dihydrorhodamine (DHR).

Results: Diethylenetriaminepentaaacetic acid (DTPA) was the standard chelator in all measurements. After 2 h of incubation in physiological buffer (HBS pH 7.4), 50% of ferric iron removal from calcein was observed for DTPA (5x molar excess over calcein) and phyt (13x excess over calcein), however isapn did not effectively compete with calcein for iron(III) up to a molar excess of 50x. DTPA and phyt blocked Fe(III)-mediated auto-oxidation of ascorbate at a chelator:iron mol ratio of 1:1, however isapn was an ineffective antioxidant in this experimental setup.
Conclusion: These results indicate that phyt is an interesting candidate for the removal of labile plasma iron (LPI), the redox-active component of non-transferrin bound iron in iron overloaded patients. The lack of activity of isapn may be due to the fact that its diimine chelating moiety would be more suited for the binding of ferrous iron, the prevalent species in traffic in the cytosol.

Poster #104
VARIABLE DEGREE OF MEAN CELL VOLUME (MCV) AND MEAN CORPUSCULAR HEMOGLOBIN (MCH) IN HEMOGLOBIN E CARRIERS IS DETERMINED BY A COMMON TMPRSS6 VARIANT (RS855791) IN THAI POPULATION
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(Presented By: Nipon Chalaow)

Introduction: Interaction of HbE characterized by a G to A substitution and beta thalassaemia gives rise to HbE/beta thalassaemia that could be as severe as beta thalassaemia major. In Thailand, an average of 10-15% of Thai population are at risk for HbE carriers, a screening to detect HbE trait is critical for the success of prevention and control program for severe thalassaemia syndromes. However, screening of HbE heterozygotes is hampered by the fact that up to 20% of HbE carriers have a borderline or lower normal range of mean cell volume (MCV, >78 fL) and mean corpuscular hemoglobin (MCH, >26 pg). Therefore the cut-off criteria using these parameters that generally are applicable for beta and alpha thalassaemia, could miss a number of HbE traits. To date, molecular basis underlying this red blood cell variability has not been understood.
Methods and Materials: To determine the role of basal iron status and genetic control of red cell indies in HbE heterozygotes, 53 HbE carriers who were been identified through our on-going Thalassaemia Screening Project were analysed. Approximately 300 healthy individuals at their reproductive age (10-50 yrs) were comprehensively studied for their CBC, hemoglobin (Hb) chromatography and molecular testing for common thalassaemia mutations. Iron study including serum iron (SI), transferring saturation (Tsat), serum ferritin (SF) and total iron binding capacity (TIBC) were determined using standard method. In addition, a common TMPRSS6 variant (RS855791; C>T) previously shown to determined Hb, MCV, serum iron and Tsat from several GWAS studies was also genotyped. In addition another 261 Thai controls from different region of the country were also analysed to determine allele frequency of TMPRSS6 variants. Five cases with HbE trait were excluded due to clinical anemia and/or iron deficiency while the rest (n=48) had Hb and iron parameters at the normal levels for age and sex. Eight individuals with HbE (16%) had basal Hb and MCH above the cut-off.
Results: No correlation between age, sex, interaction of alpha thalassaemia genes (3.7 and 4.2 type deletions) and serum ferritin with red cell parameters was identified. Only the T allele of TMPRSS6 variant was found to be a strongest factor to determine MCV and MCH in HbE carriers. Individual with T/T alleles had significant lower MCV (mean 73.1±4.1 vs. 75.8±2.7 fL) and MCH (25±1.4 vs. 25.8±0.9 pg) comparing between genotypes T/T vs. T/C. A lower level of SI and Tsat in T/T individuals was also observed although they did not reach statistically significant. Interestingly, allele frequency of T allele in Thai population was higher than those of reported Caucasian population (T allele; 0.6 vs. 0.34) and this result was confirmed by our population study.
Conclusion: This data provide the first human evidence that TMPRSS6 variant can determine red blood cell phenotype, possibly through iron regulatory control by hepcidin -ferroportin mechanism, in hemoglobin disorder. It remains to be seen whether such variant and other hitherto TMPRSS6 mutants could be a genetic modifier for a more severe form of thalassaemia syndrome as it has been suggested recently from a murine model.

Poster #105
IN SILICO AND FUNCTIONAL ANALYSES OF GENES OF THE IRON METABOLIC PATHWAY
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(Presented By: Natalie Strickland)

Introduction: Transcriptional regulation is the first step in the process of gene expression and is governed by the presence of specific *cis*-regulatory regions (*cis*motifs), residing within the promoter region of genes, and the functional interactions between the products of specific regulatory genes (transcription factors-TFs) and these *cis*-motifs. Accurate bioinformatic analyses of *cis*-motif architecture could offer insights into complex mechanisms governing transcriptional regulation, serving as a refined

approach for prediction and the study of regulatory targets such as specific genes or groups of genes, such as the iron metabolism pathway.
Methods and Materials: The DNA sequence of the upstream non-coding region (~2 kb) of 18 genes (*ACO1*, *CP*, *CYBRD1*, *FTH1*, *FTL*, *HAMP*, *HEPH*, *HFE*, *HFE2*, *HMOX1*, *IREB2*, *LTF*, *SLC11A2*, *SLC40A1*, *STEAP3*, *TF*, *TFRC*, *TFR2*) known to be involved in the iron metabolism pathway was retrieved from the human Ensembl database. These sequences were subjected to *in silico* analyses to identify regions of conserved nucleotide identity utilising specific software tools.
Results: The sequences of nine (*CYBRD1*, *FTH1*, *HAMP*, *HFE*, *HFE2*, *HMOX1*, *IREB2*, *LTF*, *TFRC*) of the 18 genes when examined were found to contain a genomic region that demonstrated over 75% sequence identity between the genes of interest. This conserved region (CR) is approximately 145 bp in size and is common to each of the promoters of the nine genes. This finding adds strength to the hypothesis that genes with similar promoter architecture, and involved in a common pathway, may be co-regulated. The CR was further examined using comparative algorithms from specific platforms for motif detection. Specific combinations of *cis*-motifs were discovered within the CR identified in the promoter regions. *In silico* analysis of putative transcription factor binding sites revealed the presence of numerous binding motifs of interest that were detected by more than one bioinformatic tool.
Validation of the bioinformatic predictions was performed in order to fully assess the relevance of the results in an *in vitro* setting. The nine CRcontaining genes were functionally investigated following the design of luciferase reporter constructs containing: 1) the 2 kb promoter, 2) a synthetic 1.86 kb promoter with the CR removed and 3) the 145 bp CR element. These three reporter gene constructs were transfected into HepG2 and COS-1 cell lines and expression levels were monitored with a dual-luciferase reporter assay under standard culture conditions and simulated iron overload conditions. Results of the luciferase assays indicate that the synthetic promoter constructs displayed statistically significant variation in expression values when compared to the untreated control constructs. Further, the CR appears to mediate transcriptional regulatory effects *via* an ironindependent mechanism. With a few exceptions, the trends in expression observed for each of the constructs for the respective genes were consistent in both of the cell lines utilized and represent repeatable results across the different experiments performed. It is therefore apparent that the bioinformatic predictions were shown to be functionally relevant in this study and warrant further investigation.
Conclusion: The requirement for tight molecular control of iron homeostasis makes this biochemical pathway an ideal candidate for the study of gene promoters and transcriptional regulation utilizing a combinatorial bioinformatic and experimental system. Results of these experiments represent a unique and comprehensive overview of novel transcriptional control elements of the iron metabolic pathway.

Poster #106
A VALIDATED ELISA FOR QUANTITATING HUMAN SERUM HEMOJUVELIN
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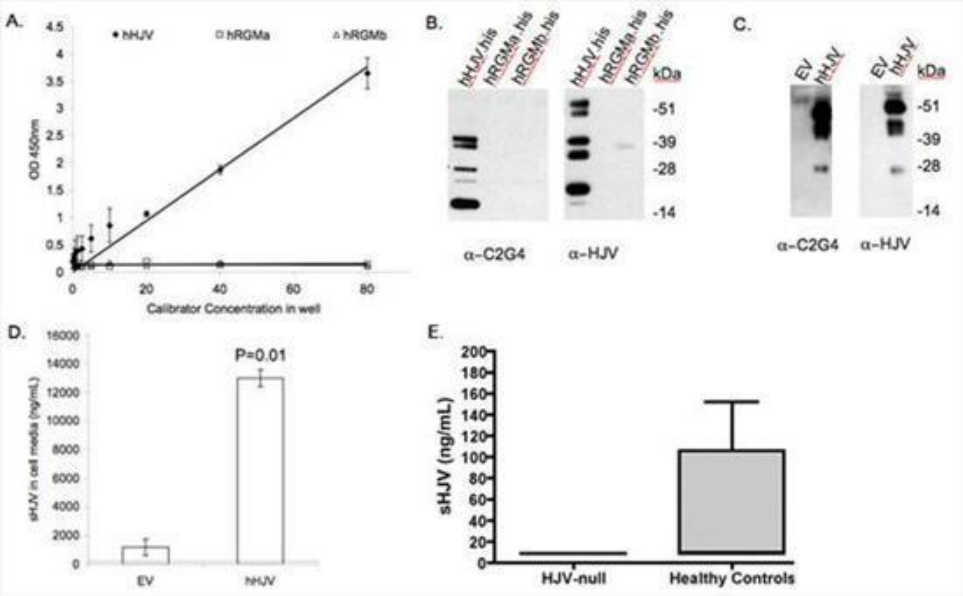
(Presented By: Chia Chi Sun)

Introduction: Recent studies have suggested the utility of soluble hemojuvelin (sHJV) as a biomarker for iron deficiency in iron deficient-diet fed rodents and in human patients with anemia of chronic disease and anemia of chronic kidney disease. Assessing whether circulating sHJV has a role in physiology and in regulating hepcidin and iron status in humans requires a reliable validated sHJV assay.
Methods and Materials: We have recently shown that the commercially available two-site HJV ELISA (USCN Life Sciences Inc., China) does not recognize recombinant human HJV protein (R&D Systems) or sHJV in conditioned media from cells transfected with human HJV cDNA (O.Gutierrez, et. al. 2012). A competitive one-site ELISA used by Brasse-Lagnel CG, et al. measured 780-1140 ng/mL of sHJV in normal healthy volunteers and 300 ng/mL of sHJV in a *HJV*-null patient, indicating that this assay has a high background.
Results: We have developed a novel, specific and reliable two-site ELISA to measure sHJV concentrations in human serum. Our laboratory characterized a novel monoclonal anti-HJV antibody (C2G4), which we used as capture antibody and a polyclonal anti-HJV (RGMc) antibody (R&D Systems) as detection antibody. Our HJV ELISA has a low background and in contrast to the commercially available HJV ELISA, our assay can specifically detect human recombinant HJV/RGMc (R&D Systems) but not the closely related human RGMa or RGMb proteins (Figure 1 A, B). It was also able to detect sHJV in conditioned media from cells transfected with human HJV cDNA (Figure 1 C, D). Furthermore, as a negative control and to set the baseline, we validated the assay using serum from a *HJV*-null (G171R HJV mutation) patient. The lower limit of detection (LLOD) of our assay in human serum was 9 ng/mL. Our *HJV*-null patient sample had undetectable levels of sHJV while serum from our normal healthy volunteers had measured sHJV levels ranging from 58 to 218 ng/mL, averaging 48 + 62 ng/mL (Figure 1 E).
Conclusion: Thus, our two-site assay has improved sensitivity and specificity for sHJV in humans. Measurement of sHJV may provide insight into the physiological role of sHJV in hepcidin and iron regulation and may be useful to determine iron status in humans.

Figure 1. Specificity of anti-HJV (C2G4) ELISA to detect sHJV in normal human serum. (A) Generation of ELISA standard curve using hHJV.His (calibrator) diluted in TBS-Casein buffer; closed diamonds (n=4). No detectable OD 450nm reading for hRGMa (open squares) and hRGMb (open triangles) standard curves at the same concentrations. (B) Immunobloting with mAb anti-HJV C2G4 and pAb anti-HJV specifically detected HJV fragments from the calibrator but not related RGMa or b proteins. (C) Specific sHJV bands were also detected in cell-conditioned media from Hep3B cells transfected with pcDNA3.hHJV (hHJV) but not with empty vector (EV). (D) The same cell-conditioned media quantified by ELISA (n=4). Pvalue was calculated using the student's t test. Dotted lines indicate LLOD. (E) Measurement of sHJV levels in healthy volunteers by ELISA, *HJV*null sHJV levels were undetectable and reported at LLOD of the assay (9ng/mL).



Poster #107

NOBEL QUANTIFICATION SYSTEM OF NON-TRANSFERRIN-BOUND IRON (NTBI) UTILIZING AUTOMATIC ANALYZER

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(Presented By: Katsuya Ikuta)

Introduction: Iron is an essential metal, but its overload causes various organ damages through free radical productions. When the balance of iron metabolism is collapsed by such as a prolonged transfusion, transferrin (Tf) is fully saturated and non-Tf-bound iron (NTBI) will appears in the serum. NTBI has been reported to relate to cellular and organ damages. Therefore, monitoring serum NTBI level is important to assess clinical status of patients with iron overload. However, the quantification of NTBI was so complicated that very limited institutions can measure NTBI at the present. We utilized high-performance liquid chromatography (HPLC)-based NTBI assay based on the previous report (Gosriwatana I. Anal Biochem, 1999), and improved the sensitivity of the method by reducing background iron contamination (Sasaki K. Mol Med Report, 2011). However, HPLC method still needs improvements, because pre-treatment of samples requires long time and high cost, so that it is difficult to handle many samples. Therefore, the novel quantification system utilizing such as automated analyzer should be desired. In the present study, we aimed to develop the novel NTBI measuring reagent corresponding to the automatic analyzer by modifying the HPLC method.

Methods: We developed the novel NTBI quantification system modifying HPLC method. NTBI was initially chelated using nitrilotriacetic acid (NTA) as same as HPLC system, but then Nitroso-PSAP was used as the chromogen instead of CP22 that was used in HPLC system. We examined the basic performance of this novel NTBI reagent without any pre-treatment of serum samples, and compared to HPLC method. Data analyses were performed using HITACHI 7700 auto analyzer. The following points were examined: (1) iron recovery from BSA , citrate-Fe, and human holo-Tf, (2) detection limit, (3) relation between the Tf saturations and NTBI values, (4) comparison of the value measured by the HPLC method and that measured by novel NTBI quantification system, (5) the effect of deferasirox (DFX) on NTBI value in iron-overloaded serum.

Result and Conclusion: Iron bound to BSA and citrate was quantified with the recovery rate of more than 90%. On the other hand, iron was not detected from holo-Tf, meaning Tf-bound iron is not measured in this system. Lower detection limit was defined as 0.34 μM. NTBI value increased markedly as Tf saturation reached 100%.The data obtained by HPLC method correlate positively with those determined by novel quantification system (R=0.923). The level of NTBI was dropped as the concentration of DFX increased, indicating this system can monitor NTBI during iron-chelation therapy. These should indicate

that our novel NTBI quantification system possess comparable efficiency compared with the HPLC method. Moreover, our new method should have quite attractive advantages: (A) that can measured approximately 800 samples in 1 hour, (B) that does not need any pre-treatment of sample serum, and (C) that does not need any expensive consumables, meaning excellent cost-benefit. Therefore our new method for NTBI quantification must be useful and powerful for the research to elucidate the clinical importance of NTBI in the near future.

Poster #108

DIAGNOSTIC ALGORITHM FOR HIGH LIVER IRON OVERLOAD

José María Alústiza[1], Agustin Castiella, MD[2], Jose Ignacio Emparanza, PhD[3], Iratxe Urreta, MD[3], Camino Martinez, MD[4], M. Dolores De Juan, MD[3] and Elena Zavala, MD[3]
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(Presented By: José María Alústiza)

Aim: To develop and validate a diagnostic algorithm for high iron overload (HIO) based on laboratory and genetic variables
Material-Methods: We collected a retrospective cohort with all consecutive patients between 2001-2008 studied by Magnetic Resonance Imaging (MRI) to determine liver iron concentration (LIC). This cohort served as the derivation set. We analyzed all variables using univariate statistics with the MRI acting as the gold standard. We studied the best combination of the diagnostics variables to build the algorithm. We validated the algorithm in a prospective cohort, collecting all patients referred to our hospital for study of iron metabolism alteration since 2009 onwards. We estimate the sensibility, specificity and predictive values with 95% CI. HIO is considered if the Hepatic iron index> 1.9 (estimated by MRI)
Results: Retrospective cohort: 242 patients (198 men/44 women), mean age 52,4 (SD 13,3). Thirty six of them had HIO. Nearly half of the patients (117/242=48,4%) had both Transferrin saturation index (TSI) and Ferritin elevated and 28 (11,5%) were C282Y homozygous. The final algorithm was as follows: We consider a patient as having HIO with the simultaneous occurrence of TSI and Ferritin elevated and C282Y homozygosis. HIO is discarded if TSI or Ferritin are within normal values. The rest should be studied by MRI.
Prospective cohort: 177 patients (148 men/29 women), mean age 56 (SD 13,9). The nosological characteristics of the algorithm in this validation study are:
 5 out of 177 (2,8%) were TSI and Ferritin elevated with C282Y homozygosis. Four of them proved to have HIO by MRI, PPV=80% (37,6-96,4)
Specificity= 99,4% (96,8-99,9)
 131 (74%) had TSI or Ferritin within normal values. Two of them had HIO NPV=99,2% (95,8-99,9), Sensitivity=83,3% (43,6-97)
 Less than a fourth (23,2%) of our sample needed to have a diagnostic MRI for HIO.
Conclusion:
MRI is not necessary in 77% of the patients for HIO diagnosis. MRI is indicated inpatients not C282Y homozygous with raised TSI and Ferritin.

Poster #109

EVALUATION OF FERRITIN >1000 CUTOFF POINT FOR DIAGNOSING TO DIAGNOSE LIVER IRON OVERLOAD

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(Presented By: José María Alústiza)

Aim: To establish the nosologic characteristics of ferritin >1000 ng/ml to diagnose high liver iron overload (hepatic iron index> 1.9) (HIO) and for the diagnosis of significant iron overload in liver (> 60 micromol Fe/g) (HIGT60) .
Material-Methods: Cohort of consecutive patients studied by MRI for quantification of liver iron concentration (LIC). Variables: age, sex, ferritin and LIC. We calculate the mean and standard deviation for quantitative variables and absolute and relative frequencies for qualitative variables. Patients with politransfusional history or diserithroietic disorders were not included in the cohort. The relationship between ferritin and LIC is analyzed using a simple linear regression model. To establish the nosologic characteristics of ferritin we estimated the sensitivity (S), specificity (Sp), positive predictive value (PPV) and negative (NPV) with their 95% CI.
Results: Total number of patients was 538 (449 men), with a mean age of 53.6 (SD 13.4). Mean ferritin value was 804.5 (SD 655.2). 56 patients (10.4%) had HIO and 59 (23.2%) had (HIGT60). Mean LIC in patients with ferritin> 1000 was 55.9 micromol Fe /g. The PPV for HIO is 27.1% (19.9 to 35.8) and NPV of 94.3% (91.6 to 96.1). With our prevalence of 10.4%, the expected results by chance alone would have been: PPV = 10.7% (5 to 21.5) and NPV = 89.6 (86.6 to 92), close to the values obtained with ferritin > 1000. To diagnose HIGT60, PPV of ferritin >1000 is 50% (41.1 to 58.9) and NPV of 84.3% (80.5 to 87.5). In this case, the expected results by chance would have been: PPV 24.6% (17.7 to 33.1) and NPV 77.1% (72.9 to 80.9).
Conclusion: Ferritin> 1000 has a low value for the diagnosis of HIO or for HGT60.

Poster #110
DIAGNOSTIC ALGORITHM FOR SIGNIFICATIVE LIVER IRON OVERLOAD
José María Alústiza[1], Jose Ignacio Emparanza, PhD[2], Iratxe Urreta, MD[2], Agustin Castiella, MD[3], Dolores De Juan, MD[2], Elena Zavala, MD[2], Camino Martinez, MD[4] and Elena Zubillaga, MD[2]
[1]Osatek; [2]Donostia Hospital; [3]Mendaro Hospital; [4]Zarauz OZ
(Presented By: José María Alústiza)

Objective: To develop and validate a diagnostic algorithm for Hepatic Iron greater than 60 micromol/gr (HIGT60) based on laboratory and genetic variables.
Material and Methods: We collected a retrospective cohort with all consecutive patients between 2001-2008 studied by Magnetic Resonance Imaging (MRI) to determine liver iron concentration (LIC). This cohort served as the derivation set. We analyzed all variables using univariate statistics with the MRI acting as the gold standard (60 micromol/gr). We studied the best combination of the diagnostics variables to build the algorithm. We validated the algorithm in a prospective cohort, collecting all patients referred to our hospital for study of iron metabolism alteration since 2009 onwards. We estimate the sensibility, specificity and predictive values with 95% CI.
Results: Retrospective cohort: 242 patients (198 men/44 women), mean age 52,4 (SD 13,3). Seventy of them had HIGT60. Nearly half of the patients (117/242=48,4%) had both Transferrin saturation index (TSI) and Ferritin elevated and 28 (11,5%) were C282Y homozygous. The final algorithm was as follows: We consider a patient as having HIGT60 with the simultaneous occurrence of TSI, Ferritin elevated and C282Y homozygosis. HIGT60 is discarded if TSI or Ferritin are within normal values. The rest should be studied by MRI. Prospective cohort: 177 patients (148 men/29 women), mean age 56 (SD 13,9). The nosological characteristics of the algorithm in this validation study are:
 5 out of 177 (2,8%) were TSI and Ferritin elevated with C282Y homozygosis. Four of them proved to have HIGT60 by MRI, PPV=80% (37,6-96,4) Specificity= 99,4% (96,8-99,9)
 131 (74%) had TSI or Ferritin within normal values. Two of them had HIGT60 NPV=99,2% (95,8-99,9), Sensitivity=33,3% (19,8-50,4)
 Less than a fourth (23,2%) of our sample needed to have a diagnostic MRI for HIGT60.
Conclusion: MRI is not necessary in 77% of the patients for HIGT60 diagnosis. MRI is indicated inpatients not C282Y homocigotes with raised TSI and Ferritin.

Poster #111
MR QUANTIFICATION OF HEPATIC IRON CONCENTRATION
Jose Ignacio Emparanza, MD[2], Jose Artetxe, MD[2], José María Alústiza[1], Agustín Castiella, MD[3], Pedro Otazua, MD[4], Elena Zavala, MD[2] and Camino Martinez, PhD[5]
[1]Osatek; [2]Donostia Hospital; [3]Mendaro Hospital; [4]Alto Deba Hospital; [5]Zarauz OZ
(Presented By: José María Alústiza)

Objective: To evaluate the accuracy of magnetic resonance (MR) imaging in the quantification of hepatic iron concentration.
Materials and Methods: Between April 1999 and June 2001, 112 patients were recruited prospectively. All had undergone liver biopsy and hepatic iron concentration quantification with spectrophotometry, followed by MR imaging. MR imaging involved use of four gradient-echo sequences and one spin-echo sequence. Signal intensity (SI) was measured on images obtained with each sequence by means of regions of interest placed in the liver and paraspinal muscle to obtain the liver-to-muscle SI ratio. The relationship between hepatic iron concentration and SI ratio for each sequence was analyzed with multiple linear regression. Receiver operating characteristic analysis was performed to find the diagnostic thresholds.
Results: Sixty-eight patients had normal hepatic iron levels (<36 μmol/g), 23 had hemosiderosis (36–80 μmol/g), and 21 had hemochromatosis (>80 μmol/g). With all sequences, an inverse linear relationship between iron concentration and SI ratio was apparent. The authors generated a mathematic model to estimate the iron concentrations from MR imaging data (r = 0.937). For estimated concentrations of more than 85 μmol/g, the positive predictive value for hemochromatosis was 100%; for those less than 40 μmol/g, the negative predictive value for hemochromatosis was 100%. For estimated concentrations of more than 58 μmol/g, the positive predictive value for iron overload was 100%; for those less than 20 μmol/g, the negative predictive value for iron overload was 100%.
Conclusion: MR imaging is a useful and noninvasive diagnostic tool for quantification of hepatic iron concentration.

Poster #112
DIAGNOSIS OF IRON OVERLOAD IN THE LIVER BY MRI WITH IN-PHASE AND OPPOSED-PHASE T1 SEQUENCE
José María Alústiza[1], Cristina Gonzalez, MD[1], Jose Ignacio Emparanza, PhD[2], Agustin Castiella, MD[3], Iratxe Urreta, MD[2] and Elena Zavala, MD[2]
[1]Osatek; [2]Donostia Hospital; [3]Mendaro Hospital
(Presented By: José María Alústiza)

Objective: To evaluate the correlation between the signal intensity (SI) of the liver in phase (P) and in opposite phase (OP) and the SI ratio between the liver and the muscle in P with the liver iron concentration (LIC) estimated by MRI.

Material and Methods: Retrospective review of liver MRI of 74 consecutive patients with suspected iron overload. The MRI technique include two sequences for estimation of the LIC and a T1- Gradient-Echo sequence in P and OP. Quantitative analysis: 1. - Estimation of the LIC by MRI. 2. - Measurement of SI ratio liver P / liver OP . 3. - Measurement of SI ratio liver/ muscle in P. Qualitative analysis: two radiologists, blinded to estimated LIC , analyzed images and they identify 2 groups: Group 1 (signal intensity of the liver is lower in P than in OP but not lower than the muscle in P) and Group 2 (signal intensity of the liver in P is lower than the muscle in P).
Results: In 11 patients the LIC is <30 mmol / g, in 35 between 30 -80 mol / g and in 30 patients> 80 micromol / gr. Quantitative analysis: Both ratios liver P/liver OP as liver/muscle P correlate with LIC . This correlation is better for liver/muscle P (r = 0.965) than for liver P/liver OP (r = 0.585).
Qualitative Analysis: The group 1 included 3 patients (mean LIC micromol Fe / g) and the group 2 26 (mean LIC 177 micromol Fe / g). There is a high degree of correlation between the two radiologists (kappa 0.83) with 6 discrepancies, five of which were evaluating the behavior of the signal intensity of the liver F / FO. All patients in Group 2 have a LIC> 83 micromol / gr.
Conclusions: The liver P/liver OP ratio has worse correlation with LIC and it is harder for qualitative assessment. The liver/muscle P ratio has a high predictive value for high iron overload and it is easier for qualitative assessment .

Poster #113
CALIBRATION OF THE QUANTIFICATION OF IRON CONCENTRATION IN THE LIVER BY MAGNETIC RESONANCE IMAGING
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(Presented By: José María Alústiza)

Objective: To calibrate 1.5 Tesla magnetic resonance scanners for the quantification of the concentration of iron in the liver.
Material and Methods: We analyzed twenty-eight 1.5 Tesla magnetic resonance scanners using a phantom with different solutions of iron (III) chloride which reproduces, in a given model to quantify liver iron concentration by MRI, the behavior of patients with moderate or high iron overload. In each MRI unit we measured the signal intensity ratio between each iron-containing tube and the tube without iron and then we calculated the liver iron concentration for moderate and high iron overload. We compared the results of each scanner with those of the reference machine in which the model and the phantom have been designed. We calculated the percentage difference.
Results: The mean difference in the ratios compared to the reference center was 11% (0.3-39). The mean concentration of iron was 71 mol Fe/g for moderate overload and 193 mol Fe/g for high overload. The mean difference was 6% (1.2- 7%) and 3.4% (0-16%). respectively. In two scanners, we applied a correction factor so that the difference was below 25% in all cases.
Conclusion: We calibrated twenty-eight 1.5 Tesla scanners for the concentration of iron in the liver and achieved variability less than 25%.

Poster #114
QUANTIFICATION OF LIVER IRON CONCENTRATION (LIC) BY MAGNETIC RESONANCE IMAGING (MRI) WITH 1 TESLA AND 1,5 TESLA MACHINES
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(Presented By: José María Alústiza)

Objective: To validate the effectiveness of the of 1 Tesla and 1.5 Tesla MRI to determinate LIC in patients with suspected liver iron overload.
Material and Methods: From 2002 to 2010 we obtained the estimated LIC by 1-Tesla MRI (Gandon et al. method) and 1.5 Tesla MRI (Alustiza et al method), and by chemical measurement in liver biopsy in 56 consecutive patients (58 MRI) .
Results: In the 1 Tesla MRI group (35 patients) according to LIC measured on liver biopsies : 15 patients were normal (<36 µmol Fe / g): 7 were correctly assessed and 8 overestimated by MRI. 15 had moderate iron overload (36 - 80 µmol / g): 5 were correctly assessed and 10 overestimated by MRI. 5 had high iron overload (> 80 µmol Fe / g): 5 correctly assessed by MRI. The correlation was r = 0.619. There were statistically significant differences between the mean LIC obtained by liver biopsies (53.43(9-214); SD 45.67;CI 95%: 37.74 to 69.12) and MRI (76.71(0-200); SD47.31; CI 95%: 60.46 to 92.97) (p <0.05), with a overestimation by MRI. In the 1.5 Tesla MRI group (23 patients) according to LIC measured on liver biopsies: 14 patients were normal: 6 correctly assessed and 8 overestimatedby MRI. 6 had moderate iron overload: 3 correctly assessed and 3 overstimated by MRI. 3 had high iron overload: , all were correctly assessed. The correlation between LIC by liver biopsies and 1.5 Tesla MRI was r = 0.815. The mean LIC obtained by biopsy (69.34 (9-714); SD 152.09; CI 95%: 3.57 to 135.11) and 1.5 Tesla MRI (70.43(11-261); SD 57.63; CI 95%: 45.51 to 95.36).These values haven't statistically significant differences (p> 0.05).
Conclusions: 1. Determination of LIC by 1.5 Tesla MRI (Alustiza et al. method) is better than 1 Tesla MRI (Gandon et al. method). 2. There is a significant tendency to overestimate LIC by 1 Tesla MRI.

Poster #115

MEASUREMENT OF LIVER IRON CONCENTRATION BY MRI IS REPRODUCIBLE

Jose Ignacio Emparanza, PhD[2], José María Alústiza[1], Agustin Castiella, MD[3], Camino Martinez, MD[4], Emma Salvador, MD[1], Elena Zubillaga, MD[2] and Arkaitz Azkuene, MD[2]
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(Presented By: José María Alústiza)

Objective: The objectives were: i) construction of a phantom to reproduce the behavior of iron overload in the liver by MRI, and ii) assessment of the variability of a previously validated method to quantify liver iron concentration between different MRI devices using the phantom and patients.
Materials and Methods: To generate the phantom iron solutions were used to reproduce the liver/muscle ratios of two "average" patients with intermediate and high iron overload in the both sequences of the model. To assess he variability 9 patients with different levels of overload were studied in 4 multivendor devices and 8 of them were studied twice in the machine where the model was developed. The phantom was analysed in the same equipment and 14 times in the reference machine.
Results: FeCl3 solutions containing 0.3, 0.5, 0.6 and 1.2 mg Fe/mL were chosen to generate the phantom , The average of the intra-machine variability for patients was 10% and for the inter-machines 8% For the phantom the intramachine coefficient of variation was always below 0.1 and the average of intermachine variability was 10% for moderate and 5% for high iron overload.
Conclusion: The behavior of the phantom reproduces the behavior of patients with moderate or high iron overload. The proposed method of calculating liver iron concentration is reproducible in several different 1.5 T systems.

Poster #116

LIVER IRON CONCENTRATION QUANTIFICATION BY MRI: ARE RECOMMENDED PROTOCOLS ACCURATE ENOUGH FOR CLINICAL PRACTICE?

Agusrin Castiella, MD[2], José María Alústiza[1], Jose Ignacio Emparanza, PhD[3], Eva Zapata,, MD[2], Belen Costero, MD[4] and Maria Díez, MD[4]
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(Presented By: José María Alústiza)

Objective: To assess the quantification of liver iron concentration (LIC) by MRI using the Rennes University (URennes) algorithm.
Materials and Methods: In 1999-2006 the LIC calculated with the URennes model in 171 patients was compared to the liver biopsy.
Results: According to the biopsy, 107 patients had no overload, 38 moderate overload and 26 high overload. The correlation between MRI and biopsy was r=0.86. MRI correctly classified 105 patients in the various levels of LIC. Diagnostic accuracy is 61.4%, with a tendency to overestimate: 43% of patients with no overload were diagnosed as overloaded, and 44.7% of patients with moderate overload were diagnosed as having high overload. The sensitivity of the URennes method for high overload is 92.3%, and the specificity for absence of overload is 57.0% MRI values > 170 µmolFe/g revealed a PPV for hemochromatosis of 100% (n=18); concentrations < 60 µmolFe/g had a NPV of 100% for hemochromatosis (n=101). The 44 patients with intermediate values remain in uncertainty.
Conclusions: The assessment of LIC with URennes is useful in 74.3 % of the patients to rule out or to diagnose high iron overload. The method has a tendency to overestimate overload, which limits its diagnostic performance.

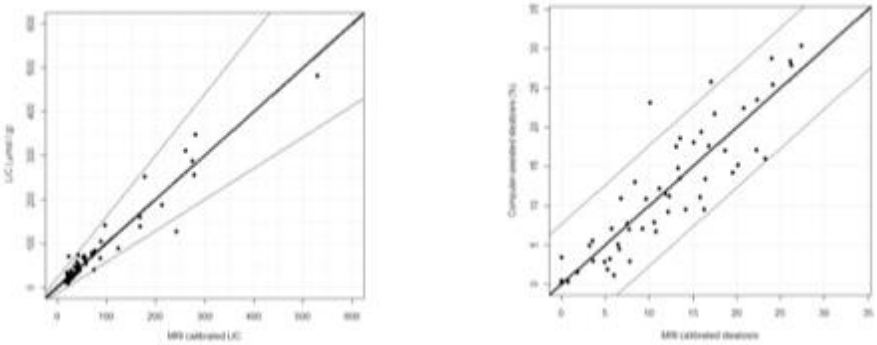
Poster #117

SINGLE BREATH-HOLD MULTIECHO MRI SEQUENCES (AT 1.5T) FOR THE SIMULTANEOUS LIPID QUANTIFICATION AND T2* MEASUREMENT IN PATIENTS WITH INCREASED SERUM FERRITIN LEVELS

Alberto Piperno, MD, Paola Trombini, MD, PhD, Irene Redaelli, Master Degree in Physics, Davide Paolo Bernasconi, PhD student, Nicoletta Paruccini, Master Degree in Physics, Filiberto Di Gennaro, MD, Sara Pelucchi, Master Degree in Biology, Nicola Zucchini, MD, Giorgio Bovo, MD and Stefania Galimberti, Associate Professor in Statistical Medicine
University of Milano-Bicocca, Monza, Italy
(Presented By: Alberto Piperno)

Introduction: Dysmetabolic Iron Overload Syndrome (DIOS) is a frequent condition characterised by hyperferritinemia and mild to moderate iron overload in association with alterations of the metabolic syndrome (MetS), insulin resistance and Non-Alcoholic Fatty Liver Disease (NAFLD). Coexistence of iron overload together with macrovescicular steatosis, lobular inflammation and insulin-resistance might contribute to the development of hepatic fibrosis in DIOS patients. Liver biopsy is often required in DIOS patients to quantify liver iron because serum ferritin is not a reliable index due to cytokine activation and hepatocellular necrosis, and to define diagnosis and prognosis of NASH. Sampling variability and invasiveness, limit the use of liver biopsy, especially when repeated biopsies are needed. Recent evidences indicate that Magnetic Resonance Imaging (MRI) is an accurate method for measuring LIC in thalassemia patients, and may provide a non-invasive tool to accurately

quantify intrahepatic lipid content. However, liver iron is a confounding factor for fat measurements by MRI, possibly limiting their use in patients with DIOS.
Methods and Results: Aim of the study was to evaluate and validate single breath-hold multiecho MRI sequences (at 1.5T) for the simultaneous lipid quantification and T2* measurement in patients with increased serum ferritin levels. This was accomplished by comparing MRI results for both iron and fat with their respective gold standards, i.e. LIC and computer-assisted image analysis for steatosis on liver biopsy specimens. The study prospectively included 67 patients (59 M and 8 F) with increased serum ferritin levels who underwent abdominal MRI examination and liver biopsy for diagnostic purposes from June 2008 and May 2010. Moreover, ten consecutive patients who performed both MRI and biopsy in 2011 were used as validation sample. Calibration equations predicting iron and fat based on MRI parameters and biopsy results were built using linear regression models. The Bland and Altman method was used to evaluate the agreement between MRI and biopsy in quantifying both liver iron and fat.
Conclusion: We demonstrated that single breath-hold multiecho MRI sequences is an efficient and rapid method to obtain a valuable measure of both liver iron content and severity of steatosis according with histological assessment in patients with different disorders leading to fat and/or iron accumulation. This is an important issue in the clinical practice because iron overload is known to occur concomitantly with NAFLD in many patients. First, MRI was able to roughly classify patients in two main different classes of severity both for iron and fat (absent-mild Vs moderate-severe), with high diagnostic performances. Second, after a proper calibration, MRI allowed an accurate quantification of iron and fat. Clinical decisions that would have been generated from MRI information, based on the findings obtained in the training set of 67 patients, are almost completely in line with those obtained from liver biopsy data of the validation sample.



Poster #118
WITHDRAWN

Poster #119
IRON BIOAVAILABILITY FROM INGESTED IRON OXIDE NANOPARTICLES
S. Chamorro[1], A. Brenes[1], A. Viveros[2], C. Romero[1], L. Gutiérrez[3], [4], G. Salas[3], [5], Y. Luengo[3], M.P. Morales[3], and F. J.Teran[5]
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(Presented By: Francisco J. Teran)

Introduction: Iron oxide nanoparticles (IONP) have become a powerful platform for several biomedical[1] and environmental[2] applications. The potential of IONP to act as contrast agent, drug and/or gene carrier, biological sensing, or hyperthermia mediator have been proved, however, the assessment of their safety and biocompatibility is mandatory prior to extending their use. Thus, the toxicity, biodistribution and excretion after long term exposure of IONP have to be elucidated.
Methods and Materials: The present study reports the iron bioavailability of ingested maghemite (gamma-Fe2O3) IONP. We use chickens as suitable animal model[3] to determine iron availability from maghemite IONP. Thus, we test the effect of a 14-days sustained ingestion of maghemite IONP (sized 6.5 and 12 nm) on plasma haemoglobin, iron excretion and accumulation in spleen and liver.Four experimental diets were fed ad libitum to chickens (12 per experimental group) from 7 to 21 days of age. One of the four groups received a basal iron-deficient diet (with no supplemental iron) while the other three groups were fed with iron-sufficient diets formulated to meet iron requirements by adding a commercial iron source (FeSO4) and two different size (6.5 and 12nm) IONP to the basal diet.
Results: After 14 days, faeces were collected and blood was sampled after an overnight fast to determine haemoglobin concentration with the cyanmethemoglobin method. Then, birds were euthanized and liver and spleen were collected, weighed and lyophilized for iron and IONP detection and quantification by atomic absorption spectrophotometry and magnetic susceptibility, respectively. Body and organ weight, and feed intake were similar among the four experimental groups. However, chickens fed the iron-deficient diet showed a reduction in plasma haemoglobin and haematocrit values and a lower iron concentration in faeces, liver and spleen than that of chickens fed ironsufficient diets, irrespective of the iron source.

Conclusion: For all parameters studied, no differences were observed among chickens fed diets supplemented with different iron sources. The latter result reflects a similar iron bioavailability from IONP than that from FeSO4, in agreement with a previous studies[4] reporting no histological accumulation of IONP in tissues of rats fed diets containing iron based nanostructures. The size of IONP does not affect the
iron bioavailability. In conclusion, our results indicate that iron from IONP is bioavailable, with no IONP accumulation in the liver or the spleen.

(1) K.M. Krishnan. 2010. IEEE Trans. Mag. 46, 2523.
(2) F.M. Koehler, et al. 2009. Chem. Commun. 32, 4862.
(3) Tako et al. 2010. Poult. Sci. 89: 514-521.
(4) Hilty et al., 2010. Nature Nanotechnol. 5, 374-380.

Poster #120

HEMODIALYSIS RESTORED IRON DISTRIBUTION WHICH WAS SEQUESTERED IN SPLEEN BY BILATERAL NEPHRECTOMY

Takeshi Nakanishi, MD, PhD, Aritoshi Kida, MD, Yasuyuki Nagasawa, MD, PhD, Yukiko Hasuike, MD, PhD, Jyunko Yamashita, Nobuhide Akaike, Yukifumi Kokuba, PhD and Takahiro Kuragano, MD, PhD
Hyogo College of Medicine
(Presented By: Takeshi Nakanishi)

Introduction: Acute kidney injury (AKI) is associated with dysregulated iron metabolism, which could play a significant role in cellular injury and several complications. In addition, the effect of hemodialysis (HD) for the therapy of AKI on iron metabolism has not been well defined. We suspected that hepcidin, the principal iron regulatory hormone, plays an important role in iron metabolism in AKI.

Methods and Materials: In the present study, iron parameters including hepcidin, were examined in the rats with bilateral nephrectomy (BNx) and the effects of HD on these parameters in these animals were tested. The rats were divided into 3 groups, 1) Sham-operated group, 2) BNx group, and 3) HD therapy was treated 2 days after BNx (HD group). Polysulfone membrane and acetate-free dialysate were used for HD. HD treatment time was 4 hours. Plasma levels of hepcidin were determined by liquid chromatography tandem mass spectrometry (LC-MS/MS). Sections of liver or spleen were stained with Berlin blue to examine the accumulation of iron. mRNA levels of hepcidin and ferroportin1 in spleen and liver were also quantified using RT-PCR.

Results: In BNx, plasma iron level and hematocrit were decreased and hepcidin was increased, while iron staining of spleen in BNx group was significantly more intense than that of Sham. After HD session, splenic iron staining was diminished to the level of Sham group, which was concomitant with the increase in plasma iron and the decrease in hepcidin. Iron stainings and mRNA levels of hepcidin and ferroportin1(FPN) in liver were not affected by BNx as well as HD. BNx remarkably decreased mRNA levels of FPN in spleen, while HD did not restore them. In summary, BNx moved iron from hemoglobin and plasma to spleen, which is associated with the increase in plasma hepcidin. A single hemodialysis session accelerated the release of iron from spleen, and plasma iron was increased which was linked with the decrease in plasma hepcidin level.

Conclusion: Our data suggested that, in AKI as well as HD, hepcidin might dynamically modulate iron metabolism in BNx as well as HD.

Poster #121

RELATIONSHIP OF SERUM IRON MARKERS TO INFECTION AFTER LIVER TRANSPLANTATION

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[1]Tufts Medical Center; [2]Lahey Clinic; [3]Intrinsic LifeSciences, LLC; [4]UCLA, David Geffen School of Medicine
(Presented By: Jennifer Chow)

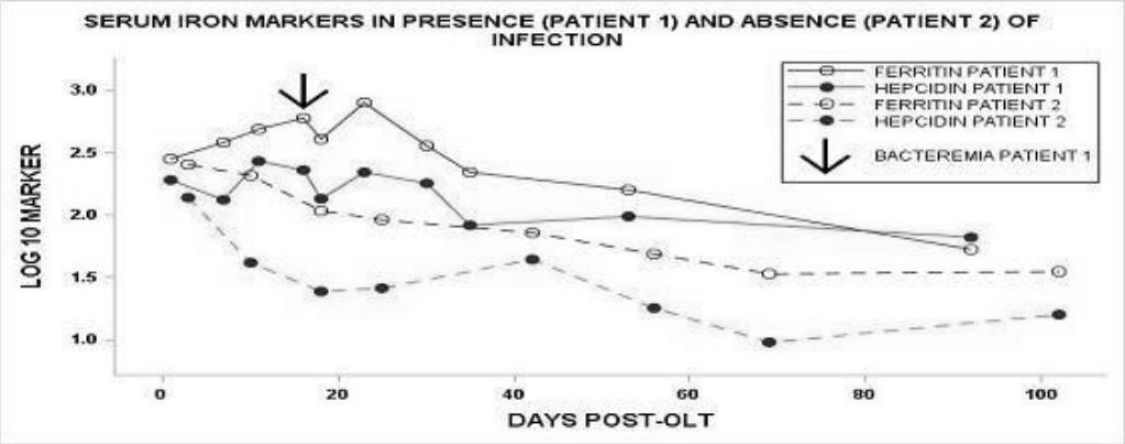
Background: Hepcidin, the iron-regulatory hormone synthesized by the liver, is induced by inflammation and infection. We explored whether serum hepcidin could have clinical utility as an early marker of infections in immunocompromised patients. We report hepcidin and other iron marker patterns in relationship to clinical infectious events in 20 patients who underwent orthotropic liver transplantation (OLT).

Methods: Baseline serum was drawn within 72 hours after surgery. Patients were prospectively followed for 6 months with additional serum drawn weekly while hospitalized and at each outpatient visit (at increasing intervals post-OLT). In addition, serum samples were captured within 72 hours of suspected infections triggered by fever, administration of empiric antibiotics, and/or ordering of microbiologic cultures. Hepcidin was measured using a validated C-ELISA assay.

Results: Overall, hepcidin and other serum iron markers (iron, transferrin saturation=TSAT, ferritin) are steady over time and reproducible. Women (N=5) had lower median hepcidin levels averaged over time than men [42.8 ng/mL (IQR 23.3-87.7) vs. 90.0 (44.2-144.7)]. In patients without infection (N=6), serum hepcidin and ferritin reach a steady-state approximately 4-6 weeks post-OLT. Immediately post-OLT, ferritin decreases more dramatically than does hepcidin. Over time, hepcidin generally has greater fluctuations over shorter periods of time than does ferritin. In patients with definite (microbiologically proven, N=5) bacterial and/or fungal infections, concurrent increases in hepcidin and ferritin were seen during 5/8 infectious episodes. The increase in hepcidin and ferritin preceded the date of the positive microbiologic culture by 3-7 days. (Graph) In patients with possible (N=5), but not microbiologically proven infections, an increase in hepcidin and/or ferritin was seen during 7/10 possible infectious episodes. In most cases, the increase in hepcidin and ferritin preceded the date of the suspected

infectious event by as long as several weeks. In nearly half of cases, hepcidin is the first to increase and reach a peak level followed by ferritin 3-7 days later. In the other half of cases, the increases in hepcidin and ferritin are simultaneous. In contrast, patients without infections had flat patterns of hepcidin and ferritin after the initial post -OLT period (1 month). A rise in ferritin and hepcidin were only seen in 1/4 patients with viral infections (recurrent hepatitis C [HCV]). The preceding rise occurred 5 weeks before HCV was diagnosed and also preceded the rise in liver function tests by 2 weeks.

Discussion and Conclusion: Hepcidin in a transplanted liver appears to be produced and regulated similarly to a healthy native liver. In most patients, a rise in hepcidin and ferritin occurs before a bacterial or fungal infection is diagnosed and decrease as the infection is treated/resolved, indicating that the two markers may be useful in early detection of these infections. Our final cohort will have 130 patients in which we will further explore the relationship between infections and hepcidin with the goal of developing predictive and prognostic tools for iron metabolism and infection.



Poster #122

EXPLORATIVE STUDIES TOWARD HEPCIDIN AS NOVEL TREATMENT FOR IRON-INDUCED KIDNEY INJURY

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(Presented By: Rachel van Swelm)

Introduction and Objectives: Chronic kidney disease (CKD) often progresses to End Stage Renal Disease necessitating renal replacement therapy. There is an unmet need for specific treatment modalities for CKD as current treatment possibilities are limited and not fully adequate. It has been well established that tubuleinterstitial injury plays a pivotal role in CKD, which may in part be mediated by iron. Moreover, increased urinary concentrations of hepcidin, an iron -regulating hormone peptide, is associated with a reduced risk of kidney injury in patients after cardiac surgery. We, therefore, hypothesize that hepcidin might have protective effects against iron-mediated kidney injury. Here, we performed explorative studies to establish the role of the kidney in hepcidin handling and the role of hepcidin in tubule cell iron metabolism.

Materials and Methods: Plasma and urinary hepcidin-1 concentrations were measured in control mice and kidney specific megalin-deficient mice (megalin lox/lox, apoECre mice on a C57Bl/6 background) by surface-enhanced laser desorption ionization time-of-flight mass spectrometry (SELDI-TOF MS). Further, human urine derived conditionally immortalized proximal tubular epithelial cells (ciPTECs) were used to demonstrate the toxicity of iron by measuring cell viability using the colorimetric formazan (MTT) assay after incubation with holotransferrin, hemoglobin, Fe(II)sulphate and Fe(III)citrate. Subsequently, hepcidin (*Hamp*) mRNA production was measured after incubation with these compounds by qPCR. For comparison, *Hamp* mRNA expression was also measured in healthy human and mouse kidney tissue.

Results and Discussion: Presence of hepcidin in healthy human and mouse kidney tissue was confirmed by immunohistochemistry. Hepcidin was observed at the apical side of proximal and distal tubules. In megalin-deficient mice (n=5), we observed that urinary hepcidin-1 concentration was 7 fold increased compared to wildtype (n=5, $p< 0.01$) suggesting megalin dependent absorption of hepcidin in the proximal tubule. We detected *Hamp* mRNA expression in healthy human and *Hamp-1* mRNA expression in healthy mouse kidney tissue, which was lower compared to liver tissue but still clearly detectable. Incubation of ciPTECs with holotransferrin led to a decreased cell viability after 24h and an induction of *Hamp* mRNA expression after 8h of incubation compared to untreated cells. Other compounds did not affect cell viability, however, incubation with Fe(III)citrate resulted in increased *Hamp* mRNA transcription after 8h, and hemoglobin increased *Hamp* mRNA concentration after 24h incubation.

Conclusion: Our explorative studies are consistent with reabsorption of filtrated hepcidin by the proximal tubule and local kidney hepcidin production, which may be affected by iron and iron-induced injury.

Grant Support: Dutch Kidney Foundation, Innovation grant no IP 12.81 “Hepcidin: a novel treatment for kidney injury”.

Poster #123

MOLECULARLY IMPRINTED NANOPARTICLES FOR HEPCIDIN CAPTURE: A SYNTHETIC ALTERNATIVE TO NATURAL ANTIBODIES

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[1]Dept. Biotechnology, University of Verona; [2]Department of Biotechnology, University of Verona, Italy; [3]Department of Public Health, University of Verona, Italy; [4]Department of Analytical Chemistry, University of Torino, Italy; [5]Department of Medicine University of Verona, Italy

(Presented By: Alessandra Maria Bossi)

Introduction: Hepcidin, the master regulator of iron homeostasis, is currently measured in biological fluids through Mass-Spectrometry (MS)-based techniques or traditional immunoassays. However, both these approaches have consistent disadvantages, including costs, need of complex instrumentation (i.e. MS), difficulties in obtaining high quality antibodies. Thus, there is a need of developing alternative approaches. Nanotechnologies have a great potential in this sense. Our aim was to produce nanoparticles (NPs) able to selectively capture hepcidin, and suitable for replacing antibodies in the future development of assays for hepcidin in biological fluids. Inherent advantages of NP-based assays are the possibility of producing high quantities of "pseudo-antibodies" at low costs, their reproducibility, their stability to harsh conditions and their stability at room temperature. These features theoretically broaden the possibilities of distributing properly assembled, cheaper, assays to every latitude, i.e. in developing countries.

Material and Methods: we prepared poly-acrylamide based NPs of sizes similar to natural antibodies, i.e. nanometer dimensions (10-80 nm) and molecular weight of 500.000-1.000.000 Daltons. The recognition abilities were imparted to the NPs by exploiting the technology of Molecularly Imprinted Polymers (MIP). In general, MIP materials are formed with a template-mediated synthesis, in which the template (here hepcidin, or part of it) is solvated together with functional monomers. The functional monomers assemble around the template according to thermodynamic forces, creating a pre-assembly that is fixed during the polymerization into the growing polymeric networks. At the end of the process, a solid material with embedded depressions complementary in size and shape to the template is formed; this is able to selectively re-bind the template. The synthetic protocol was optimized: monomer composition, polymerization temperature, initiators systems, solvents, to prepare MIP-NPs having a small number of binding sites per particle, resembling natural antibodies.

Results: A collection of MIP-NPs of 10-80 nm was prepared by setting up a precipitation polymerization method. In order to compare MIP-NPs to antibodies, the recognition abilities of the MIP-NPs were evaluated with a panel of analytical techniques, including Capillary Electrophoresis, Isothermal Titration Calorimetry, and Fluorescence. The dissociation constants of the MIP-NPs were in the range 10-6-10-8 M, binding kinetics 1-3 hours, the imprinting effect which expressed as analyte bound onto MIP-NP vs bound onto control-NP was 1.1-1.4. The good selectivity of the MIPNPs for Hepcidin was proven by the negative binding of peptides with sequences unrelated to the target hormone.

Conclusions: Nanotechnologies have been proved promising to obtain plastic "pseudo-antibodies" with recognition abilities and behavior comparable to natural antibodies. While further optimization are in progress, the data collected so far allowed to select the best protocols for the MIPNPs preparation, in view of their next integration with detection systems to obtain low-cost and good performance pseudo-immunoassays.

Poster #124

IS HEPCIDIN-25 PRODUCTION INCREASED IN PATIENTS WITH CHRONIC KIDNEY DISEASE?

Adam Rumjon and Iain Macdougall, MB, ChB, BSc, MD, FRCP

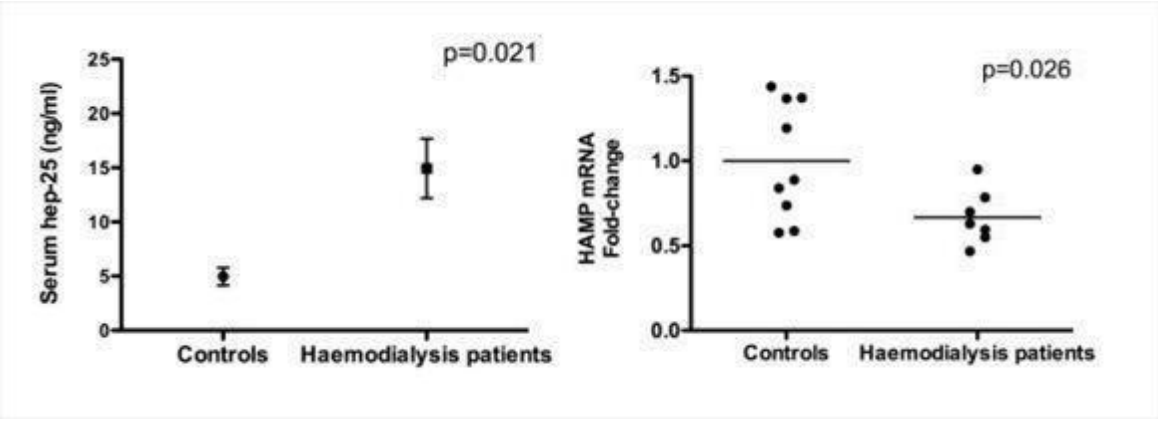
King's College London

(Presented By: Adam Rumjon)

Background: Hepcidin-25 (hep-25) plays a critical role in iron metabolism, and acts principally by blocking the transport of dietary iron through enterocytes, thus reducing the availability of iron for the production of red blood cells. Hep-25 also acts on hepatocytes and macrophages to regulate iron fluxes *in vivo*. Hep-25 levels are elevated in inflammatory conditions, but have also been shown to be elevated in haemodialysis (HD) patients. A combination of reduced filtration and the pro-inflammatory/acidic milieu of chronic kidney disease (CKD) is thought to be responsible for this, but the exact contribution of each is unknown. In this exploratory study, we sought to examine the possible pathways that are responsible for the elevated levels of hep-25 in CKD.

Methods: Patients were selected for this study on the basis of the following inclusion criteria; on HD for >3 months, haemoglobin (Hb) >10 g/dL, CReactive Protein (CRP) <20 mg/L, dialysis via an arterio-venous fistula/PTFE graft, stable IV iron and erythropoietin doses for >1 month, and no hospitalisation/antimicrobials for >1 month. A control group of healthy individuals was selected for comparison. Blood (10ml) was drawn pre-dialysis and separated by Ficoll Hypaque (GE Healthcare) centrifugation. Serum hep-25 levels were measured using liquid chromatography mass spectrometry. Peripheral blood mononuclear cells (PBMCs) were processed immediately (5-6 $x10^6$ cells/mL); total cellular RNA was extracted using RNeasy kits (Qiagen) and the extracted RNA (500 ng) was reversed transcribed using a High Capacity RNA-to-cDNA kit (Applied Biosystems. Quantitative RT-PCR was performed using the Applied Biosystems 7900HT Fast Real-Time system.

Amplification reactions were performed using Taqman HAMP (encoding hepcidin) and GAPDH (housekeeping) primers. All reactions were performed in triplicate.
Results: To date, 5 males and 2 females have been studied (age 56±20(SD) years, Hb 11.3±0.8 g/dL, CRP 10.4±5.0 mg/L, ferritin 367±90 ng/mL and albumin 39.7±1.8 g/L). The median IV iron dose was 100mg/week, and the median Eprex dose was 46.2 [IQR 18.9-114.6] iu/kg/week. The mean age of the control group was 44±15 years. Serum levels of hep-25 were significantly elevated in the HD population (14.9±2.7 vs 4.9±0.8, p=0.021). Interestingly, however, HAMP mRNA levels were significantly lower than in healthy controls (mean fold-change 0.667±0.06 in HD compared to controls (p=0.026)).
Conclusion: Despite higher circulating levels of hep-25, significantly lower transcription of HAMP mRNA was unexpectedly observed in HD patients. This counter-intuitive result, which has not been described previously, requires confirmation in a larger cohort of patients. Elucidation of this effect is also required; it is possible that negative feedback mechanisms may be in operation in HD patients to reduce hep-25 levels.



Poster #125
SIGNIFICANCE OF HEPCIDIN LEVELS ASSESSMENT IN THE DIAGNOSIS OF SELECTED TYPES OF ANEMIA IN CHILDHOOD
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Introduction: The peptide hormone hepcidin is the main factor regulating iron homeostasis. It ensures communication between places where iron is stored (hepatocytes and macrophages) and places where it is absorbed (enterocytes), utilized (erythroid cells) or recycled and released into the bloodstream (macrophages). Hepcidin synthesis is regulated by signals responding to inflammation, erythropoietic activity, iron level, iron stores in the organism and oxygen tension. An increase in hepcidin level leads to the retention of iron in enterocytes and macrophages and a drop in iron plasma level.
Objective: To assess hepcidin levels and their diagnostic contribution in pediatric patients with selected types of anemia: pyruvate kinase deficiency (PK), iron deficiency anemia (IDA) and anemia in inflammatory bowel disease (IBD).
Patients and Methods: Using the proteomic analysis, hepcidin level was assessed in 27 children (5 patients with PK deficiency, 10 patients with IDA and 12 patients with IBD) in the age of 6 months–18 years. Hepcidin levels were compared to those in a cohort of 16 healthy children examined prior to planned surgery. The levels of the putative negative regulator of hepcidin, growth differentiation factor 15 (GDF-15), were measured in selected patients using commercial ELISA kit.
Results: Hepcidin levels in patients with PK deficiency are significantly lower than in healthy controls ($p<0.02$) which corresponds to accelerated erythropoiesis. Hepcidin levels negatively correlated with increased serum levels of GDF-15 and likely contribute to a greater iron overload in PK patients. In patients with IDA, significantly lower hepcidin levels ($p<0.01$) were found in comparison with the control group, reflecting an increased iron-demand of the organism. Surprisingly, no significant difference between hepcidin levels in patients with IBD and the controls was found. However, the range of hepcidin levels in IBD patients was substantially broader from extremely low to increased values. Thus hepcidin level can clearly define IBD patients with absolute iron deficiency. In both groups, IDA and IBD, the GDF15 levels were 2- to 3-times higher than in normal controls. More samples need to be analyzed to address possible correlation between GDF-15 and hepcidin under these conditions.
Conclusion: Determination of hepcidin levels can enable more accurate diagnosis of anemia by providing information on the current status of iron metabolism. It may provide an important piece of information not only on the current deficiency of iron required for erythropoiesis and the degree of iron overload, but also on the current capacity of enterocytes to absorb iron from the intestinal lumen. It may be used as guidance when deciding on indications of oral and parenteral application of iron. The knowledge of hepcidin level, in correlation with levels of other proteins involved in the regulation of iron metabolism, may bring very important insights into the aspects of iron homeostasis.

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Poster #126

PLASMODIUM FALCIPARUM AND PLASMODIUM VIVAX GAMETOCYTE CLEARANCE IN MELANESIAN CHILDREN DETERMINED BY MAGNETIC FRACTIONATION ILLUSTRATES SPECIES-SPECIFIC DIFFERENCES IN MALARIA TRANSMISSION

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(Presented By: Stephan Karl)

Introduction: Despite recent improvements in global malaria control, there are still more than 300 million clinical episodes per year and more than 500,000 deaths. The most common parasite species world-wide are *Plasmodium falciparum* and *P. vivax*. Malaria parasites infect red blood cells and catabolize hemoglobin. The resulting free heme is detoxified by polymerization into crystalline hemozoin material. Iron in hemozoin is present in the high spin III+ state rendering infected cells containing this material susceptible to magnetic fractionation. Gametocytes are the blood stages of malaria parasites that transmit the disease to the mosquito vector. They contain high quantities of hemozoin. *P. falciparum* and *P. vivax* gametocytes occur at low densities in peripheral blood, making it difficult to investigate their production and clearance kinetics. We have previously described a method of high field gradient magnetic fractionation to simultaneously and quantitatively detect circulating *P. falciparum* and *P. vivax* gametocytes blood with high sensitivity.
Methods and Materials: In the present study we investigated gametocyte clearance in a cohort of Melanesian children with uncomplicated malaria treated with one of two artemisinin combination therapies (ACTs), namely artemether-lumefantrine and artemisinin-naphthoquine. It has been shown previously that *P. falciparum* and *P. vivax* gametocytes are cleared at different rates, presumably due to different adaptation of these parasite species to survival during prolonged periods of low transmission. Whereas *P. falciparum* relies on long-living, metabolically inactive, drug-resistant gametocytes, *P. vivax* produces dormant liver stages (hypnozoites) with gametocyte forms that are short lived and rapidly cleared.
Results: In the present study, magnetic fractionation identified 82% of children with *P. falciparum* malaria and 100% of children infected with *P. vivax* as gametocyte carriers before ACT was given. *P. falciparum* gametocytes were present in 51% of children two weeks after treatment while all children with *P. vivax* were gametocyte free at 48h after treatment. Six weeks post-ACT, 40% of children had *P. vivax* gametocytes while only 7% had *P. falciparum* gametocytes. This suggests that there is relatively frequent relapse of *P. vivax* from hypnozoites.
Conclusion: In conclusion, magnetic fractionation is a convenient and sensitive method for simultaneous detection of *P. falciparum* and *P. vivax* gametocytes in peripheral blood. The present study illustrates the different strategies that have evolved in these two malaria parasite species to overcome periods of reduced or absent transmission.

Poster #127

ANALYSIS OF IRON DISTRIBUTION AND MAGNETIC CHARACTERIZATION OF SCHISTOSOMA MANSONI AND SCHISTOSOMA JAPONICUM EGGS

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(Presented By: Stephan Karl)

Introduction: Schistosomiasis, caused by infection with bloodflukes of the genus *Schistosoma*, is a major disease of humans in tropical and developing nations. Some 200 million people are infected with the disease, with 600 million at risk of infection.
It has become increasingly recognized that elimination of hepatosplenic schistosomiasis is hindered by the absence of appropriate diagnostic techniques. With a diagnostic limit of approximately 100 eggs per gram feces, the current gold standard test, the Kato-Katz method of faecal examination, is limited by poor sensitivity. It is estimated that more than half of all infections with hepatosplenic schistosomiasis are missed in cross sectional studies. Recently an improved method for the detection of parasite eggs in fecal samples has been described involving magnetic fractionation of fecal matter after introducing magnetic particles to form parasite egg – magnetic particle conjugates. This approach enables screening of much larger volumes of fecal samples and thus increased diagnostic sensitivity. However, the mechanism for the binding of the magnetic particles to the *Schistosoma* eggs remains unexplained and may either be related to specific surface characteristics of the eggs or their inherent magnetic properties.
Methods and Materials: The aim of the present study was to characterize the magnetic properties of *Schistosoma mansoni* and *Schistosoma japonicum* eggs. Eggs were isolated from livers of infected mice and investigated using scanning electron microscopy (SEM), transmission electron microscopy (TEM) based elemental mapping, superconducting quantum interference

device (SQUID) magnetic susceptometry and inductively coupled plasma atomic emission spectroscopy (ICP-AES). Egg migration in magnetic fields was studied using light microscopy. Furthermore the binding characteristics of magnetic particles to parasite eggs where quantitatively investigated.
Results: The present study shows that iron is localized in the shell of Schistosoma eggs. The eggs exhibit distinct paramagnetic characteristics. However, these magnetic properties did not enable migration of eggs *per se* in a magnetic field. Furthermore, magnetic particles bound to the eggs even without an applied magnetic field. Interestingly, *Schistosoma japonicum* exhibited higher affinity to the particles as compared to *Schistosoma mansoni*.
Conclusion: It can be concluded that the binding of the magnetic particles to the eggs is unlikely to be caused purely by the inherent magnetism of the eggs. Further studies will aim to optimize magnetic particle – egg binding to improve the novel magnetic fractionation method for diagnosis of Schistosomiasis

Poster #128
COMPARATIVE CHARACTERIZATION OF IRON DEPOSITS IN SPLEENS AND LIVERS OF MALARIA AND SCHISTOSOMA INFECTED MICE
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(Presented By: Lucia Gutierrez)

Introduction: Malaria and schistosomiasis are two common and debilitating human parasitic diseases. Current WHO estimates of global infections range from 200 – 500 million new malaria cases each year and approximately 200 million chronic schistosomal infections. Some life cycle stages of both parasite genera circulate in the human host, catabolizing the oxygen transport protein hemoglobin to sustain their metabolism. To counteract the accumulation of free heme, both parasites have evolved a specific detoxification pathway involving conversion of highly reactive free heme to polymeric hemozoin material. The insoluble hemozoin deposits in organs of the infected host, especially the liver and spleen. The exact kinetics of hemozoin clearance from these organs is unknown but available evidence indicates that it can persist for many years post-infection and that its accumulation over a lifetime represents a potentially quantifiable integrated record of an individual's history of infection. It has also been shown that hemozoin is involved in complex immune mediatory mechanisms in both diseases, including impairment of the development and function of dendritic cells that phagocytose hemozoin, direct promotion of apoptosis of erythrocyte precursors, and activation of proinflammatory cytokines causing fever via the Toll-like receptor 9 signaling pathway. Studies of the localization of hemozoin in host immuneassociated tissues are, therefore, of potential importance. Due to the high-spin, trivalent state of the iron in hemozoin, the material exhibits distinct paramagnetic characteristics which can be detected using a variety of different techniques. In addition to hemozoin, iron-containing ferritin granules are also deposited in the liver and spleen during malaria infection. It is not known whether the same occurs in schistosomiasis. The colocalization of these two magnetic materials in host tissues may, therefore, enable the identification and differentiation of past episodes of malaria and schistosomiasis, as achieved via assessment of disease-specific magnetic signals.
Methods and Materials: In the present study we investigated livers and spleens of mice infected with *Plasmodium berghei*, *Schistosoma mansoni* and *Schistosoma japonicum* by energy filtered transmission electron microscopy (EFTEM), nuclear magnetic resonance (NMR) and superconducting interference device (SQUID) magnetometry.
Results: EFTEM revealed that all tissues contained hemozoin and ferritin. Ferritin was more abundant in the spleens of *Schistosoma* infected mice, while hemozoin was the main deposit in the malaria infected spleen tissues.
Conclusion: The different deposition patterns resulted in distinct NMR and SQUID signals for infected tissues, which were clearly different from those of uninfected control tissues.

Poster #129
THE P38 HOMOLOGUE MAP KINASE HOG1 IS INVOLVED IN THE RESPONSE OF CANDIDA ALBICANS TO HIGH IRON CONCENTRATIONS
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(Presented By: Hani Kaba)

Introduction: *Candida albicans* is an opportunistic fungal pathogen, causing most of the nosocomial fungal bloodstream infections. Iron is one of the essential elements required by pathogens for growth and spread. Binding of iron to host proteins is part of the host defense system against fungal infections. Accordingly, hosts with iron overload are highly susceptible to infections. Iron is not only an essential element, but also a potent inducer of toxicity. Thus, iron uptake is tightly regulated. During iron restricted conditions, *C. albicans* induces the reductive system for high affinity iron uptake. This system is composed of ferric reductases, multicopper ferroxidases (MCFOs) and an iron permease Ftr1. We were interested in the identification of proteins involved in the regulation of this system and studied the response of *C. albicans* towards high and low iron concentrations in the growth medium.

Methods and Materials: We used single gene deletion mutants of *C. albicans* in this study. We determined the formation of intracellular reactive oxygen species (ROS) and of iron concentrations in the culture supernatants as indicators of iron uptake. Additionally we detected MCFO levels as well as ferric reductase activity. We observed that elevated iron concentrations (≥ 5 µM Fe3+) in the growth medium induced a flocculent phenotype, which we quantified via the determination of sedimentation rates.
Results: This phenotype was dependent on the mitogen activated protein (MAP) kinase Hog1, as well as on the synthesis of new proteins. High (30 µM) but not low (1 µM) iron concentrations induced intracellular ROS accumulation in *C. albicans* wild-type (WT). We used increased ROS accumulation together with the removal of iron from culture supernatants as indicators of iron uptake. We confirmed that iron was taken up by both the Δ*hog1* mutant and the WT strain. This indicated that in the Δ*hog1* mutant, the response towards high iron concentrations, leading to induction of flocculation, was not activated. Indeed, we proved that high but not low iron led to a significant hyper-phosphorylation of Hog1 in WT cells. In line with this, viability of Δ*hog1* mutant was decreased under high iron conditions compared to the WT. The MAP kinase Hog1 is activated by various stress conditions, among which is oxidative stress. Therefore, we incubated iron treated *C. albicans* with the ROS scavenger N-acetylcysteine (NAC). NAC could reverse the iron induced ROS accumulation but not the iron induced flocculation. Thus, the Hog1-dependent iron induced flocculation was not related to intracellular ROS accumulation.
Conclusion: Deletion of *HOG1* led to increased MCFOs expression and to increased cell surface ferric reductase activity compared to WT cells, even under otherwise repressive, iron sufficient conditions. Activation of Hog1 by fludioxonil abolished induction of MCFO expression and the increase of ferric reductase activity by iron restriction. However, the effect of fludioxonil on components of the *C. albicans* reductive iron uptake system was partly independent of Hog1.
Thus, fludioxonil may be a useful tool to prevent *C. albicans* from full utilization of the reductive pathway during iron limiting conditions present in healthy hosts.

Poster #130
IRON ACQUISITION BY MYCOBACTERIUM TUBERCULOSIS RESIDING WITHIN MYELOID DENDRITIC CELLS
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(Presented By: Bradley Britigan)

Introduction: The pathophysiology of *Mycobacterium tuberculosis* (M.tb) infection is linked to the organism's ability to grow within a macrophage phagosome. Essentially all successful pathogens, including M.tb, have evolved strategies to acquire host Fe. Pathogens such as M.tb that live within cells must acquire Fe from their intracellular locale. In vivo, extracellular Fe is chelated to transferrin (TF) and lactoferrin (LF) and we previously reported that M.tb replicating in human monocyte-dervied macrophages (MDM) can acquire Fe initially bound to TF, LF, and citrate, as well as from the MDM cytoplasm. However, the magnitude of M.tb Fe uptake varied with the nature of the Fe chelate.
Methods and Results: Lung myeloid dendritic cells are a newly recognized reservoir of M.tb during infection. We hypothesized that Fe acquisition by M.tb growing within dendritic cells and macrophages may differ. Human myeloid dendritic cells were generated by incubating peripheral blood monocytes with IL-4 and GM-CSF for 6 days, which was confirmed by surface marker expression: CD80+ (5.4 +/- 1.4%); CD83+ (0.0 +/- 2.1%); CD86+ (10.8 +/-7.9%); CD11c+ (75.3 +/- 6.2%); and HLA ABC+ (82.7 +/- 6.6%). When dendritic cells or MDM were infected with M.tb (strains H37Ra and Erdman) showed similar sustained growth rates regardless of which extracellular Fe chelate was added to the culture media: Fe-citrate, Fe-LF, or Fe-TF. As measured using ^{59}Fe, Fe acquired by M.tb within dendritic cells varied with the nature of external Fe provided: citrate > LF > TF. Fe acquired/bacteria/day was similar over 7 days post-infection. The magnitude of Fe acquired by dendritic cells from the three Fe sources also varied: citrate > LF = TF. The hierarchy of Fe acquisition by dendritic cells from the different Fe chelates was similar to that of MDM, although ^{59}Fe acquired/cell by the dendritic cells was ~20-50% of that with MDM. Whereas infection of MDM with M.tb resulted in a ~50% decrease in the magnitude of Fe acquired by these cells from each chelate, infection of dendritic cells with M.tb had no effect on the magnitude of dendritic cell Fe uptake.
Conclusion: The above results indicated that M.tb within dendritic cells gain access to extracellular Fe. However, the route by which this occurs was not clear: does Fe traffic directly to the bacterium or first move to a cytosolic pool of the cell to which the organism has access? To examine these possibilities, we measured the ability of M.tb to acquire ^{59}Fe from dendritic cells whose Fe pool was labeled by incubating the cells with ^{59}Fe bound to TF, LF, or citrate for 24 hours prior to infection with M.tb. ^{59}Fe uptake by M.tb under these conditions was significantly less than when the ^{59}Fe was added extracellularly during infection, although the relative magnitude of ^{59}Fe uptake from the chelates remained citrate>LF>TF. These data suggest that the nature of Fe available within the local environment may influence M.tb biology within macrophages and myeloid dendritic cells. Future studies should focus on the role of this interaction in M.tb infection in vivo.

Poster #131

GALLIUM EXHIBITS ANTIMCROBIAL ACTIVTY IN VITRO AND IN VIVO; ROLE OF INHIBITION OF CRITICAL IRON-DEPENDENT ENZYMES

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Introduction: Acquiring iron (Fe) is critical to the metabolism and growth of most microbes. Disruption of Fe metabolism is a potential approach for novel antimicrobial therapy. Gallium (Ga) has many similarities to Fe. Biologic systems are often unable to distinguish Ga^{3+} from Fe^{3+}. Sequential reduction and oxidation is critical for Fe to function at the active site of various enzymes/cytochromes. Unlike Fe^{3+}, Ga^{3+} cannot be physiologically reduced to Ga^{2+}. Thus, substituting Ga for Fe in the active site of enzymes may render them non-functional. Ga concentrates within human macrophages.
Methods and Materials: Thus, we hypothesized that Ga could prove efficacious in treating bacterial pathogens. We previously demonstrated that systemic administration of Ga(NO3)3 is effective in murine models of pulmonary infection with *Pseudomonas aeruginosa* (P.a; *J.Clin.Invest. 117:877-888, 2007*) and *Francisella tularensis* (F.t; *Antimicrob.AgentsChemother. 54: 244-253, 2010*). We now report that Ga(NO3)3 shows efficacy in murine tuberculosis models. Three groups of Balb/C SCID (3 mice/group) were infected with 100 colony forming units (CFU) of *Mycobacterium tuberculosis* (M.tb, strain H37Rv) intratracheally, following which they received daily intraperitoneal saline, 10 mg/kg $Ga(NO_3)_3$, or $NaNO_3$ at the same molar equivalent of NO3 – as provided in Ga(NO3)3.
Results: All mice receiving saline or $NaNO_3$ died 25 – 40 days post-infection. All $Ga(NO_3)_3$-treated mice survived to 6 weeks, at which time they were sacrificed. M.tb CFU in the lungs, liver, spleen and kidney of the saline- and $NaNO_3$-treated mice were significantly higher than in Ga-treated mice. When the M.tb inoculum was increased to 10^6 CFU, all control mice died between days 20 and 24. All mice receiving $Ga(NO_3)_3$ remained alive at 28 days, exhibiting a significant decrease in tissue CFU relative to control. When Balb/C mice were substituted for Balb/C SCID mice as a chronic (non-lethal) infection model using 10^6 M.tb CFU, $Ga(NO_3)_3$ significantly decreased lung CFU.
Conclusion: The specific mechanism(s) whereby Ga inhibits bacterial growth is not clear. We previously reported that Ga interferes with Fe acquisition by P.a, F.t, and M.tb, suggesting Ga may interfere with key Fe-dependent bacterial enzymes. Therefore, we examined the effect of growth in the presence of sub-growth inhibitory $Ga(NO_3)_3$ concentrations on bacterial ribonucleotide reductase (RR, a key enzyme in DNA replication) and aconitase activities. Ga decreased P.a, F.t, and M.tb RR activity by 50-60%, but no additional inhibition was seen at Ga concentrations that completely inhibited growth. Aconitase activity decreased by 40-50% in M.tb, but was not seen with F.t. or P.a aconitase. Consistent with prior effects in F.t, Ga decreased P.a catalase activity 50%. However, no effect on SOD was seen. Thus, Ga inhibits RR, aconitase, and catalase activity of some bacterial species, but the magnitude of inhibition and dose response curves do not match the growth inhibition. $Ga(NO_3)_3$ shows efficacy in murine models of P.a, F.t, and M.tb infection, inhibits bacterial Fe acquisition and leads to a decrease in activity of Fedependent enzymes. Additional work is warranted to further define Ga's mechanism of action and to optimize delivery forms for possible therapeutic uses in humans.

Poster #132

RHODAMINE LABELING OF 3-HYDROXY-4-PYRIDINONE IRON CHELATORS IS AN IMPORTANT CONTRIBUTION TO TARGET MYCOBACTERIUM AVIUM INFECTION

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(Presented By: Tania Moniz)

Introduction: Infectious diseases still remain a serious problem and the increasing development of bacterial resistance to traditional antibiotics has reached alarming levels. Consequently, the development of new antimicrobial agents is imperative and novel modes of action and/or different cellular targets should be considered. Iron is an essential element for most living organisms, namely pathogenic bacteria. To obtain this crucial micronutrient from the environment these microbes synthesize high affinity hexadentate iron(III) chelators known as siderophores. Considering this need of iron it seems possible to fight infectious diseases by means of iron deprivation induced by synthetic iron(III) chelators able to compete with the natural siderophores. Iron acquisition is thus one of the possible pathways that can be successfully targeted to design new antibacterial drugs by pharmacological intervention via administration of iron chelators. In this field, hydroxypyridinones were recently selected as "privileged" chelating structures for the design of antibacterial agents.
Methods and Results: Our group is particularly interested in mycobacterial infections. We have recently demonstrated that tripodal hexadentate chelators, based on 3-hydroxy-4-pyridinone units, can limit the access of iron to these bacteria and have a significant inhibitory effect in the intramacrophagic growth of Mycobacterium avium [1, 2]. These results showed that the chelation of iron is a determinant although not sufficient property of these compounds for antimicrobial activity. A rhodamine B isothiocyanate labelled chelator (MRH7) exhibited the strongest inhibitory activity and was identified as a lead compound since a dose response effect was observed. Significant inhibition of M. avium growth was achieved at a concentration as low as 1 μM. To identify key molecular features essential for the biological activity we designed parent hexadentate and bidentate chelators, in which different structural groups are introduced in the molecular framework. Herein, we report the work concerning three novel fluorescent chelators: a hexadentate ligand labelled with 5(6)-carboxytetramethylrhodamine (MRH8)

and two 3-hydroxy-4-pyridinone fluorescent bidentate ligands labelled with rhodamine B isothiocyanate (MRB7) and 5(6)-carboxytetramethylrhodamine (MRB8).

Conclusion: The results show that all fluorescent chelators are capable of restricting the intramacrophagic growth of M. avium and that the inhibitory effect is dependent on the fluorophore. In fact, for compounds bearing the same fluorophore the results obtained with the hexadentate or bidentate chelator (MRH7/MRB7 or MRH8/MRB8) are identical as long as the appropriate stoichiometric amount of chelator is used. The inhibitory effect of the rhodamine B isothiocyanate labelled compounds (MRH7 and MRB7) is significantly greater than that observed for the other two chelators, thus pointing out the significance of the rhodamine B isothiocyanate molecular fragment.

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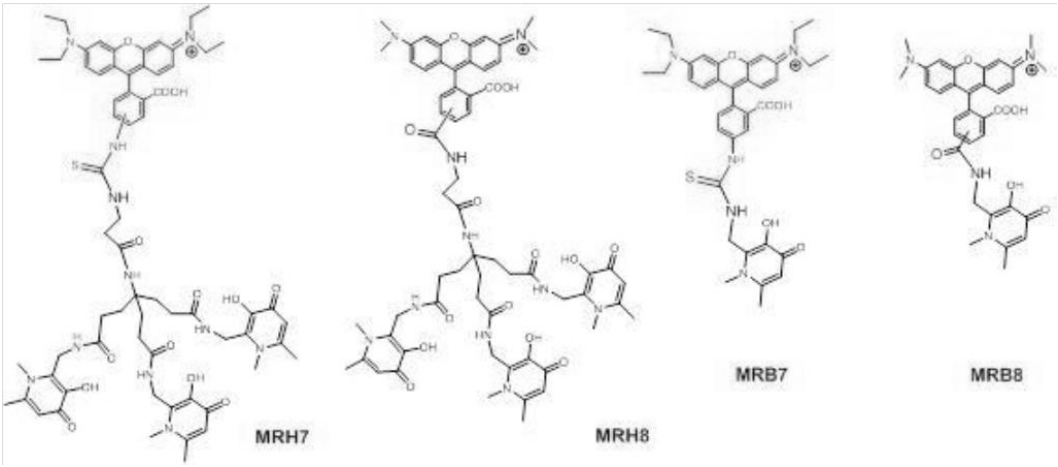

Poster #133

BIOAVAILABLE IRON CONTENT OF PLASMODIUM FALCIPARUM PARASITIZED ERYTHROCYTES INCREASES WITH PARASITE MATURATION AND IN RESPONSE TO EXTRACELLULAR IRON

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(Presented By: Martha Clark)

Introduction: Malaria is a significant public health concern; causing 250 million infections a year and 1 million deaths. Iron deficiency is co-endemic with malaria, and clinical and epidemiological studies have revealed an unequivocal association of host iron status with susceptibility to malaria infection. During its erythrocyte life cycle, *Plasmodium falciparum* requires iron for DNA synthesis, glycolysis, pyrimidine synthesis and electron transport. It is currently unknown what host iron sources erythrocyte stage *P. falciparum* is able to access and utilize. The host red blood cell (RBC) contains 100fg (20mM) of iron; which is predominantly incorporated into heme within hemoglobin. The malaria parasite lacks the heme oxygenase activity required to release host iron from heme. In addition to RBC iron sources, the malaria parasite may additionally be able to access serum iron.

Methods and Materials: We developed a flow cytometry based method for determining the bioavailable iron content of parasitized erythrocytes using the nucleic acid dye SYTO 61 and the iron sensitive dye Calcein AM. This approach has allowed us to systematically study changes in bioavailable iron in parasitized cells through the course of the erythrocyte life cycle and in response to the addition of extracellular iron sources.

Results: We observed that the bioavailable iron content increases with the development of the malaria parasite from early ring to the schizont stage, and that the addition of either transferrin or ferric citrate to culture media increases the bioavailable iron found in late stage trophozoites.

Conclusion: Based upon these results, we hypothesize that bioavailable iron content of late stage parasitized erythrocytes increases to meet the iron demands of the parasite as it increases metabolic activity and commences DNA replication. Furthermore, our results suggest that erythrocyte stage *P. falciparum* is able to incorporate both transferrin bound and nontransferrin bound iron (NTBI) into its bioavailable iron pool. Our method for detecting bioavailable iron within malaria parasitized RBCs provides an important tool for elucidating the mechanisms by which the malaria parasite senses, acquires, stores, and regulates iron during the erythrocyte stage of its life cycle.

Poster #134

IRON STATUS AND IRON SUPPLEMENTATION MODULATE LOAD OF INFECTION AND SURVIVAL IN A MALARIA MURINE MODEL

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(Presented By: Daniel Carapau)

Introduction: Iron availability and distribution in the mammalian host is known to regulate infections by many different pathogens. In the case of *Plasmodium*, the causative agent of malaria, our laboratory recently showed that hepcidin up-regulation and iron re-distribution during blood-stage infection regulate the liver load of infection upon a secondary challenge (Portugal et al. 2011). However, the role of iron metabolism during blood-stage infection, the phase when disease arises, has not been properly characterized.
Methods and Materials: In order to investigate the impact of the iron status of the host on *Plasmodium* infection and disease, we investigated how differences in dietary iron influence the course of malaria in a self-resolving, non-lethal murine model of infection (*P. yoelii* 17XNL).
Results: Our data clearly shows that parasite load correlates positively with the amount of iron in the diet. Surprisingly, survival curves correlate negatively with both the amount of iron in the diet and the parasite load. Most importantly, oral iron supplementation during infection could significantly rescue the lower survival rates of animals under iron-deficient diet, in spite of the fact that supplementation led to a significant increase in parasitemia (% of infected red blood cells).
Conclusion: Taken together, our results imply that iron content in the diet, as well as iron supplementation, have dramatic effects on the course and outcome of malaria infections. This is of special relevance in the context of the current debate about the effects of iron supplementation in malaria endemic areas, which arose from conflicting results in clinical trials. Our data suggest that, at least in the context of a self-resolving murine model of infection, iron supplementation might be beneficial to avert pathology, even if it might increase the load of infection.
We will further study the relations between iron metabolism and murine malaria models of infection and disease, in order to shed some light on the discrepancies between epidemiological studies of iron supplementation in different areas of malaria prevalence.

Poster #135

MALARIA-INDUCED ALTERATIONS IN MACROPHAGE IRON HANDLING: AN EXPLORATIVE IN VITRO STUDY

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(Presented By: Susanne van Santen)

Objectives: Iron supplementation remains controversial in malaria endemic areas since the balance between beneficial versus possible harmful effects has not yet been set. To increase our insights in the potential mechanisms by which iron status could affect parasite replication and hence the clinical sequelae of malaria, we aimed to explore the effect of *P. falciparum* infected erythrocytes (pRBCs) on macrophage- derived hepcidin and other components of cellular iron homeostasis including the gene expression of iron handling genes ferroportin, heme-oxygenase 1 (HO-1), H-ferritin, divalent metal transporter 1 (DMT1), natural resistance-associated macrophage protein 1 (Nramp1) and intracellular iron assessment.
Methods: The gene expression of the iron regulatory genes hepcidin (Hamp), ferroportin, HO-1, H-ferritin, DMT1 as well as intracellular iron concentrations were assessed in human M1 differentiated macrophages and in mouse RAW macrophages after challenge with either pRBCs or uninfected red blood cells (uRBCs). In addition we primed and co-stimulated with either IFNγ or hepcidin and primed with an iron chelator. Intracellular iron concentrations were measured with an endosomal iron probe, the calcein AM method and by Energy-dispersive X-ray microanalysis (EDX). Since erythrophagocytosis is an important feature in malaria with significant consequences for the macrophage iron status, erythrophagocytosis was induced by adding immuunglobulins to the pRBCs and uRBCs.
Results: 1. Erythrophagocytosis of uRBCs and/ or pRBCs had significant influences on macrophage iron homeostasis, involving heme- mediated upregulation of ferroportin and HO-1 transcripts, increased the labile iron pool and cellular H-ferritin expression. 2. Malaria- mediated inflammation does not induce significant effects on important iron handling genes of macrophages, neither by direct interaction of infected RBCs with macrophages, nor by priming and co-stimulation with hepcidin or IFNγ. In addition, whereas systemic hepcidin is of importance in malaria infections, we observed no increase in macrophage hepcidin gene expression after a challenge with pRBCs. Next, we showed a (non-significant) relative suppression of the increase in the gene expression of ferroportin by pRBCs in comparison to uRBCs, which agreed with more intracellular iron and ferritin protein induced by parasitized RBCs. 3. Iron deprivation through the use of an iron chelator did not change the Hamp expression in macrophages upon challenge with pRBCs.
Conclusion: Our in vitro data support the hypothesis that malaria affects iron homeostasis of macrophages, however, erythrophagocytosis of infected as well as uninfected red blood cells seem much more important compared to *P falciparum* induced inflammatory alterations. Erythrophagocytosis was associated with an increase of intracellular iron, ferroportin and HO-1 gene expression.

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Poster #136

HIV-1 INFECTION AND IRON: ROLE OF HEME-OXYGENASE-1 AND FERROPORTIN

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(Presented By: Sergei Nekhai)

Introduction: HIV-1 transcription is activated by HIV-1 Tat protein, which recruits CDK9/cyclin T1 and other host transcriptional co-activators to the HIV-1 promoter. Tat itself is phosphorylated by cell cycle kinase 2 (CDK2) and inhibition of CDK2 by small interfering RNA or iron chelators inhibits HIV-1 transcription. HIV-1 transcription is also activated by NF-kB that binds to HIV-1 LTR independent to Tat but can also be recruited Tat-dependently by CDK9/cyclin T1. Previously induction of heme oxygenase-1 (HO-1) by hemin was shown to inhibit HIV-1 in vitro and in vivo. We also showed previously that expression of ferroportin inhibits HIV-1 transcription.

Methods and Materials: We show here that treatment with hemin induces both HO-1 and ferroportin expression and potently inhibits HIV-1. We also show that hemin induces expression of IkBα, an inhibitor of NF-kB, and p21, a CDK2 inhibitor.

These effects were replication with iron chelators, PpY-eT and PpY-aT, which both chelators efficiently inhibited HIV-1 and induced the expression of IkBα and p21. Ferroportin Q248H mutation has an allele frequency of 2.2-13.4% in African populations and is associated with a mild tendency to increased serum ferritin in the general population. Ferroportin Q248H mutation associates with lower hepcidin levels in HIV-1 infected Rwandese.

Results: We also recently showed that ferroportin Q248H mutant has reduced sensitivity to physiologic hepcidin concentrations. In the absence of hepcidin, both WT ferroportin and Q248H ferroportin efficiently inhibited HIV-1 transcription and replication. Hepcidin treatment induced HIV-1 transcription and replication inhibited by WT ferroportin but not Q248H mutant ferroportin. Also, HIV-1 replication was reduced in primary monocytes from ferroportin Q248H subjects as compared to controls.

Conclusion: In conclusion, HIV-1 can be inhibited through the induction and HO-1 and ferroportin likely through the upregulation of IkBα and 21. An expression of ferroportin Q248H that has a decreased sensitivity to hepcidin may offer an additional protection from HIV-1.

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Poster #137

β-CAROTENE AND VITAMIN A REVERSE ABNORMALITIES IN IRON METABOLISM IN INFLAMED CACO-2 CELL-LINE

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(Presented By: Oksana Katz)

Objectives: The inflammatory process can enhance the development of anemia of inflammation as a result of iron sequestration inside intestinal epithelial cells resulting in a reduced bioavailability of iron. Administration of iron supplements can aggravate the inflammation, resulting in increased sequestration of iron, which further increases the anemic condition. Anti-inflammatory agents like β-carotene or vitamin A may reduce the anemia associated with iron release from storage proteins and decrease the inflammation improving the effects of the iron supplements. The current study presents the fate of iron following induction of inflammation in vitro, and the role of iron-related proteins (transferrin receptor (TfR), L-and H-ferritin, ferroportin (FPN)) in releasing the sequestrated iron after administration of vitamin A or β-carotene in order to reduce the inflammatory state.

Methods: An in vitro model of IL1 β stimulation of Caco-2 cells was used to induce inflammation the inflammatory state was validated by measuring IL8 release. The impact of iron supplementation on the cells was investigated in a time and dose dependent manner while β-carotene and vitamin A were used as anti-inflammatory compounds, verified by the suppression of IL8 release. The effects on iron related proteins (H-, L-ferritin, ferroportin, transferrin receptor) were compared in inflamed Caco-2 cells with or without β-carotene and vitamin A treatment.

Results: Increasing concentration of iron (0, 10, 100, 200, 250, 350 µmol/L) and incubation period (4h, 12h, and 24h) lead to a significant increase in IL8 release. Applying β-carotene resulted in reduction of IL8 (from 1306.2pg/ml to 253.75pg/ml) while L- and H-ferritin levels were increased to 45.7%, 215.7%, 118.2%, and 70% respectively, and ferroportin were decreased by20%, 44.4%, 47.7%, 40.8% respectively ($P<0.05$). Vitamin A alone had no effect on the stabilization of iron metabolism. A strong correlation between pro-inflammatory cytokine IL8 and L-, H-ferritin was found.

Discussion: Iron supplementation induces oxidative stress, elevates IL8 secretion, and induces L- and H-ferritin production. The iron binding to ferritin ensures its safe storage and prevents production of oxidative specimens, while it may interfere with

the exit of iron outside the cells. Another factor that may contribute to the epithelial iron sequestration may be the decreased expression of the iron exporter ferroportin (FPN). β-carotene may decrease free-radicals production and possesses a more potent anti-inflammatory capacity then vitamin A. In fact, β-carotene, fully annulled IL8 secretion and its influence on L-,H-ferritin, FPN, TfR following iron application, while vitamin A only partially diminished IL8 release and the accompanied intracellular protein consequences.
Conclusion: β-carotene and to a lesser extent vitamin A stabilized the main iron related proteins (ferroportin, L-, H-ferritin and transferrin receptor) and diminished pro-inflammatory cytokine production (IL8). These results suggest that by applying anti-inflammatory compounds, in chronic inflammatory conditions, less iron is locked in intestinal epithelial cells, leading to increased iron bioavailability. This may be a possible approach to combat iron deficiency anemia associated with inflammation. Further studies are needed to see whether these findings can be applied in vivo.

Poster #138
INVESTIGATING THE ROLE OF CYTOKINES AND HEPCIDIN IN ANEMIA OF INFLAMMATION
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(Presented By: Sara Gardenghi)

Introduction: Anemia of inflammation (AI) is commonly observed in patients with chronic illnesses and is associated with poor health outcomes. It is characterized by hepcidin-induced iron restricted erythropoiesis, blunted erythropoietin production and efficacy, and shortened red cell lifespan. Together with the well-known interleukin-6 (IL-6)/hepcidin (Hamp) pathway, a complex pattern of pro-inflammatory cytokines contributes to AI, directly affecting erythropoiesis and iron metabolism.
Methods and Materials: To elucidate the role of Hamp and iron as well as IL-6 and other inflammatory cytokines in AI, we generated models of acute and chronic inflammation. Acute inflammation was induced with one intraperitoneal injection of heat-killed *Brucella abortus* (HKBA, 5x108 particles/mouse) in wild-type (WT), IL-6 knockout (*IL-6*-KO) and *Hamp*-KO mice (n≥15/group), followed by weekly CBCs to observe the development of anemia and the subsequent recovery. We collected serum samples from the same mice (untreated and injected with HKBA) after 6 hours, and analyzed serum iron concentration, transferrin saturation, and the levels of 58 cytokines, as well as erythropoiesis, (the last parameter described in the companion abstract by Gardenghi et al).
Results: The model of chronic AI was generated in WT mice with a loading dose of HKBA ($3x10^8$ particles/mouse), followed by $2x10^8$ particles/mouse doses, began at day 14 and performed weekly. *IL-6*-KO and *Hamp*-KO mice responded less severely to HKBA compared to WT mice in the acute model. In fact, while WT mice hemoglobin (Hb) values were lowest 2 weeks after injection (8.3±1.4 g/dl) and recovered over 7 weeks, *IL-6*-KO mice Hb was similarly affected after 2 weeks (7.5±1.8 g/dl) but recovered in only 3 weeks. *Hamp*-KO mice were the least affected after 2 weeks (10.9±0.8 g/dl) and recovered by week 4. Iron-depleted *Hamp*-KO mice were still less sensitive to HKBA administration, suggesting that protection against anemia was independent of iron overload and dependent on the intrinsic lack of *Hamp* expression. Hamp, IL-6, and additional cytokines were elevated 6 hours after HKBA administration, whereas serum iron and transferrin saturation were reduced. However, all these parameters quickly normalized afterward. Cytokines analysis showed upregulation of interleukins, growth factors, and molecules involved with vascularization 6 hours after HKBA injection in serum of WT, *IL-6*-KO and *Hamp*-KO mice. Studies are undergoing to discriminate specific differences in the expression of inflammatory molecules in these mice. Preliminary experiments on the chronic model featuring repeated HKBA administration showed that Hb values were reduced (10±1.2 g/dl) for up to 35 days in WT mice. All HKBA models were characterized by resistance to treatment with either erythropoietin or erythropoietin stimulating agents.
Conclusion: In conclusion, our data demonstrates that IL-6 and Hamp contribute separately to the pathophysiology of AI, since lack of *IL-6* or *Hamp* is protective but does not completely prevent anemia. The model of chronic AI is under investigation to determine whether anemia is either due to iron-restricted erythropoiesis produced by high and long-lasting levels of Hamp, or a direct effect of the inflammatory cytokines on erythroid cell cycle and survival, or both. Nevertheless, this modified approach will be utilized to further explore AI.

Poster #139
THE HEREDITARY HEMOCHROMATOSIS PROTEIN HFE INHIBITS MHC I ANTIGEN PRESENTATION
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CRCHUM
(Presented By: Alexandre Reuben)

Introduction: HFE is a β2-microglobulin (β2-m)-dependent protein involved in the regulation of iron absorption from the intestine. Mutations in this gene are highly prevalent in the population of European descent and may lead to the development of hereditary hemochromatosis (HH), an iron overload disease. HFE is a structural homolog of major histocompatibility complex class I (MHC I) molecules that are involved in presentation of cellular antigens to the immune system. Unlike classical antigen presenting MHC Ia molecules, HFE interacts with transferrin receptors (TfR) 1 and 2, senses iron body status and activates downstream signaling pathways that regulate iron homeostasis. In addition, several lines of evidence suggest that the cellular immune system may contribute to HH. For example, immunological defects have been observed in patients with

HH while Hfe-deficient mice lacking lymphocytes develop a more severe form of iron overloading. Furthermore, it has been shown that HFE can be recognized by T lymphocytes and that this interaction influences the development of the T cell repertoire. We therefore hypothesized that HFE may have a distinctive function in the process of MHC I antigen presentation.
Methods and Materials: In order to assess the effects of HFE expression on T cell recognition, we used an *in vitro* model of antigen presentation in which we expressed wildtype HFE (HFEWT) and the most common HH-related HFE variants (HFEH63D and HFEC282Y) in HEK293 cells over-expressing HLA-A*0201 (HLAA2) as antigen presenting cells (293-A2/APC), and tested their ability to present various epitopes to antigen-specific CD8+ T lymphocytes by measuring the ensuing production of soluble factors and expression of T cell activation markers.
Results: We found that HFEWT expression in 293-A2/APC and, to a lesser extent, pathology-related HFE mutants, substantially affects the ability of HLA-A2 molecules to present the endogenously-processed gp100 melanoma epitope (gp100209-217). Similar results were obtained when testing melanoma MART-1 and influenza M1 antigens, as well as epitopes with low- and high-affinity for MHC I molecules, indicating that HFE-mediated inhibition of T lymphocyte recognition is independent of antigen origin or affinity. In addition, MHC I presentation was suppressed to similar levels in the presence of HFEWT and the HFEV100A mutant which prevents TfR binding, indicating that the effect is not mediated through HFE interaction with TfR. We further show that β2-m co-transfection in the presence of HFEWT, HFEH63D or HFEC282Y successfully restored HLA-A2 surface expression, yet β2-m complementation was manifestly unable to revert the inhibition of MHC I antigen presentation by HFEWT, demonstrating that competition for β2-m binding is not responsible for decreasing T lymphocyte antigen recognition. Finally, external peptide-pulsing of 293-A2/APC demonstrated that HFEWT specifically inhibited recognition of antigens requiring intracellular processing to be presented on MHC I molecules, indicating that HFEWT inhibits CD8+ T lymphocyte activation through interference with the classical MHC I antigen presentation pathway.
Conclusion: Taken together, our results suggest that HFEWT may interfere with antigen processing and presentation. A better understanding of the molecular mechanisms of action of HFE on MHC I presentation may lead to a better control of the cellular immune response in autoimmunity and against pathogens.

Poster #140
CHARACTERIZATION OF ERYTHROPOIESIS IN A MURINE MODEL OF ANEMIA OF INFLAMMATION DEVELOPED WITH HEAT-KILLED BRUCELLA ABORTUS TREATMENT
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(Presented By: Sara Gardenghi)

Introduction: Anemia of inflammation (AI) is an immune-driven condition associated with chronic diseases. Hallmarks of AI are the limited availability of iron for erythroid progenitor cells (iron-restricted erythropoiesis) and reduced red cell survival, resulting from disturbances of iron homeostasis and decreased synthesis and effectiveness of erythropoietin.
Methods and Materials: Wild-type (WT), interleukin-6 knockout (*IL-6*-KO) and hepcidin knockout (*Hamp*-KO) (n≥15/group) murine models of acute inflammation, generated with one intraperitoneal injection of heat-killed *Brucella abortus* (HKBA), were analyzed weekly for hemoglobin (Hb), RBC and reticulocytes number, as well as mean corpuscular hemoglobin (MCH) and hemoglobin reticulocyte content (CHr) with CBCs. We discriminated the different stages of erythroid differentiation in the bone marrow (BM) and spleen of the same mice, using CD44 and Ter119 antibodies, and determined the lifespan of NHS-biotin/streptavidin-labeled RBCs.
Results: As shown in detail in the companion abstract by Gardenghi et al., all HKBA-injected mice developed acute anemia in 1-2 weeks. *Hamp*-KO mice were the most protected since their Hb dropped significantly less compared to WT and *IL-6*-KO mice. The RBC number was decreased in all the murine genotypes. However, while MCH and CHr were reduced in WT and *IL-6*-KO mice, they increased in *Hamp*-KO. This latest factor could be significant in explaining *Hamp*-KO highest Hb values of all the HKBA-treated mice. Reticulocytosis characterized the recovery phase of all the mice. FACS analysis one week after HKBA administration showed that erythroid progenitors were reduced in the BM of *IL-6*-KO mice, but absent in WT and *Hamp*-KO mice, compared to untreated animals. Interestingly, even if HKBA-treated *Hamp*-KO mice had higher Hb compared to WT mice (see previous CBC data), both genotypes presented disruption of erythropoiesis in their BM. Concurrently, we observed increased production of erythroid precursors in the spleen of all mice (stress erythropoiesis), which was more apparent in *Hamp*-KO mice although not sufficient to completely justify their mild anemia. To investigate additional causes responsible for the protection of *Hamp*-KO and *IL-6*-KO mice against HKBA, we analyzed the RBC lifespan of these mice and that of WT mice following administration of HKBA. We showed that erythropoiesis is disrupted for 4 – 7 days, after that *Hamp*-KO and *IL-6*-KO mice recover earlier than WT mice, due to an increased and faster production of new RBCs. However, the lack of RBC production is not the only mechanism responsible for anemia in our HKBA mouse model. We are also investigating the destruction of RBC, which may occur by erythrophagocytosis. Finally, we are examining the macrophage populations profile in all murine genotypes, before and after HKBA injection. Macrophages, in fact, not only support inflammation, but also are essential in producing anti-inflammatory cytokines, as well as in sustaining stress erythropoiesis and recovery from anemia.
Conclusion: In conclusion, we showed that erythropoiesis is severely affected in our mouse model of AI. Our data suggest that, in addition to iron restrictederythropoiesis, an acute inflammatory effect on erythropoiesis is occurring in the HKBA model of AI, affecting erythroid cell survival and/or proliferation.

Poster #141

SERUM HEPCIDIN IN INFLAMMATORY BOWEL DISEASES: BIOLOGICAL AND CLINICAL SIGNIFICANCE

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(Presented By: Natascia Campostrini)

Introduction: Crohn's disease (CD) and ulcerative colitis (UC) are inflammatory bowel diseases (IBD) due to the interplay between genetic predisposition, dysregulated immune response, and environmental factors. Anemia is a common feature of IBD, caused by either inflammation or iron deficiency. Hepcidin, a peptide hormone mainly produced by hepatocytes, regulates body iron homeostasis. Increased hepcidin production during inflammation plays a role in the pathogenesis of anemia of inflammation, and hepcidin determination may prove useful in the differential diagnosis of anemias during inflammatory diseases.
Material and Methods: We used surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) to measure serum hepcidin-25 and hepcidin-20 isoforms in 54 patients with IBD (22 with CD, 32 with UC) and 54 sex- and age-matched reference subjects (36 healthy controls and 18 patients with anemia not associated with inflammation or renal failure). Disease activity, complete blood counts, iron status and erythropoiesis-related parameters were obtained for all study subjects.
Results: In IBD hepcidin-25, the iron bioactive isoform, correlated positively with C-reactive protein (CRP) and serum ferritin; an inverse correlation was observed with transferrin, soluble transferrin receptor (sTfR) and the sTfR/Log(ferritin) ratio. Similar correlations with indices of iron status and erythropoiesis were found in reference subjects. IBD patients with anemia of inflammation had higher hepcidin-25 levels than patients with iron deficiency anemia (IDA) or a combination of IDA and inflammation (P=0.0061). In patients with inflammation and serum ferritin concentration from 100 to 200 ng/mL, hepcidin-25 was low, suggesting that during inflammation iron deficiency can occur with ferritin values up to 200 ng/mL. A serum hepcidin-25 concentration below 2.0 nM correctly differentiated 85% of patients with IDA (with or without inflammation) from patients with anemia of inflammation. Hepcidin-25 was the main correlate of hepcidin-20 in reference subjects, whereas in IBD both hepcidin-25 and CRP correlated with hepcidin-20, suggesting the presence of an inflammation dependent regulation of hepcidin-20, which is not linked to hepcidin-25 regulation.
Conclusions: In IBD serum hepcidin-25 is influenced by iron stores, inflammation and iron requirement for erythropoiesis. Hepcidin-25 determination can be useful in the differential diagnosis of IBD-associated anemias. Hepcidin-20 is linked to hepcidin-25, but inflammation appears to have an independent regulatory role on its concentration; thus, hepcidin-20 may have a biological function during inflammation.

Poster #142

NITROGEN MONOXIDE INHIBITS HEME SYNTHESIS IN MOUSE ERYTHROID CELLS

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(Presented By: Marc Mikhael)

Introduction: Anemia of infection (AI) often manifests in patients with chronic immune activation due to cancer, chronic infections, autoimmune disorders, rheumatoid arthritis and other diseases. The pathogenesis of AI is complex and involves cytokine-mediated inhibition of erythropoeisis, insufficient erythropoietin production and diminished sensitivity of erythroid progenitors to this hormone, and retention of iron in hemoglobin-processing macrophages. Nitric oxide (NO) is a gaseous molecule produced by activated macrophages that has been identified as having numerous effects on iron metabolism.
Methods and Materials: Here we explore the possibility that NO affects iron metabolism in reticulocytes and our results suggest that NO may also contribute to AI. We treated reticulocytes with the NO donor, sodium nitroprusside (SNP).
Results: Our results indicate that NO inhibits heme synthesis dramatically and rapidly at the level of erythroid specific 5-aminolevulinic acid synthase 2, which catalyzes the first step of heme synthesis in erythroid cells. We also show that NO leads to the inhibition of iron uptake via the transferrin (Tf)-transferrin receptor pathway.
Conclusion: In addition, NO also causes an increase in eIF2α phosphorylation levels and decreases globin translation. The profound impairment of heme synthesis, iron uptake and globin translation in reticulocytes by NO raises the possibility that this gas may also contribute to AI.

Poster #143

ACTIVATION OF NATURAL KILLER T CELLS BY ALPHA-GALACTOSYLCERAMIDE AFFECTS IRON METABOLISM

Hua Huang, Gabriela Fragoso, PhD and Manuela Santos, PhD
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(Presented By: Hua Huang)

Introduction: Natural killer T cells (NKTs) are a subset of T lymphocytes that express surface receptors characteristic of the T and NK cell lineages. Like conventional T lymphocytes, NKTs express T cell receptors (TCR), but unlike conventional T cells, which react with peptide antigens in the context of MHC-I or MHC-II molecules, NKTs react with lipid or glycolipid antigens presented by the MHC class I-related glycoprotein CD1d. NKTs recognize the CD1d-restricted glycosphingolipid antigen alpha-galactosylceramide (alpha-GalCer), a marine sponge-derived compound that has potent immunoregulatory potential.
Methods and Materials: Our interest in studying the impact of NKT cell activation in iron homeostasis was prompted by the following observations: 1) Most NKTs are found in the liver, which is the major organ producing hepcidin, the major regulator of systemic iron homeostasis; and 2) Activated NKTs rapidly release large amounts of cytokines, which are known to affect many genes involved in iron metabolism, including hepcidin and the cellular iron exporter ferroportin. To evaluate the impact of NKTs on iron metabolism, we activated NKTs in vivo using alpha-GalCer.
Results: We found that NKT activation resulted in major changes in iron homeostasis, summarized as follows: 1) an early phase, up to ~24h, which resembles the reported effects of toll-like receptor (TLR)-mediated signaling with LPS and is characterized by slight hypoferremia, hepcidin induction and ferroportin suppression; 2) a second phase, coinciding with the peak of splenic cell proliferation, during which hepcidin levels are suppressed while ferroportin levels normalize despite the raised levels of iron in the serum and increased total spleen iron. These findings contrast with the well-characterized effects of iron-loading, known to induce the expression of hepcidin and ferroportin in the liver, and indicate that NKT activation may have very specific effects in iron homeostasis that are very distinct from activation of immune responses through TLRs. Although alpha-GalCer is a highly specific ligand for CD1d, we wished to confirm that the effects observed were dependent on CD1d. In fact, CD1d-/ - mice had no variations in serum iron or hepcidin levels in the liver after alpha-GalCer administration. Because these results could be interpreted as either caused by the absence of CD1d on an antigen-presenting cell and/or caused by the lack of a T-cell population such as NKT cells, which do not develop in CD1d-/- mice, the effect on iron homeostasis of alpha-GalCer was also assessed in Rag1-/- mice, which lack both T and B lymphocytes. Despite the presence of CD1d in Rag1-/- mice, alpha-GalCer did not evoke any changes in serum iron or hepcidin expression. In vivo engagement of NKT cells rapidly induces a cascade of cellular activation that also involves elements of innate immunity, including NK cells. Therefore, to examine the role of NK cells we used antibody-mediated depletion of NK cells with anti-asialoGM1 antibody. Deletion of NK cells however, did not affect the effects of alpha-GalCer in iron homeostasis, indicating the NK cells are not involved.
Conclusion: Taken together, these results imply that the effect of alpha-GalCer on iron homeostasis requires CD1d and NKT cells, and does not occur through a direct effect on the hepatocytes or some other CD1d-bearing cell type alone, suggesting that the NKT/CD1d system affects iron homeostasis.

Poster #144

FRIEND OF GATA AND GATA-6 MODULATE THE TRANSCRIPTIONAL UP-REGULATION OF HEPCIDIN IN HEPATOCYTES DURING INFLAMMATION

Edward Bagu, PhD, Antonio Layoun, PhD and Manuela Santos, PhD
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(Presented By: Edward Bagu)

Introduction: Hepcidin is an antimicrobial peptide hormone that plays a central role in the metabolism of iron. It is encoded for by the HAMP gene and is expressed mainly in hepatocytes and at lower levels in other cells, such as macrophages. Hepcidin expression in the liver can be induced through at least two major pathways: the inflammatory pathway, mainly via IL-6; and the iron-sensing pathway, mediated by BMP6. GATA-proteins are group of evolutionary conserved transcriptional regulators that bind to the consensus motif -WGATAR- in the promoter region. In hepatoma cells, GATA- proteins 4 and 6 in conjunction with the co-factor Friend of GATA (FOG) were shown to modulate the transcription of *HAMP*. However, it is unclear as to which of the GATA- proteins drive the expression of *HAMP in-vivo*.
Methods and Materials: In this study, using *in vitro* and *in vivo* approaches, we investigated the relevance of GATA and FOG proteins for hepcidin expression in response to IL-6 and BMP6. We found that, as expected, treatment of hepatoma Huh7 cells with either IL6 or BMP6 increased the *HAMP* promoter activity.
Results: The *HAMP* promoter activity following treatment with IL6 or BMP6 was further increased by co-transfection of the promoter with GATA proteins 4 and 6. However, co-transfection of the *HAMP* promoter with FOG proteins 1 or 2 repressed the promoter response to treatments with either IL-6 or BMP6. The effects of both GATA and FOG proteins on the promoter activity in response to IL6 or BMP6 treatment were ablated by mutation of the GATA response element -TTATCT- in the *HAMP* promoter region -103/-98. *In vivo*, treatment of mice with *lipopolysaccharide* (LPS) led to a transient increase of *Gata-6* expression in the liver that was positively correlated with the expression of hepcidin. The peak expression of *Gata-6* was observed 3 hours post treatment with LPS, while *Gata-4* and *Fog 1* and *2* were transiently repressed for 12 hours (*Fog-1*) and 24 hours (*Gata-4* and *Fog-2*) post-treatment with LPS.
Conclusion: Taken together, our results indicate that the concordant up-regulation of GATA-6 along with the repression of FOG 1 and 2 may be crucial for the endogenous transcriptional up-regulation of hepcidin during inflammation.

Poster #145

ROLE OF TOLL-LIKE RECEPTORS AND MYD88 ADAPTOR PROTEIN IN THE DEVELOPMENT OF HYPOFERREMIA AND THE REGULATION OF IRON HOMEOSTASIS

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(Presented By: Antonio Layoun)

Introduction: Hepcidin plays the role of a negative regulator of iron absorption, resulting in the inhibition of iron export from the intestine and macrophages. During inflammation, the level of hepcidin is greatly increased. Thus, when the inflammation persists, hepcidin expression continues to be activated leading to hypoferremia. In infectious and inflammatory diseases, hypoferremia is considered as host defense to deprive microorganisms of iron. Moreover, using the Toll-like receptors (TLRs), cells such as macrophages play a crucial role in host defense by recognizing molecular patterns associated with pathogens, named (PAMPs). In previous studies we found that hepcidin induction in the liver by lipopolysaccharide (LPS) is dependent on the signaling pathway mediated by toll-like receptor 4 (TLR4). Also, we recently found that hepcidin expression in macrophages can be regulated through multiple TLRs. In the present study, we investigated the role of TLRs and their adaptor proteins MyD88 and Trif in the development of hypoferremia and the regulation of iron metabolism in mice.
Methods and Results: To assess whether the TLRs are able to induce hypoferremia, C57BL/6 mice were treated by a single injection of Zymosan (TLR2 ligand), Poly I:C (TLR3 ligand) or LPS (TLR4 ligand). We found that treated mice developed acute hypoferremia compared to saline-treated control group. To determine which TLR signaling pathway is involved in inducing hypoferremia, MyD88-/- and Trif-/- mice were treated with LPS. We found that MyD88-/- mice failed to develop full hypoferremia unlike Wt and Trif-/- mice. Using real-time RT-PCR, we found that unlike Wt mice, MyD88-/- and Trif-/- mice failed to induce hepcidin expression. Furthermore, we also found that MyD88-/- mice were unable to induce ferritin after LPS stimulation. Using atomic absorption spectroscopy, we confirmed that MyD88-/- mice were unable to store iron in the spleen after LPS stimulation. Given the crucial role of MyD88 in the development of hypoferremia, we also investigated its role in regulating iron homeostasis. Using atomic absorption and Perl's staining we show that, when placed for 2 weeks on normal and carbonyl iron diet, MyD88-/- mice stored more iron in both liver and spleen when compared to Wt mice. Moreover, we confirmed by ELISA that basal ferritin levels in MyD88-/- mice are significantly higher than Wt mice in both control and treated groups. To study the phenotype observed in MyD88-/- mice, we measured hepcidin expression and found that despite the higher iron levels in MyD88-/- mice, hepcidin levels were equal or slightly lower than Wt mice, indicating that MyD88 is additionally involved in the iron-sensing pathway.
Conclusion: The development of acute hypoferremia during LPS response seems to occur via a MyD88-dependent mechanism that is dissociated from peripheral cytokine production and hepatic hepcidin induction. On the other hand, hepcidin regulation by LPS is linked to cytokine production through MyD88 and Trif signaling. Moreover, the adaptor protein MyD88 is required for ferritin induction and iron storage in the spleen during LPSinduced hypoferremia. Furthermore, MyD88 is necessary for optimum response to BMP6 signaling and adequate hepcidin expression, thus playing a crucial role in maintaining normal iron absorption and liver and spleen iron storage levels.

Poster #146

IDENTIFICATION OF NOVEL REGULATORS OF FERROPORTIN EXPRESSION

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(Presented By: Claudia Guida)

Introduction: The transmembrane protein Ferroportin (Fpn) is the only known mammalian iron exporter. It is mainly expressed in hepatocytes, duodenal enterocytes, macrophages and placental trophoblasts. The iron-regulated hormone hepcidin binds to Fpn at the cell surface and triggers its internalization and degradation. Hence the hepcidin/Fpn regulatory system controls dietary iron absorption, iron release from macrophages, mobilization of iron from hepatic stores and iron transfer across the placenta. Misregulation of the hepcidin/ferroportin (Fpn) regulatory circuitry causes frequent disorders of iron overload (e.g. hereditary hemochromatosis) or iron deficiency (e.g. the anaemia of chronic inflammation). Here, we report the identification of novel cellular regulators of ferroportin-mediated iron export, a fundamental process that controls systemic iron homeostasis.
Methods and Materials: We generated a stable, inducible Hela cell line expressing a hFPN-renilla fusion protein and applied a RNAi kinome screen (smart pool library targeting around 800 genes by Dharmacon) to systematically identify factors that affect ferroportin internalization and degradation. For the screen cells were reverse transfected with a pool of four siRNAs per gene in 384-well plates, treated with doxycycline to induce the expression of the hFPN-Renilla fusion protein and incubated in the presence or absence of hepcidin. Renilla luciferase activity provided a sensitive, fast and hepcidin-responsive quantitative readout for FPN expression. As a measure of the phenotypes, Z-scores were calculated using the cellHTS2 software package from Bioconductor.
Results: Correlations between Z-scores identified hepcidin-dependent and -independent FPN regulators. The kinome screen yielded 70 candidate regulators of ferroportin expression. Validation experiments were performed to (1) assess the knockdown

efficiency of each Fpn regulator (2) analyze effects on cell viability/proliferation and non-specific readouts and (3) confirm the phenotype of RNAi pools by applying individual siRNAs. Based on these analyses, we selected 15 genes for further characterization in murine bone marrow-derived macrophages.
Conclusion: Interestingly, most validated regulators of ferroportin expression confer hepcidin-independent FPN regulation and are associated with immune processes. Specifically, we identified a Toll-like receptor as an effective regulator of Fpn expression in bone marrow-derived macrophages, further corroborating the relationship between iron homeostasis and the inflammatory response.

Poster #147
IL-28 POLIMORPHISM AND IRON HOMEOSTASIS DISTURBANCES IN CHRONIC HEPATITIS C: PRELIMINARY POLISH SINGLE CENTER STUDY
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(Presented By: Katarzyna Sikorska)

Introduction: Several parameters, both viral and host, determining the efficacy of treatment of hepatitis C virus (HCV) infected individuals have been described so far. Among them, recently discovered single-nucleotide polimorphism (SNP) of IL-28 in infected with HCV genotype 1, most resistant to currently available treatment, proved to be one of the major determinants and CC homozygosity in rs12979860 locus being the favourable prognostic factor of HCV-related liver disease. From the other hand, iron overload that is frequently diagnosed in chronic hepatitis C (CHC), is considered as to be a negative prognostic factor.
Methods and Materials: The aim of presented study was to investigate the possible influence of IL-28 polymorphism on development of iron overload, and in future to provide the answer whether the iron burden worsens the rate of antiviral treatment efficacy among carriers of good-response IL-28 polymorphism. 33 individuals, with confirmed HCV infection, have been enrolled in this preliminary study. The frequency of CC genotype, verified by RFLP method, was at 27% (9), CT - 61% (19) and TT at 12% (5).
Results: The iron overload parameters (serum iron, transferrin saturation, serum ferritin and presence of iron deposits in liver tissue) as well as histopathological parameters of liver injury (fibrosis, inflammation and hepatic steatosis) were assessed. Interestingly, biochemical, serum markers of iron overload were more frequently observed among CT and TT carriers, with 5/24 [21%] of CT/TT patients having ubnormally elevated transferrin saturation (seen as over 45% saturation), 8/24 [33%] having evevated iron in serum, while none of these parameters were increased in CC carriers. Elevated above normal values ferritin concentrations were observed only in 1/9 [11%] of individuals with CC polimorphism and in 7/24 [29%] of ones carrying CT or TT SNP. CC carriers compared only to TT presented significant lower concentration of hemoglobin ($p=0.02$), lower serum iron concentration ($p=0.03$) and lower transferrin saturation ($p=0.04$). Iron deposition in liver biopsy specimens was equally distributed within CC and CT/TT carriers with 22% and 25%, respectively. Intensity of hepatic steatosis and inflammation activity was similar in compared groups. Severe liver fibrosis, however, was more pronounced among CT/TT carriers (9/24 [37.5%] for CT/TT and only 1/9 [11%] of CC).
Conclusion: Despite small numer of cases analysed in the study so far, there is an interesting correlation between the iron homeostasis disturbances and CT/TT SNP at IL-28.
We do hope that ongoing study of ours will serve for better understanding of some aspects of CHC pathogenesis.

Poster #148
PARENTERAL IRON ALONG WITH A HIGH FAT DIET RESULTS IN INCREASED HEPATIC IMMUNE CELL ACCUMULATION, INFLAMMATION, OXIDATIVE STRESS AND APOPTOSIS BUT DECREASED STEATOSIS IN AN OBESE, DIABETIC MOUSE MODEL OF NON-ALCOHOLIC FATTY LIVER DISEASE
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(Presented By: Priya Handa)

Aim: To examine the effects of parenteral iron loading in an obese, diabetic murine model of non-alcoholic fatty liver disease.
Methods: Leprdb/db mice were fed either a high unsaturated (61%) fat diet (HF) or normal chow (NC) for 8 weeks. A subset of mice on both diets were administered a single dose of 1.25 mg/g wt Fe-dextran by IP injection at the start of the study. Histologic features of non-alcoholic steatohepatitis (NASH) and iron deposition were scored using previously established scoring criteria. Malondialdehyde (MDA), iron levels, TUNEL, hydroxyproline and gene expression profiles were assessed in liver tissue.
Results: Iron-loaded mice had a mixed pattern of hepatocellular and Kupffer cell iron deposition assessed by staining as well as higher hepatic iron content determined by biochemical measurement in addition to higher hepcidin gene expression ($p<0.0002$). Mice treated with high fat plus iron diet (HFI, mean steatotic grade, 1.33) and chow plus iron diet (CI, 1.25) had decreased hepatic steatosis compared to HF (2.5) and NC (2.2), or HF mice, respectively ($p<0.04$). CI and HFI mice demonstrated impaired hepatic lipid metabolism and mitochondrial biogenesis indicated by reduced ACOX1, SCD1, CPT1α

and TFAM gene expression compared to NC and HF ($p<0.05$). Hepatocellular ballooning was not observed in any iron-treated mice. HFI and CI mice had greater lobular inflammation compared to NC and HF ($p<0.015$), which were primarily in zone 3 monocytes and lymphocytes. Consistent with this observation, gene expression levels of macrophage/dendritic cell markers such as CD11b, CD11c, CD68, INOS and F480 were upregulated in hepatic tissue in the HFI group relative to the HF group ($P<0.05$). HFI mice also showed increased hepatic gene expression of macrophage M1 cytokines such as IL6 and TNFα, and chemokine MCP-1 compared to HF mice ($P<0.05$). Interestingly, liver gene expression profiles of macrophage M2 markers such as Arginase-1, Ym1 and IL-10 were also enhanced in HFI mice compared to HF ($P<0.05$). Furthermore, increased liver gene expression of T cell cytokine, interferong and regulatory T cell marker, Foxp3 were also observed in HFI mice ($P<0.05$, relative to HF). Both HFI and CI mice had increased oxidative stress compared to either NC or HF mice as indicated by higher levels of hepatic MDA and hemeoxygenase-1 (HO-1) gene expression ($p<0.02$). Increased hepatic hydroxyproline levels were also observed in HFI compared to either NC or HF mice. Hepatic microRNA analysis revealed that microRNAs associated with liver diseases and iron homeostasis, mIR122 and miR146a, were elevated upon iron treatment. TUNEL analysis revealed that parenteral iron treatment caused apoptosis relative to NC and HF mice.

Conclusions: Parenteral iron administration induces hepatic immune cell accrual and activation, marked inflammatory response, greater oxidative stress, collagen production and apoptosis, when combined with a high unsaturated fat diet. However, decreased steatosis and a lack of hepatocellular ballooning was also present in mice administered iron. Taken together, our studies show that iron may have diverse effects on NAFLD pathogenesis in this murine model.

Poster #149

NRF2 PARTICIPATES IN THE UP-REGULATION OF FERROPORTIN EXPRESSION BY NITRIC OXIDE

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(Presented By: Agnieszka Stys)

Introduction: Nitric oxide (NO) is widely recognized as a molecule strongly altering iron metabolism. Numerous and complex interventions of NO into cellular iron homeostasis include regulation of genes critical for iron uptake, storage and export. This function of NO is largely dependent on its ability to coordinately control the expression of transferrin receptor 1, ferritin and ferroportin by post-transcriptional mechanisms involving the iron regulatory protein (IRP)/iron responsive element (IRE) system. In addition the modulation of gene expression by NO occurs also through the redox regulation of transcription factors.

Methods and Materials: We focused our study on Nrf2, a transcription factor known to bind to the ARE/MARE (antioxidant-response element/Maf recognition element) sequences and activate the transcription of antioxidant genes in response to redox signaling. In addition nitric oxide has also been shown to potently activate Nrf2 (Buckley *et al.*, 2003) and thus potentially mediate the cytoprotective effect of NO. Of importance among iron metabolism genes both *ftl* and *fth* encoding for the heavy (H) and light (L) ferritin chains as well as *slc40a1* encoding for the ferroportin (Fpn), the sole identified cell iron exporter, contain MARE/ARE promoter sequences (Marro *et al.*, 2010; Hintze and Theil, 2005). Recently diethyl maleate and sulforaphane, two potent Nrf2 activators, were also shown to up-regulate Fpn expression and counteract lipopolysaccharide (LPS)-induced ferroportin mRNA
suppression in macrophages (Harada *et al.*, 2011).

Results: Here, using mouse bone marrow-derived macrophages (BMDM) exposed to the slowly releasing NO donor diethylenetriamine NONOate (DETA/NO) or bacterial lipopolysaccharide (LPS) we unveil a key players in the intrinsic regulation of ferroportin transcription in response to LPS (immunological stimuli). We demonstrate that LPS-dependent Fpn suppression is completely abolished not only in TLR4-/-macrophages, but also in macrophages deficient for the two adaptor molecules, namely MyD88 and TRIF. Furthermore, we show a transient (between 0.5 and 8 hour) upregulation of Nrf2 in BMDMs after DETA/NO treatment. In our experiments this increase preceded a 3-fold elevation in Fpn mRNA levels, which was completely ablated in DETA/NO-treated BMDM obtained from Nrf2-deficient mice (Nrf2-/-). Interestingly, Ft-H expression was induced to an even greater extent (5-fold) in wt BMDM exposed to DETA/NO, however, in contrast to Fpn mRNA expression, NO also significantly increased Ft-H level in Nrf2-/- BMDM.

Conclusion: Our results suggest that NO produced by macrophages in response to LPS stimuli is crucial for the activation of Nrf2 signaling pathway that prevents excessive TLR4/MyD88/TRIF-dependent Fpn down-regulation.

Poster #150

FLUORESCENT IRON CHELATORS WITH ANTIMICROBIAL ACTIVITY: INSIGHT ON STRUCTURE-ACTIVITY RELATIONSHIPS FROM LIPOSOME PARTITION STUDIES

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(Presented By: Maria Rangel)

Introduction: Our group has been developing work on the design of iron chelators to be used in two main applications: (a) imaging of intracellular iron and (b) new strategies to fight infection based on the concept of iron deprivation. [1-3]
For imaging iron we are developing “turn-on” and “turn-off” fluorescent chelators, which allow the detection of iron by lighting up or fading upon coordination of iron. To fight infection we are developing chelating units with higher affinity for iron(III) when

compared with mycobacterial siderophores. To enhance the activity of the chelators their units are being combined with other molecular frameworks, which are known to target bacterial membranes.

Methods and Materials: The interaction process of drugs with a lipid bilayer is crucial to understand the mechanism of drug action and a significant contribution to the development of new bioactive molecules. The group has developed liposome preparation methodologies and has been using steady-state fluorescence, fluorescence anisotropy, EPR and more recently NMR to access permeation and partition properties of new drugs. [4, 5]

Results: Herein we report the results obtained for the set of fluorescent hexadentate and bidentate chelators that have been tried as potential anti-mycobacterial agents. The compounds are ligands of the 3-hydroxy-4-pyridinone family and were conjugated with xanthene derivatives thus providing compounds with distinct colour, hydrophilic/lipophilic balance and charge at physiological pH. We performed comparative studies of the partition of the compounds and the corresponding fluorophores in large unilamellar liposomes and the results provide evidence that the biologically active compounds strongly interact with the lipid phase while the less-active do not. The results obtained for liposomes composed by the lipids DMPC and DMPG suggest that a surface effect is quite important for the interaction with the membrane. Considering the results obtained in the infection model and the partition properties of the chelators tested we speculate that the most active rhodamine labelled chelators are targeting the phagosomal compartment and that the phagosomal membrane is one potential site where the fluorophore may tether the chelator to allow successful competition with mycobacterial siderophores.

Conclusion: Our hypothesis, outlined in Figure 1, is that the role of the rhodamine part of the molecule is to anchor the chelators in the outer and inner parts of the phagosomal membrane. In such a way, the chelators are targeting the cellular niche where the bacteria multiplies and efficiently restricting the iron supplies thus compromising the survival of bacteria.

Figure 1

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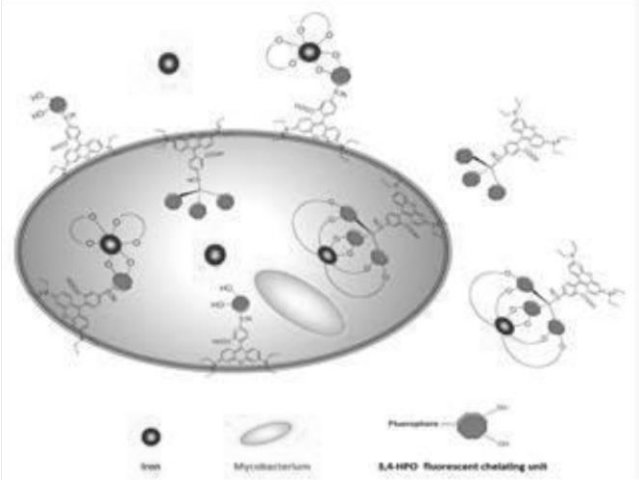

Poster #151

STRONG INDUCTION OF ACTIVIN B IN ESCHERICHIA COLI SEPSIS

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(Presented By: Hélène Coppin)

Introduction: We recently showed that activin B had an unexpected but crucial role in the induction of hepcidin by inflammation. Indeed, there was a dramatic induction of Inhbb mRNA, encoding activin βB-subunit, in the liver of mice challenged with lipopolysaccharide, slightly preceding an increase in Smad1/5/8 phosphorylation and Hamp mRNA. Interestingly, activin B induced Smad1/5/8 phosphorylation in human hepatoma-derived cells and, synergistically with IL-6 and STAT-3 signaling, markedly upregulated hepcidin expression, an observation confirmed in mouse primary hepatocytes.

Methods and Materials: To test whether the increase in activin B expression was part of the host antimicrobial defense mechanisms, we injected C57BL/6 mice with Escherichia coli K1, a gram-negative bacterium frequently causing septic bacterial infections. Twenty-eight hours after injection, Inhbb mRNA expression was strongly induced in the liver of E. Coli infected mice, and this coincided with an increase in Smad1/5/8 phosphorylation and Hamp mRNA.
Results: Infection of mice with less virulent mutated E. Coli strains showed that the upregulation of activin B persisted as long as bacteria could be detected in the spleen of infected animals. It is thus likely that Inhbb expression is induced by a metabolic product or a heat-labile structure of E. Coli, for instance via pattern-recognition receptors such as TLR4. In contrast to activin B, expression of the hemojuvelin and ferroportin genes were both strongly downregulated in E. Coli infected mice.
Conclusion: Infection of mice with different parasites or injection of turpentine to induce sterile abscesses are currently ongoing and will determine whether the contribution of activin B to hepcidin upregulation is limited to bacterial or parasitic infections, or whether it is a more general response to inflammation.
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Poster #152
EFFECT OF NITRIC OXIDE ON IRON HOMEOSTASIS IN AN INTESTINAL EPITHELIAL CELL LINE
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(Presented By: Shirly Moshe-Belizowski)

Background: Inflammatory bowel diseases (IBD) are chronic inflammatory disorders of the gastrointestinal tract. Nitric oxide (NO) is a vital physiological agent, which plays important roles in a variety of processes, including the immune response. In IBD, reactive oxygen and nitrogen species (ROS/RNS) such as NO are produced locally at high levels, causing elevated oxidative stress and cellular damage. In order to better understand the influence of NO on iron homeostasis and regulation in IBD, intestinal inflammation was mimicked by exposure of the human intestinal epithelial cell line Caco-2, to an NO donor at physiological oxygen tension.
Methods: The effect of NO on the iron regulatory protein- (IRP1 and 2) activities was evaluated by Electromobility Shift Assay and immunoblot analysis of IRP2. Regulation of iron importers transferrin receptor 1 (TfR1) and divalent metal transporter 1 (DMT1), the iron exporter ferroportin and the iron storage protein ferritin was examined at both mRNA and protein levels by real-time PCR and immunoblot analysis respectively. In addition, short and long-term changes in cellular iron homeostasis were measured by tracking 55Fe-transferrin, Inductively Coupled Plasma analysis (ICP-MS) and by measuring of the labile iron pool (LIP) by Calcein.
Results: Our findings showed that shortly after the NO release iron was secreted from the cells. We found that Caco-2 cells secret ferritin and therefore we believe that the NO induced iron secretion may partly involve ferritin secretion. The reduced intracellular iron levels combined with the increased NO levels, result in a strong activation of IRP1. IRP2, which is up-regulated in low iron conditions and down-regulated in conditions of oxidative stress, is only moderately activated. The IRP targets reacted to this activation as expected, with increased expression of TfR1 and decreased ferritin and ferroportin levels. The protein levels of DMT1 were unchanged. Accordingly, TfR1 mRNA levels were elevated whereas mRNA levels of ferritin and ferroportin variants were unchanged. In addition, after continuous NO exposure total cellular iron levels and LIP were both decreased.
Discussion and Conclusions: The decreased total cellular iron levels and LIP after long-term NO exposure suggest that the up-regulation of the iron import (via TfR) in combination with the down-regulation of the iron export (via ferroportin) are not able to overcome the NO induced iron secretion. Our results also imply that in intestinal epithelial cells, the low iron levels are the dominant regulators of IRP2 during inflammation. Previous studies showed an increase in mucosal iron in IBD biopsies and a recent study showed iron accumulation in macrophages following NO induced inflammation. Taken together we suggest that the elevated iron levels described in the IBD tissues are due to iron accumulation in the local macrophages and not in the inflamed intestinal epithelial cells. We further speculate that the iron secreted from the inflamed intestinal cell may be the source of iron overload in local macrophages. This iron shift from one cell type to another may be the molecular basis to the accumulation of reactive iron in inflamed tissue, which might further worsen the inflammation.

Poster #153
ROLE OF HEPCIDIN IN THE SETTING OF HYPOFERREMIA DURING ACUTE INFLAMMATION
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(Presented By: Sophie Vaulont)

Introduction: One of the most potent pathogen-derived inflammatory signals is the Gram-negative bacterial cell wall component lipopolysaccharide, LPS, which induces a massive release of cytokines and other inflammatory mediators in the infected host. This release is critical for the integration of the innate immune response, but requires tight regulation to limit the resulting inflammation. This release is critical for the integration of the innate immune response, but requires tight regulation to

limit the resulting inflammation, which can lead to endotoxic shock. Endotoxic shock is not only associated with an extreme proinflammatory response but is also often accompanied by hypoferremia, a primitive defensive mechanism allowing reducing circulating iron, limiting its availability to pathogens. Hepcidin has emerged as the possible pathogenic mediator of hypoferremia. Hepcidin functions as the master regulator of cellular iron export by controlling the amount of cell surface ferroportin, an iron exporter present predominantly on the basolateral surface of intestinal enterocytes and macrophages. Hepcidin binding to ferroportin induces its internalization and degradation, resulting in cellular iron retention, decreased iron export and hypoferremia.

Methods and Materials: While hepcidin was found up-regulated by LPS in several species, the requirement of hepcidin for hypoferremia in response to LPS has not yet been demonstrated. To address this question, we treated wild type (WT), heterozygous and homozygous (Hepc-/-) mice for 6h with sublethal doses of LPS. Ferroportin mRNA and protein levels were assessed in duodenum and spleen (the two predominant tissues contributing to circulating body iron), as well as other iron-regulatory genes, and plasma iron was determined. We show that after LPS injection, ferroportin mRNA and protein levels were totally repressed in the duodenum (as well as DMT1 and Dcytb) in both WT and Hepc-/- mice. In contrast, in the spleen, although ferroportin mRNA levels were also largely decreased regardless the genotype, ferroportin protein levels were decreased only in WT mice and not in Hepc-/- mice, suggesting a different sensitivity of ferroportin to hepcidin in these tissues during acute inflammation.

Results: We show that LPS injection in WT mice resulted in a two-fold increase of liver hepcidin expression and a 75% decrease of plasma iron. In the Hepc-/-, hypoferremia was largely blunted but LPS still induced a 15% reduction of plasma iron, suggesting that both hepcidin-dependent and –independent mechanisms are contributing to the development of hypoferremia during acute inflammation. Finally, we show that in the LPS-treated Hepc+/- mice, while hypoferremia was similar to that in WT mice, hepcidin gene expression was surprisingly not up-regulated. We identified that the livers of the Hepc+/- mice were significantly iron loaded with a concomitant decrease of the iron BMP/HJV/SMAD signaling pathway and suggested that the reduced activity of this pathway may explain the absence of hepcidin response to inflammation.

Conclusion: In conclusion, our results demonstrate that hepcidin is crucial, but not the sole mediator of LPS-mediated acute hypoferremia, its major contribution relying mainly on decreased ferroportin protein levels in the spleen. Furthermore, we establish that LPS-mediated repression of iron-absorption gene expression in the duodenum is independent of hepcidin. Finally, our results in the hepc+/- mice indicate that elevated hepcidin gene expression is not a prerequisite for the setting of hypoferremia during early inflammatory response and highlight the intimate cross talk between inflammatory and iron -responsive pathways for the control of hepcidin.

Poster #154

DOES HELICOBACTER PYLORI INFECTION AFFECT DUODENAL MUCOSAL EXPRESSION OF IRON TRANSPORTERS?

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(Presented By: Molly Jacob)

Introduction: Iron deficiency anemia is often associated with gastric infection with *Helicobacter pylori* (*H. pylori*). We hypothesized that such an infection may affect duodenal mucosal expression of divalent metal transporter (DMT1)and ferroportin, which are involved in the absorption of non-heme iron in the duodenum. The present study was undertaken to evaluate this hypothesis.

Methods and Materials: Forty patients undergoing upper gastrointestinal endoscopy were recruited after informed consent. *H. pylori* infection was diagnosed by rapid urease test of antral biopsies.Gene expression of DMT1 and ferroportin was determined in duodenal mucosal biopsies, by reverse transcription and quantitative polymerase chain reaction (qPCR). Routine haematological parameters and serum levels of ferritin and C-reactive protein were estimated. Analysis of variance and Pearson's correlation coefficient were used to test significance of differences seen.

Results: Based on the results of the rapid urease test and the presence or absence of anemia, patients recruited were categorized in to those with both *H. pylori* infection and anemia, those with only *H. pylori* infection, those with only anemia and those with neither condition. Ferroportin mRNA levels were significantly up-regulated in patients with both *H. pylori* infection and anemia (8.71 ± 3.71; n=10), when compared with those who had only *H. pylori* infection (1.93 ± 1.71; n= 10) or only anemia (2.78 ± 1.95; n=10) or neither (3.94 ± 2.70; n=10) (p=<0.01 in all cases). DMT1 expression was also up-regulated in these patients when compared with those with only the infection (6.46 ± 4.78 vs 0.54 ± 0.74; p=0.003).The expression of these 2 genes were significantly positively correlated with each other (R=0.697, p=0.00). DMT1 and ferroportin negatively correlated with mean corpuscular volume (R=-0.337, p=0.034 and R=-0.324, p=0.042 respectively). In addition, DMT1 also negatively correlated with hemoglobin (R = -0.321, p = 0.043).

Conclusion: The results of this preliminary study show that co-existence of anemia and *H. pylori* infection was associated with up-regulation of duodenal DMT1 and ferroportin gene expression.

Poster #155

NITRIC OXIDE-DEPENDENT REGULATION OF FERROPORTIN-1 CONTROLS MACROPHAGE IRON HOMEOSTASIS AND IMMUNE FUNCTION IN SALMONELLA INFECTION

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(Presented By: Manfred Nairz)

Introduction: The transcriptional expression of nitric oxide (NO) synthase-2 (NOS2) is controlled by iron, while NO affects the binding activity of iron regulatory proteins and cellular iron homeostasis. We therefore postulated that iron homeostasis and the formation of NO are closely linked during host immune responses to microbial pathogens.

Methods: We examined the reciprocal interactions between NO production and iron homeostasis using *Nos2*$^{-/-}$ mice and primary peritoneal macrophages, pharmacological NO donors, iron chelators and transient transfections. To induce an immune response, infection with *Salmonella enterica* serovar Typhimurium was used.

Results: We found that NO upregulated the expression of ferroportin-1 (Fpn1), the major cellular iron exporter. *Nos2*$^{-/-}$ macrophages displayed an increased iron content due to reduced Fpn1 expression and allowed for an enhanced iron acquisition by intracellular *Salmonella* Typhimurium. In vivo, *Nos2* gene disruption or inhibition of NOS2 activity led to a significant accumulation of iron in the spleen and its macrophages. Mechanistically, lack of NO formation resulted in impaired nuclear factor erythroid 2-related factor-2 (Nrf2) expression, whereas pharmacological NO donors enhanced the binding activity of Nrf2 in peritoneal macrophages, subsequently leading to increased Fpn1 transcription and cellular iron egress. Following infection of *Nos2*$^{-/-}$ macrophages with *Salmonella* Typhimurium, the iron accumulation was paralleled by a reduced cytokine (TNF-α, IL-12 and IFN-□) expression and an impaired pathogen control, all of which were restored upon administration of the iron chelator deferasirox or hyperexpression of Fpn1 or Nrf2.

Discussion: These data illustrate that the accumulation of iron in the abscence of Nos2 counteracts a proinflammatory and antibacterial host immune response and suggest that part of the protective effect of NO results from its ability to prevent an iron overload in macrophages.

Conclusion : NO-dependent Nrf2 activation results in Fpn1 induction, linking macrophage iron homeostasis and immune effector function.

Poster #156

UROPATHOGENIC ESCHERICHIA COLI DOWN-REGULATES HEPCIDIN EXPRESSION IN KIDNEY

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(Presented By: Dounia Houamel)

Introduction: Urinary tract infection (UTI) is mainly due to uropathogenic Escherichia coli (UPEC) that have the ability to move up the urinary track and to reach renal epithelia. UPEC require iron for growth particularly in the urinary tract environment where iron is limiting. In fact, the high molecular weight iron-transferrin complex is hardly eliminated by the kidney. In addition, we have recently shown that the iron regulatory hormone, hepcidin strengthens the barrier of the urinary excretion of iron by controlling its reabsorption in distal nephron tubules. Hepcidin is a 25 amino acid peptide that regulates negatively iron homeostasis but it has also been shown to exhibit an antimicrobial activity. Interestingly, beside to being predominantly produced by the liver, we and others have shown that this peptide is expressed in renal distal tubules. In addition, hepcidin is expressed in macrophages where it is supposed to act through an autocrine/paracrine pathway to decrease iron release and where it is stimulated by lipopolysaccharide (LPS) through TLR4 signaling. In hepatocytes, hepcidin is induced in response to high systemic iron via BMP/Smad1/5/8 -signaling pathway and to infection/inflammation via IL-6 and LPS through activinB and Smad1/5/8- signaling.

Aim: we proposed to investigate the role of renal hepcidin in UTI. Indeed, since distal tubules are the preferential site of UPEC adhesion and colonization in kidneys, we hypothesized that the local synthesis of hepcidin in these segments might represent an effective defence system against infection.

Methods: Two different strains of pathogenic Escherichia coli (CFT073 and HT7) were tested and the impact of the bacterial inoculation was analyzed in two genetic backgrounds of host mice (CBA/J, and C57BL/6J). Mice were analyzed 24h and 48h after infection. Pathogen growth and invasion were estimated by titration of bacteria in infected bladder and kidney homogenates. RT-qPCR was used to analyze the expression level of renal hepcidin1 and 2, the sensitive BMP-responsive

gene Id1,Toll-like receptor 4 (TLR4), interleukin IL-6, tumour necrosis factor alpha (TNFa) and the siderophore-binding protein, lipocaline (Lcn2).
Results: 1- bacterial count showed significant infection in the bladders and in the kidney (<109 CFU/ml and <106 CFU/ml, respectively) in different mice and with the two UPEC.
2- Despite a significant increase in the mRNA expression of TLR4, IL-6 and TNFa, hepcidin 1 and hepcidin 2 mRNA levels were decreased in infected kidney. This effect was observed only at 48h UTI. Lcn2 transcript, in contrast was markedly up regulated.
3- Id1 gene was induced suggesting that BMP-signalling pathway was not altered.
4- The expression of the Na+/H+ exchanger 3 (NHE3), the water channel Aquaporin (Aq2) and the Na+/K+/2Cl- co-transporter (BSC1) was unchanged, indicating that hepcidin decrease is not a consequence of epithelial renal cell damage.
Conclusion: These data strongly suggest that renal hepcidin may be one of the targets that UPEC trigger to become pathogenic and evade host defences during UTI. Further experiments are in progress including UTI in host mice pre-treated with hepcidin and In vitro studies to highlight the molecular mechanism by which UPEC affect hepcidin expression.

Poster #157
HEPCIDIN REGULATION DURING ACUTE INFECTIONS
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(Presented By: Andrew E. Armitage)

Introduction: Hepcidin controls uptake and distribution of iron. An evolutionary involvement in immunity is suggested since hepcidin is an IL-6/Stat3-pathway regulated acute phase protein that bears structural resemblance to the antimicrobial defensins. Hepcidin is hypothesized to aid host defence against certain pathogens by modulating iron distribution. Here, we investigated how hepcidin is regulated during acute murine systemic Candidiasis, and during acute human HIV-1, HCV and HBV infections.
Materials and Methods: *Murine Candidiasis*: Male C57BL/6 mice were infected intravenously with increasing doses of *Candida albicans* SC5314 for up to 48 hours. Kidney CFU, weight, serum transferrin saturation and expression of liver hepcidin and related genes (qRT-PCR) were quantified. *Human acute viral infections*: Sequential samples had been taken every 2-5 days from plasma donors who were subsequently found to have acquired HIV-1, HBV or HCV infection during the course of donation. Plasma hepcidin levels (Bachem hepcidin-25 EIA) were measured and correlated with viraemia, cytokines (Luminex and ELISA), ferritin and CRP (Abbott analyser). Hepcidin and cytokine levels were also measured in plasma samples from a cross-sectional cohort of HIV-1 infected individuals, 6 months post-infection.
Results: Liver hepcidin was strongly induced within 48 hours of highly pathogenic systemic murine *Candida albicans* infection, accompanied by reduced ferroportin mRNA expression and dramatically lowered transferrin saturation. In concert, expression of the Stat3-dependent gene Fga, encoding fibrinogen alpha chain, was induced. However, transferrin saturation was also reduced in mice infected with lower *Candida* doses, in the absence of detectable hepcidin upregulation, suggesting hepcidin-independent mechanisms may also contribute. Following the escalation of acute HIV-1 viraemia, hepcidin was upregulated in parallel with increases in IL-6. Hepcidin levels were also elevated in a separate cohort of HIV-1 infected individuals at 6 months post-infection. However, hepcidin was not notably upregulated during acute HCV and HBV infections, which are characterized by weaker or non-detectable systemic inflammatory responses; indeed, during acute HBV viraemia, the very low levels of hepcidin observed suggest hepcidin might be actively suppressed.
Discussion and Conclusions: Induction of hepcidin appears to be a component of the innate response to some but not all acute infections. Liver ferroportin mRNA downregulation may also contribute to systemic iron control during acute infection. The murine *Candida* model should facilitate investigation of the contribution of the hepcidin-ferroportin axis and iron redistribution to control of an acute infection in vivo. Alterations in iron status have previously been observed during HIV-1 infection; our data suggest that hepcidin upregulation may contribute to these effects. In contrast, the apparent lack of hepcidin induction during acute HCV and HBV infection may indicate different effects on hepcidin regulation during these infections.

Poster #158
IRON CHELATING PROPERTY OF DIETARY GRAPE POLYPHENOLS AND ITS ROLE IN THE PREVENTION OF LIPID OXIDATION
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(Presented By: Gorka Salas)

Introduction: Dietary factors as polyphenols and fiber might affect iron bioavailability. Grape polyphenols are thought to possess health-related properties, mainly attributed to its antioxidant and metal chelating activities. The intake of these bioactive substances may reduce the plasma iron content[1,2], and consequently should be reduced in iron deficiency situations but might be useful in situations characterized by oxidative stress[3]. The reduction of iron absorption may protect tissues against damage caused by oxygen free radicals and ion dependent metal lipid peroxidation. Free iron is the most probable catalyst for the initiation of peroxidation of polyunsaturated fatty acids (PUFA)[4]. Chickens provide an accurate in vivo model for

iron bioavailability[5] and also for lipid peroxidation studies because poultry meat is rich in PUFA and susceptible to oxidative deterioration.
Methods and Materials: Thus, we investigate the effect of dietary grape pomace (GP) rich in polyphenols and fiber on the plasma iron content and antioxidant status in chickens. Three diets containing 0, 5 and 10% of GP were formulated. Birds (30/diet) were fed experimental diets during 21 days, then 6 birds per treatment were randomly selected and after an overnight fast blood was collected by cardiac puncture and plasma Fe was determined by inductively coupled plasma optical emission spectroscopy. Another 6 birds per treatment were euthanized and thigh meat was collected to determine the extent of lipid oxidation by measuring the thiobarbituric acid reacting substances (malondialdehyde) generated at 1 and 4 d of storage.
Results: Dietary inclusion of GP both at 5 and 10% caused a significant reduction in the concentrations of plasma iron (up to 14%; $P<0.001$).We recently reported a reduction on plasma iron content in chickens after the intake of a concentrate grape seed extract rich in polyphenols[2]. Polyphenol compounds possess hydroxyl and carboxyl groups able to bind metal ions bearing positive charges such as iron. In vitro studies[6] using intestinal cells reported that polyphenols from grape seed might interact with iron creating a non-transportable polyphenol-iron complex preventing the iron exit across the basolateral membrane. This reduction in plasma iron content observed with dietary GP was correlated with a lower lipid oxidation of meat.
Conclusion: Accordingly, previous studies[7],[8] also reported an increase in the oxidative stability of tissues of chickens fed diets containing GP related to an increase of plasma α-tocopherol content. In conclusion, this study indicates that a reduction of plasma iron content obtained with dietary intake of grape polyphenols might protect tissues against lipid peroxidation.

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Poster #159
EFFECT OF IRON CHELATION ON T CELL RESPONSES IN RHEUMATOID ARTHRITIS
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(Presented By: Damini Tewari)

Introduction: Rheumatoid Arthritis (RA) is a systemic auto-immune disorder affecting 1% of the adult UK population and having significant financial and social costs. It is characterised by chronic inflammation, which is in part mediated by the proliferation of pro-inflammatory, autoreactive CD4+ T cells. Current therapies for RA include steroidal and non-steroidal anti-inflammatory drugs, Disease modifying drugs, TNF inhibitors amongst others. The need for new therapies is highlighted by the fact that most of the existing therapies are either very expensive or not very effective. Our approach is based on the observation that chronic inflammation leads to sequestration of iron within synovial cells via the actions of hepcidin, leading to increased cell proliferation and tissue damage. Previously, a clinically approved iron chelator, Desferrioxamine (DFO), was used in a clinical trial for RA, but the trial was halted due to toxicity, although RA symptoms did improve. We are investigating a novel class of iron chelators generated at KCL which have a greater specificity for iron.
Methods and Materials: Our laboratory has tested these chelators in a mouse model of RA. C57/BL 6 mice were primed with Type II Collagen and ELISA was used to study the effect of iron chelators on cytokine production from Antigen Presenting Cells (APCs) and T cells from the spleen and lymph nodes. 3H incorporation was used to analyse the effect of chelator treatment on cell proliferation. Results showed that the chelators were able to significantly reduce disease symptoms both prophylactically and therapeutically in a murine model of collagen induced arthritis at doses significantly lower than those used in previous clinical trials with DFO. The most prominent effect, both *in vitro* and *in vivo*, is on proliferation of, and IFN-y production from, CD4+ T cells. A comparison with structurally related compounds lacking the ability to chelate iron have confirmed that this is an iron dependent mechanism.
Results: Furthermore, *in-vitro* experiments on human CD4+ T cells stimulated with Tetanus Toxoid, confirm that the chelators reduce T cell proliferation by more than 50% and IFN-y production by 30%. Moreover extensive experiments have demonstrated that the chelators exert their effects on CD4+ T cells rather than APC. Preliminary experiments investigating possible mechanisms of action of the chelators indicate that they may be exerting their effect on the IL-2 pathway by reducing the expression of pSTAT5, which is essential for proliferation.
Conclusion: In conclusion, it is evident that iron is an essential nutrient in the body. It is known to play a vital role in cell growth and survival due to its association with DNA synthesis enzymes such as Ribonucleotide reductase, as well as its role in cell cycle progression. The importance of the IL-2 proliferation pathway in RA is also well established and several JAK3 inhibitors, such as CP690550, are in Phase II clinical trials for RA. Our study demonstrates that intracellular iron has the ability to modulate inflammatory responses in Rheumatoid arthritis and results indicate that the use of novel iron chelators, which interfere with the down-stream signalling of the IL-2 pathway, could prove to be a potential model for RA therapy.

Poster #160

DETAILED CHARACTERIZATION OF THE MOUSE MODEL OF ANEMIA OF INFLAMMATION CAUSED BY HEAT-KILLED BRUCELLA ABORTUS REVEALS MULTIPLE CAUSES OF ANEMIA AND PARTIAL DEPENDENCE ON HEPCIDIN

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(Presented By: Airie Kim)

Introduction: Anemia is a well-known complication of inflammatory diseases but the few mouse models of this condition are inadequately characterized. We describe in detail the anemia induced in C57BL/6 mice by intraperitoneally (IP)-injected heat-killed Brucella abortus (HKBA) and test the contribution of hepcidin and iron by comparing the severity of anemia in wild-type vs. hepcidin-1 KO mice.
Methods: After two weeks on an iron-adequate diet, the mice were injected with HKBA or saline, then euthanized and analyzed at 0, 3h, 6h, 12h, 1d, 2d, 3d, 7d, 14d, 21d and 28 days for iron, hematological, and inflammatory parameters.
Hepcidin-1 knockout mice on C57BL/6 background were also analyzed and compared to WT. Those mice were placed on a low-iron diet for several weeks to normalize their iron stores, then injected with HKBA. Full analysis was performed at 0, 6h, 7d, 14d, 21d and 28d.
Results: Acute inflammation was seen in HKBA mice, with hepatic SAA-1 and hepcidin expression peaking at 6 h after injection, followed by a chronic phase with leukocytosis and hepatic perivascular inflammatory cell infiltrates. The mice developed a significant anemia by 7d (Hb 9) with a nadir by 14d (Hb 7), followed by partial recovery of Hb over the next two weeks. Hypoferremia persisted throughout the time course except for a transient increase in serum iron during a period of suppression of erythropoiesis around 3d, manifested as a trough in reticulocyte counts (RPI 2%). Blood smears showed schistocytes by 7d, and RBC life span was shortened 2.5-fold as measured by RBC biotinylation assay. With worsening anemia, erythropoietin levels increased to a maximum on days 7-14, initiating reticulocytosis by 14d and hemoglobin recovery starting by 21d. Hepcidin was increased compared to saline-treated mice from 3h to 2d but by 14d it was lower than controls presumably due to the suppressive effect of erythropoiesis. Nevertheless, from 14d to 28d erythropoiesis was iron-restricted in HKBA mice as indicated by dramatic increase in RBC zinc protoporphyrin. Iron accumulation was seen in the liver and the spleen, likely due to hemolysis and increased hepcidin. In HKBA-treated hepcidin KO mice, anemia was milder (nadir Hb 10) with quicker recovery. HKBA-treated hepcidin KO mice had increased serum iron levels, likely a result of hemolysis and free efflux of recycled iron from the liver and the spleen, and only trace elevation of zinc protoporphyrin, indicating the absence of significant iron-restricted erythropoiesis. Correspondingly, the liver and spleen iron were decreased compared to controls.
Hepcidin KO mice also had a significantly increased early mortality compared to the WT mice (21% vs. 0%).
Conclusions: We characterized a mouse model of anemia of inflammation that manifests multifactorial pathogenesis including transient suppression of erythropoiesis, shortened erythrocyte lifespan due to hemolysis, and increased hepcidin and iron restriction. In hepcidin KO mice, anemia was milder and recovery was accelerated, likely due to the absence of the iron-restriction component. However, inflammation caused significant mortality in hepcidin KO compared to none in WT mice indicating that the lack of hepcidin dysregulated inflammation or exacerbated tissue injury.

Poster #161

COMPARISON OF METHODS TO ASSESS THE INFLUENC OF CHRONIC INFLAMMATION ON INTESTINAL IRON ABSORPTION IN MICE

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(Presented By: Christiane Becker)

Introduction: Inflammation reduces intestinal iron absorption, thought to be mediated by increased hepcidin impairing ferroportin in enterocytes' basolateral membranes. Studies in ferritin-H knock-out mice suggest ferritin-H being prerequisite for enduring reduction of iron-absorption. In contrast, iron-deficiency increases iron absorption due to reduced hepcidin concentrations. Both mechanisms (reduced ferroportin and high ferritin concentration in enterocytes) should increase iron retention in enterocytes. More recently, it has been proposed that high hepcidin concentrations may also impair intestinal iron uptake via DMT-1 which should reduce intestinal iron retention.
Ligated duodenal loops (LDL) and ^{59}Fe-gavage (GAV) are established methods to test ^{59}Fe-retention in intestinal tissue and in the body *in vivo*, however, with different time frames: LDLs yield linear ^{59}Fe-transfer rates, a dynamic parameter usually tested for 10 – 15 min, while GAV assesses intestinal and whole body ^{59}Fe-retention under steady state condition after several days. Our project aims at comparing both methods under inflammatory and iron-deficient conditions. This may be of interest, as equilibration of iron *in statu transferandi* with enterocyte ferritin iron stores, and re-absorption of iron from digested exfoliated enterocytes in more distal intestinal areas should not show up within 10 – 15 minutes.
Methods: Conventionally raised male wild-type (WT) and homozygous IL-10$^{-/-}$ with a 129 SvEv TAC background (origin: E. Balish, University of Wisconsin, USA) were fed an iron-adequate diet. Duodenal and whole body iron retention were determined in LDL in 14 animals (7 WT, 7 IL-10$^{-/-}$) offering ^{59}Fe as Fe-NTA(1:2)-complex (10 µmol Fe/L) for 15 minutes. ^{59}Fe-absorption was determined in a whole body counter for small animals (type AW3, mab solutions, Dettenheim, Germany) and

expressed as pmol^{59}Fe/cm/min. To analyze whole body iron retention 19 mice (11 WT, 8 IL-10$^{-/-}$) were gavaged with 30 µl ^{59}Fe as $FeSO_4$ (10 mmol Fe/L in 10 mM HCl saline). After gavage and 7 days later, ^{59}Fe-whole body iron retention was measured in the whole body counter and expressed as percentage of the baseline ^{59}Fe-load.
Results: Duodenal LDL ^{59}Fe-retention showed no significant differences between IL-10$^{-/-}$mice and corresponding WT controls (1.22 vs. 1.66 pmol ^{59}Fe/cm). 7 day ^{59}Fe-whole body retention (IL-10$^{-/-}$mice: 2.6 %; WT mice 2.5 %) as well as duodenal ^{59}Fe retention (IL-10$^{-/-}$mice: 0.033 %; WT mice 0.019 %) did not differ significantly between genotypes.
Conclusion: These preliminary data are in the same order of magnitude as observed in earlier controls. Neither method revealed a significant impact of inflammation on ^{59}Fe- duodenal retention. Ongoing molecular biological investigations may help to clarify the corresponding responses of proteins engaged in iron absorption in inflammation.

Poster #162
MACROPHAGE-HFE IS CRITICAL FOR LIPOPOLYSACCHARIDE (LPS)-CONTROLLED HEPATIC HEPCIDIN ACTIVATION
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(Presented By: Christoph Metzendorf)

C. Metzendorf and M. Vujić Spasić contributed equally to this work.
Introduction: Systemic iron homeostasis is disrupted in the common, potentially fatal iron overload disorder Hereditary Hemochromatosis (HH) due to mutations in the Hfe gene. HH is hallmarked by inadequate expression of hepcidin, the key regulator of systemic iron homeostasis. While inappropriately low hepcidin levels contribute to iron overload, increased hepcidin expression in response to cytokines plays an important role in the anaemia of inflammation (AI). In addition, Hfe is required for a complete hepcidin response to the inflammatory stimulus LPS (Roy et al., 2004) as Hfe-/- mice fail to appropriately elevate Hepcidin mRNA expression in response to the injection of LPS (5µg).
Methods and Materials: Interestingly, this finding is replicated in a mouse model with macrophage-specific Hfe ablation, while it appears unaltered in mice lacking hepatocytic HFE, showing for the first time a functional role of Hfe in macrophages and innate immunity.
Results: By applying transcriptome- and proteome-wide (Eichelbaum, Winter, Diaz, Herzig, &Krijgsveld, 2012) analyses, we have identified 8 differentially expressed mRNAs and 12 proteins potentially secreted exclusively by LPS-treated bone marrow derived macrophages from wild type or Hfe-/- mice.
Conclusion: Functional studies are ongoing to elucidate the mechanism(s) of how macrophage Hfe controls hepatic hepcidin levels.
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Poster #163
EXPRESSION OF LACTOFERRIN-DERIVED PEPTIDES IN E.COLI AND STUDY OF THEIR ANTIMICROBIAL PEPTIDE ACTIVITY
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(Presented By: Sebastien Farnaud)

Introduction: Multidrug resistance is widespread among gram-negative bacteria. One way proposed to overcome the problem of the emergence of resistance is the use of new antimicrobial compounds and/or combination therapy. The iron-protein lactoferrin has been proposed to have antimicrobial activity. Whereas some studies suggest that this antimicrobial activity is iron-dependent, other results have shown that some N-terminus derived peptides, named lactoferricins, are responsible of this antimicrobial activity. In recent years, many positively charged polypeptides called antimicrobial cationic peptides have been isolated and are thought to be a major factor in antimicrobial defense. Different modes of action such as hydrophobicity, cationicity and secondary structure have been proposed as important features of antimicrobial effect.
Methods and Materials: In this study, antimicrobial activity was obtained with synthetic peptides derived from the bovine lactoferricin sequence and peptides corresponding to chimeras of human and bovine sequences.
Results: The results underline the importance of tryptophan and arginine residue content, and their relative location for antimicrobial activity.
Conclusion: To further explore the antimicrobial activity of these peptides and understand their mode of action *in situ*, several expression systems were designed to express the peptides derived from human and bovine Lactoferricins in *E.coli*. In order to improve their stability, the peptides were expressed as native and fusion peptides. In addition, to understand their mode of action, different cellular spaces were targeted. The preliminary results which describes antimicrobial activity when the peptides are expressed inside the cells, suggest some internal targets and challenges the previously proposed mode of action where the main target of the peptide is the outer membrane.

Poster #322

HEPCIDIN-DEPENDENT AND HEPCIDIN-INDEPENDENT REGULATION OF ERYTHROPOIESIS IN A MOUSE MODEL OF ANEMIA OF CHRONIC INFLAMMATION

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(Presented By: Cindy Roy)

Introduction: The anemia of inflammation (AI) occurs in many disease states including infection, autoimmune disorders, chronic kidney disease and cancer. While anemia is defined based on hemoglobin concentration, additional features of AI include hypoferremia (despite adequate iron stores) and impaired erythropoiesis. Increased expression of hepcidin antimicrobial peptide (Hepc) is correlated with hypoferremia and anemia in various disease states, but the requirement of Hepc for AI has not been fully demonstrated. Overexpression of Hepc results in microcytic hypochromic anemia and redistribution of iron stores in mice and humans. However this iron-restricted erythropoiesis is not entirely consistent with the normocytic, normochromic anemia that is often associated with AI. The turpentine-induced sterile abscess model of AI exhibits Hepc-mediated hypoferremia in the early stages of inflammation, but also exhibits a normocytic, normochromic anemia. Anemia is correlated with IL-6 in multiple disease settings. While IL-6 may drive Hepc expression, IL-6 has also been shown to have direct inhibitory effects on erythroid progenitors. Thus, AI is likely to be multifactorial, resulting from inhibitory effects of Hepc and inflammatory cytokines.
Methods and Results: We investigated the individual requirements of Hepc 1 or its positive regulator, Interleukin-6 (IL-6) for the development of AI in a rodent model. We used turpentine-induced sterile abscesses to model AI in Hepc1(-/-) or IL-6(-/-) mice to determine whether these genes are required for AI. We assessed the development of inflammation, features of red blood cells and markers of erythroid development. Hepc1 (-/-) mice exhibited an inflammatory response, as circulating neutrophils increased from 0.74 (0.17, 0.91; median, range) in untreated Hepc1(-/-) mice to 1.98 (1.74, 3.26) K/mcL in Hepc1(-/-) mice with abscesses (p=0.013). While hemoglobin levels did not decline significantly in Hepc1(-/-) mice with abscesses, erythrocyte numbers were significantly reduced from 10.24 (10.02, 10.26) in untreated Hepc1(-/-) mice to 8.97 (8.28, 9.50) M/mcL in Hepc1(-/-) mice with abscesses (p=0.014). To compensate for the reduction of erythrocytes in Hepc1(-/-) mice with abscesses, mean cell volume (MCV) increased from 46.3 (45.8, 48.7) to 51.9 (50.6, 53.2) fL (p=0.001) and mean cellular hemoglobin (MCH) increased from 15.1 (14.4, 15.4) to 16.5 (16.2, 17.6) pg (p=0.006) to maintain hemoglobin concentration. We also observed a shift in the distribution of erythroid progenitors in the bone marrow of Hepc1 (-/-) mice with sterile abscesses, with fewer late progenitors and an increase in early progenitors (p=0.05). This observation is presumably related to the decline in circulating erythrocyte number. IL-6(-/-) mice also responded to sterile abscess with an increase in circulating neutrophils from 0.47 (0.08, 0.72) to 1.17 (0.91, 1.79) K/mcL (p<0.001). In contrast to Hepc1(-/-) mice with abscesses, hemoglobin concentration declined significantly from 13.9 (13.1, 14.6) g/dL in untreated IL-6(-/-) mice to 13.3 (12.8, 13.8) in IL-6(-/-) mice with sterile abscesses (p=0.016). Consistent with the decline in circulating red blood cells, IL-6(-/-) mice with sterile abscesses also exhibited a shift in the distribution of erythroid progenitors in the bone marrow (p=0.001).
Conclusion: Our results demonstrate the requirement of Hepc1 for the development of anemia in the turpentine-induced sterile abscess mouse model of AI. Simultaneously, our results demonstrate hepcidin-independent effects of inflammation on the suppression of erythropoiesis.

Poster #165

THE ROLE OF HYPOXIA INDUCIBLE FACTORS IN IRON TRANSPORTER EXPRESSION IN CACO-2 CELLS

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(Presented By: Katayoun Pourvali)

Introduction: Iron deficiency anaemia (IDA) leads to local tissue hypoxia. Many of the effects of hypoxia are mediated by stabilization of the transcription factors HIF1α and HIF2α. Recent work has suggested that HIF-2α is the main regulatory factor in intestinal enterocytes where is controls the expression of iron and copper transport genes. Recently, we showed that HIF2α also controls the basal expression of the copper transporter Ctr1 under normoxic conditions suggesting a wider regulatory role. The aim of this study was to investigate the role of HIFs on iron transporter expression in intestinal Caco-2 cells grown under hypoxic and normoxic conditions.
Methods and Materials: Fully differentiated Caco-2 cells were exposed to either 1% or 19% O2 and were treated for 24 h with N,NO-(2,5-dichlorosulfonyl) cystamine (20 μM), which suppresses cellular HIF1α levels or the HIF 2α inhibitor Methyl-3-(2-(cyano(methylsulfonyl) methylene)hydrazino) thiophene-2-carboxylate (10 μM). The mRNA expression of iron transporter genes (DMT1, ferroportin (FPN), and Hephaestin) was measured using qPCR. The data was analysed by one-way ANOVA and Dunnett's post-hoc test. Differences between test groups and the untreated controls were considered significant at $p < 0.05$.
Results: Hypoxia induced the expression of DMT1, FPN and hephaestin. In normoxic condition, both the HIF1α and HIF2α inhibitors reduced DMT1 mRNA expression significantly (HIF-1α (0.49±0.16, p<0.001) and HIF-2α (0.46±0.03, p<0.001)). FPN mRNA was decreased by 30% (p<0.05) following HIF2α inhibitor treatment in normoxia. There was no effect of either inhibitor on transporter expression in hypoxia. None of the treatments altered the expression of the ferroxidase hephaestin.

Conclusion: Our study demonstrates that HIFs play a key role in regulating the basal iron transporter expression under normoxic conditions in intestinal Caco-2 cells. Taken together these data suggest that additional factors may be required to mediate the effects of hypoxia on iron transporter gene expression.

Poster #166

REGULATION OF INTRACELLULAR IRON HOMEOSTASIS UNDER HYPOXIA

Qianqian Luo, Medicine Master, Chunming Cheng, Medicine Master in reading, Dan Wang, Medicine Master, Xian Cao, Medicine Master in reading and Li Zhu, PhD
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(Presented By: Li Zhu)

Introduction: Iron and oxygen are both essential elements for life metabolism. Iron levels are stringently regulated within cells by a host of iron-related proteins that keep iron levels in check. These proteins contain transferrin (Tf), transferrin receptor (TfR), divalent metal transporter 1 (DMT1), ferritin (Ft), ferroportin (FpN), and iron regulatory proteins (IRPs, including IRP1 and IRP2). As cytosolic RNA-binding protein, IRP1 and IRP2 could sense and control the level of intracellular iron by binding to iron response elements (IREs) located in the untranslated region (UTR).
The influence of hypoxia on cells is extensive and hypoxia-inducible factors (HIFs, including HIF-1, HIF-2 and HIF-3) play the key role. Some iron-related proteins involved in iron homeostasis are directly or indirectly regulated under hypoxia. Concurrently, HIF-1 has been shown to regulate intracellular iron by binding to hypoxia responsive elements (HREs) that are located within the genes of iron-related proteins such as DMT1 and TfR1.
Methods and Materials: Our research focused on the interaction between the IRP1/IRE and HIF1/HRE systems and how cells utilize these intricate networks to regulate intracellular iron levels under hypoxia.
Results: The results of atomic absorption method showed intracellular total iron in cultured cell lines were enhanced after hypoxia for 6h. Due to IRPs was the main regulator of intracellular iron homeostasis, the expression of IRPs was detected using real time quantitative PCR and Western blots. The results suggested IRP1 was decreased time-dependently under hypoxia. Using Genomatix MatInspector software, three putative HREs were found in the regulatory region of IRP1. Through electrophoretic mobility shift assay (EMSA), supershift and promoter activity studies, we proofed HIF-1 could bind specially with HREs of IRP1 and consequently inhibit IRP1 expression. Besides, we observed the expression of DMT1, TfR1, Ft, and FpN. The results showed all the four proteins were increased time-dependently during hypoxia, which hinted the ability of iron absorption, storage and export was strengthened. As IRE had been identified in the 5'-UTR of both Ft and FpN mRNA, the reduction of IRP1 under hypoxia was no doubt induced the expression of Ft and FpN.
Previous studies revealed the expression of DMT1 and TfR1 were regulated by both HIF-1 and IRP1 respectively. To investigate whether IRP1 played role in regulating DMT1 and TfR1 under hypoxia, small interfering RNA (siRNA) against IRP1 was designed. DMT1 and TfR1 expression was reduced significantly after knockdown IRP1 under normoxia, which indicated IRP1 could steady DMT1 and TfR1 mRNA and further stabilize its proteins under normoxia. Nevertheless, siIRP1 under hypoxia increased DMT1 and TfR1 expression weakly but not reduced both of them. It suggested that the effect of IRP1/IRE system on stabilizing DMT1 and TfR1 was weaker than that of HIF-1/HRE system on promoting DMT1 and TfR expression under hypoxia. Compared to the expression under normoxia after siIRP1, the level of DMT1 and TfR1 proteins were enhanced obviously after siIRP1 under hypoxia, which further testified HIF-1 greatly facilitate DMT1 and TfR expression without IRP1.
Conclusion: Taken together, these results indicated both HIF-1/HRE system and IRP1/IRE system take part in the regulation of intracellular homeostasis under hypoxia. IRP1/IRE system may play a negative feedback role in this process.

Poster #167

INCREMENTAL IRON AND ITS EFFECTS ON HYPOXIA PATHWAYS

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(Presented By: Nicole Bart)

Results: Iron and oxygen are both essential for life. Despite this, too much of either substance can lead to cell toxicity. Consequently, both are tightly regulated. Iron is closely linked to the hypoxia inducible factor (HIF) pathway, which is activated in response to a decrease in oxygen levels. In normoxia, HIF is ubiquinated and rapidly degraded by the proteasome. This interaction requires hydroxylation of HIF-α by iron-dependent prolyl hydroxylases. The hydroxylation requires oxygen as a substrate and iron as a cofactor. In hypoxia, HIF is stabilised leading to angiogenesis, red blood cell changes and alteration in the pulmonary arteries. This study will investigate the effect of incremental iron on healthy individuals exposed to hypoxia.
Methods and Materials: In this study, 18 healthy individuals are being given 6 months of incremental intravenous iron. They are placed in the hypoxic chamber at the beginning and end of the study. During their time in the chamber, hourly echocardiograms are performed to investigate changes in their pulmonary arteries. Red cell mass is also examined through a closed circuit breathing technique to assess long term changes.
Conclusion: There could be several implications that might benefit patients with pulmonary hypertension and airways disease and to people exposed to high altitudes.

Poster #168

IRON AND HYPOXIA LINK: DOWNREGULATION OF LUCIFERASE ACTIVITY OF HEPCIDIN PROMOTER BY HYPOXIC SERA IN HUH-7 CELLS

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(Presented By: Federico Greni)

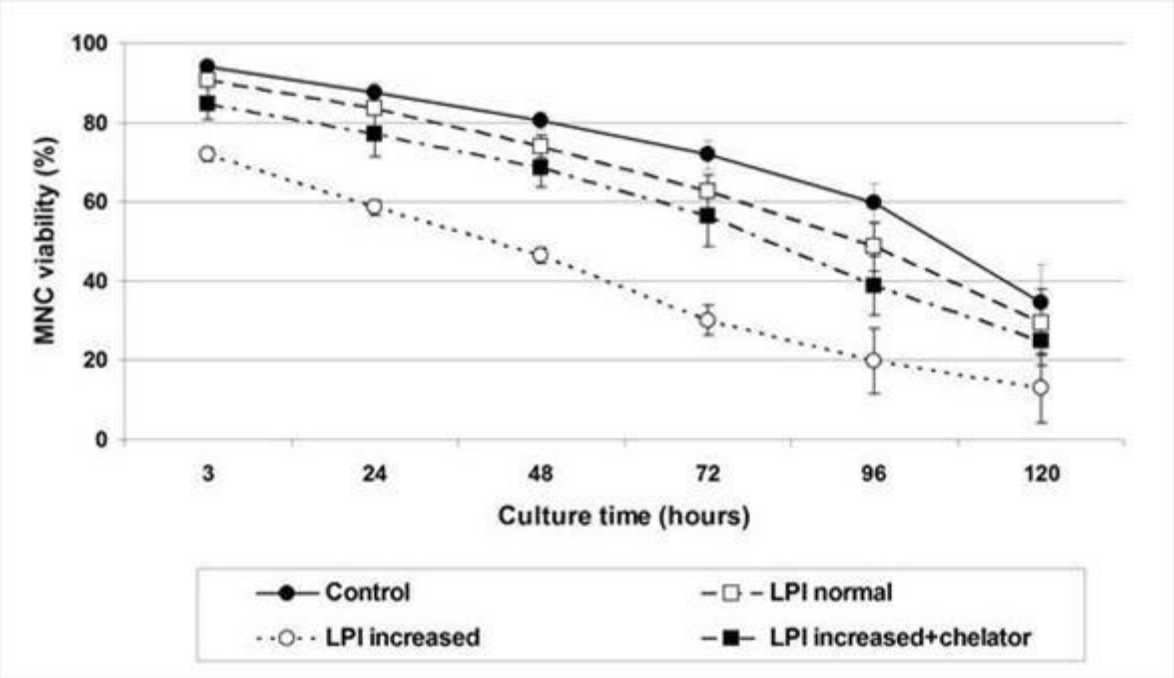
Introduction. Hepcidin is 25 residues peptide produced by hepatocytes, which acts by binding to ferroportin promoting internalization and degradation. This process limits intestinal iron absorption and macrophages release. Hepatic hepcidin expression is inhibited by hypoxia in vivo and in vitro. Mechanism of hepcidin downregulation mediated by hypoxia is unknown and controversies exist whether hepcidin down-regulation is directly caused by hypoxia or indirectly through the release of circulating factors by the activated erythroid bone marrow. The aim of our work is to clarify some of the mechanisms of hypoxia-induced hepdicin downregulation.
Subjects and Methods. 16 volunteers (Group HC) participating to the HighCare2008 project on Himalaya (Mt. Everest Base camp: 5400 meters above the sea level (asl)) and 11 volunteers (Group MR) participating to a study performed on Mount Rosa in Italy at the Capanna Margherita (4544 meters asl) were studied. Sera were collected at sea level (Milan: 140 meters asl) (normoxic serum) and after high altitude exposure (hypoxic serum). Huh-7 cell line was taken as in vitro model to evaluate the effect of hypoxic sera by using both luciferase assays and RT-PCR. For luciferase assays cells were transfected with the wildtype (2997 bp) or the 1207bp fragment hepcidin promoter. Transfected and non-transfect huh-7 cells were stimulated with normoxic or hypoxic sera for 48h and luciferase activity or hepcidin mRNA expression were measured.
Results. Luciferase assay (wildtype promoter) in Group HC and MR showed a significant hepcidin down-regulation in cells exposed to hypoxic sera (1805+1580 RLU at Sea Level and 822+776 RLU at High Altitude, $p < 0.001$). In addition, luciferase reporter activity in Huh-7 transfected with the 1207 bp fragment showed a dramatic reduction independent to hypoxia (76.4+36 RLU at Sea Level and 94.2+90 RLU at High Altitude). To evaluate the molecular pathway involved in hepdicin down-regulation, mRNA expression of *HAMP, ID1, BMP6,* and *TMPRSS6* has been evaluated, and preliminary data suggest concordant *HAMP* and *ID1* regulation.
Discussion and Conclusion. These results suggest the existence of circulating factors in hypoxic sera of volunteers exposed to high altitude able to down-regulate hepcidin synthesis in hepatic cell lines. Further studies are needed to clarify the molecule and the molecular pathway involved in this process.

Poster #169

IMPACT OF LABILE PLASMA IRON ON VIABILITY OF CULTURED MONONUCLEAR CELLS

Flávio Naoum, MD, PhD, Idiberto Zotarelli, Breno Espósito, PhD, Ana Carolina Abreu and Oswaldo Grecco, MD, PhD
Academia de Ciência e Tecnologia
(Presented By: Flávio Naoum)

Introduction: Myeloablative conditioning for hematopoietic stem cell transplantation (HSCT) leads to a fast increase of non-transferrin bound iron, including the labile plasma iron (LPI) pool, mainly due to suppression of erythropoiesis and release of iron from liver and bone marrow cells. It has been suggested that increased LPI, the redoxactive and toxic form of iron, may cause cell damage and ultimately lead to tissue toxicity and other complications commonly observed in the early post-HSCT period. However, this assumption has not been proved yet.
Methods and Materials: In order to evaluate if increased LPI levels can cause cell toxicity, we evaluated its impact on viability of mononuclear cells obtained from six patients undergoing autologous HSCT for multiple myeloma (n=4) and lymphoma (n=2). Firstly, LPI was measured in plasma samples obtained from each patient before (baseline) and after conditioning (on day 0), revealing normal levels (<0.5µM) in all baseline samples (mean:0,17µM; range:0-0.4µM) and increased levels in all day 0 samples (mean:1.7µM; range:1,1-4.0µM). Then, mononuclear cells were separated from an aliquot of hematopoietic progenitor cells collected by apheresis, and cultured in duplicates for five days in four experiments: without addition of plasma (control) and with addition of autologous plasma containing normal LPI levels (baseline sample), increased LPI levels (day 0 sample) and increased LPI levels (day 0 sample) with addition of an iron chelator (deferiprone).
Results: Viability of cultured mononuclear cells was determined by trypan blue method at 3, 24, 48, 72, 96 and 120 hours. Cell viability decreased over time in all experiments ($p < 0.001$; **Figure**). There was no difference in cell viability between control cultures and cultures with normal LPI levels at all time points. Cells cultured with increased LPI levels presented the lowest viability in relation to the other three experiments at all time points ($p < 0.001$). The viability of cells cultured with increased LPI levels in the presence of iron chelator was similar to that of cells cultured with normal LPI levels, also at all timepoints ($p > 0.05$).
Conclusion: In conclusion, increased LPI levels can decrease substantially the viability of mononuclear cells, an effect that can be prevented or at least attenuated by the use of iron chelators.
Figure. Changes on viability of mononuclear cells (MNC) cultured without and with autologous plasma containing normal LPI levels, increased LPI levels and increased LPI levels with the addition of the iron chelator deferiprone. (Values represent mean±SEM).



Poster #170
NOVEL HETEROZYGOUS MISSENSE MUTATIONS IN GLRX5 GENE OF A CHINESE PATIENT AFFECTED BY SIDEROBLASTIC ANEMIA
Gang Liu, BS[2], Shanshan Guo, BS[3], Bing Han, MD[4] and Guangjun Nie[1]
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(Presented By: Guangjun Nie)

Introduction: Sideroblastic anemia is characterized by the emergence of ring sideroblasts in the bone marrow. There are two forms of sideroblastic anemia, namely inherited sideroblastic anemia and acquired sideroblastic anemia. Inherited sideroblastic anemia is a heterogeneous disease caused by mutations of genes involved in heme biosynthesis, iron–sulfur (Fe/S) cluster biogenesis, Fe/S cluster transport and mitochondrial metabolism. GLRX5 is a 156–amino acid mitochondrial enzyme that plays an essential role in the synthesis of Fe/S clusters. Deficiency of GLRX5 causes severe microcytic anemia phenotype in zebrafish mutants. However, sideroblastic anemia caused by GLRX5 deficiency is extremely rare in human.
Methods and Materials: In this study, we identified the first two heterozygous missense mutations in GLRX5 gene of a Chinese patient affected by sideroblastic anemia.
Results: Clinical presentation of this patient is more severe than the described case caused by a homozygous silent mutation in the first exon of GLRX5 gene that interferes RNA splicing. Functional analysis of these two mutations is going to present in human erythroid leukemic cell line K562.
Conclusion: Our current study further confirms the role of GLRX5 in human mitochondrial iron metabolism.

Poster #171
RENAL ERYTHROPOIETIN PRODUCTION IS IMPAIRED WITH IRON OVERLOAD AND HEPCIDIN LOSS
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(Presented By: Boualem Moulouel)

Introduction: Erythropoietin (EPO) is produced almost exclusively in the kidney (90% versus 5% in the liver), particularly in interstitial fibroblasts localized at the cortico-medullary junction. EPO synthesis is predominantly regulated by oxygen homeostasis. Of interest, it has been shown that the production of EPO is regulated by iron as well but the mechanism of this regulation remains unclear. Nevertheless, EPO gene transcription is under positive control by the nuclear factor HIF2α, which is increased whenever iron or oxygen is low. Iron homeostasis is controlled by hepcidin, a 25 amino acid peptide that regulates negatively both intestinal absorption and macrophage iron release. Hepcidin is produced predominantly by the liver

and at a lesser extent in the tubular cells of the kidney. In addition, we have recently shown that hepcidin regulates renal handling of iron and that hepcidin knockout mice (hepc-/-) exhibited a marked iron deposition within the thick ascending limb of Henle. In this study we investigated whether EPO synthesis is monitored by the intrarenal hepcidin-dependant regulation of iron. We hypothesized that since the interstitial fibroblasts that produce EPO are immersed in a faintly vascularized environment, their iron content may be modulated by the degree of iron reabsorption in the tubular cells.
Methods: Localization of iron deposits in renal fibroblasts was analyzed by electron microscopy. Blood and iron-related parameters in wild type (WT) and Hepc-/- mice were analyzed under steady state conditions and in response to iron restricted diet (ID; 5 weeks with 5 mg Fe/Kg) or to phlebotomy (300 µl blood loss every 2 days for 10 days). EPO mRNA expression was quantified by RT-qPCR. In vitro studies on renal EPO-producing cells (REPC) were performed to investigate the effect of iron and/or hepcidin.
Results: 1- Electron microscopy analysis showed that significant iron deposits were present in renal interstitial fibroblasts of the kidney medulla in hepc-/- mice. 2- Renal EPO mRNA was reduced in hepc-/- mice as compared to WT (ratio to GAPDH: 0.28 *vs* 0.55 for hepc-/- *vs* control mice respectively, *p<0,02*). ID induced a strong expression of EPO in WT (95 fold increase, p<0,03) but not in hepc-/- mice (2.5 fold p=NS). Similar data were obtained after phlebotomy suggesting that iron deposits in interstitial fibroblasts may impair renal EPO production. 3- Hepcidin-mRNA level in liver was significantly decreased in WT ID mice versus controls (ratio to GAPDH: 0.18 *vs* 1.48 for ID *vs* control mice respectively, *p<0,05*). However in kidney, hepcidin-mRNA expression was slightly increased (ratio to GAPDH: 0.06 *vs* 0,04 for ID *vs* control mice respectively, *p<0,05*), which may probably potentiate iron depletion of interstitial fibroblasts and therefore EPO production. 4- Treatment of REPC cells with the iron chelator DFO increased EPO-mRNA level whereas iron excess (ferric nitrilotriacetate; Fe-NTA) decreased EPO-transcript. However, when REPC cells were incubated with 300 nM hepcidin, EPO synthesis remained unchanged suggesting no direct effect of the hormone on renal fibroblasts.
Conclusion: Our data indicate that hepcidin, by controlling renal tubular iron transport, may exert a feedback control on EPO production. Thus a crosstalk between renal tubular cells and interstitial fibroblasts may be required for an appropriate EPO production in normal and pathological situations.

Poster #172
MUTATION SPECTRUM IN CHINESE PATIENTS AFFECTED BY CONGENITAL SIDEROBLASTIC ANEMIA AND A SEARCH FOR GENOTYPE-PHENOTYPE RELATIONSHIP
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(Presented By: Gang Liu)

Introduction: Sideroblastic anemia is a group of diseases characterized by the emergence of ringed sideroblasts in the bone marrow. There are two forms of sideroblastic anemia, namely congenital sideroblastic anemia (CSA) and acquired sideroblastic anemia. Congenital sideroblastic anemia is a group of heterogeneous diseases caused by mutations of various genes involved in heme biosynthesis, iron–sulfur (Fe-S) cluster biogenesis and transport and mitochondrial metabolism.
Methods and Materials: In this study, we investigated the mutation spectrum in Chinese patients affected by sideroblastic anemia and identified pathogenic mutations in *ALAS2*, *SLC19A2* and *SLC25A38* genes in a cohort of 33 patients. Our results suggested that X-linked sideroblastic anemia (XLSA) represents the most common form in Chinese CSA patients, thus we searched for a genotype-phenotype relationship in 15 patients carrying *ALAS2* mutations.
Results: Our results suggested that the patients with ALAS2 deficiencies close to the binding site or directly involved in the binding of substrate (glycine and succinyl-coenzyme A) or pyridoxal 5'-phosphate (PLP) showed more severe anemia and earlier onset of disease. We have identified four homozygous mutations in *SLC19A2* gene, which is responsible for thiamine-responsive megaloblastic anemia (TRMA). Surprisingly, yet none of them has developed diabetes, a hallmark of TRMA in patients of other ethnic origins.
Conclusion: Three novel mutations in *SLC25A38* gene of two patients were also identified, thus this is the first report of mutations in *SLC25A38* gene in East Asian patients.

Poster #173
FUNCTIONAL ANALYSIS OF FLVCR1 MUTANTS IN POSTERIOR COLUMN ATAXIA AND RETINITIS PIGMENTOSA
Izumi Yanatori, DDS, PhD, Yumiko Yasui, PhD, Koshiro Miura, MD, PhD and Fumio Kishi, MD, PhD
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(Presented By: Izumi Yanatori)

Introduction: The biological roles of heme are diverse and range over most areas of cell metabolism and gene regulation. However, excess heme is toxic, as it promotes oxidative cell membrane damage through lipid peroxidation or DNA damage by generation of reactive oxygen species. Therefore, free cellular heme levels must be tightly regulated to provide an adequate supply, while avoiding heme toxicity. The feline leukemia virus subgroup C receptor 1 (FLVCR1) is a heme exporter that maintains the intracellular heme concentration. FLVCR1 was previously assumed to be involved in Diamond-Blackfan anemia, and it was recently reported that mutations in the FLVCR1 gene are found in patients with posterior column ataxia and retinitis pigmentosa (PCARP).

Methods: Four FLVCR1 mutations in PCARP (Asn121Asp, Cys192Arg, Ala241Thr, and Gly493Arg) are located within putative transmembrane domains. In this study, we made MDCK cells lines expressing FLVCR1 mutants for functional analysis. We analyzed the heme export activity by using a fluorescent heme analog ZnMP and by comparing the sensitivity against heme toxicity.
Results: The results obtained from ZnMP export test and heme toxicity assay showed that all of the four FLVCR1 mutants lost their heme export activity. To investigate the mechanism responsible for this loss of activity, we determined the subcellular localization of FLVCR1 mutants. The mutant molecules did not localize to the plasma membrane, but were observed in intracellular structures, including lysosomes. We examined the half-life of FLVCR1 in the cells, which was 2 – 4h for the mutants compared with >16h for wild-type.
Discussion: We hypothesize that the loss of function of FLVCR1 mutants is caused by their mislocation in the cell. Based on these results, we propose that FLVCR1 mutants failed to fold properly in the ER, were rapidly degraded in the lysosomes, and could not export heme out of the cells. Thus, accumulation of heme in FLVCR1-mutant cells could cause cellular toxicity.

Poster #174
DISTINCT ROLES OF FLVCR1 ISOFORMS IN PRIMITIVE AND DEFINITIVE ERYTHROPOIESIS
Sonia Mercurio, Sara Petrillo, PhD Student, Chiabrando Deborah, PhD, Giulio Valperga, Student, Lorenzo Silengo, Prof., Fiorella Altruda, Prof., Massimo Santoro, Assistant Prof. and Emanuela Tolosano, Prof.
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(Presented By: Sonia Mercurio)

Introduction: Erythropoiesis comes in two waves, primitive and definitive with distinct origin and requirement of specific transcription factors. Flvcr1, a member of the major facilitator superfamily of transporters, is the cell surface receptor for feline leukemia virus, subgroup C. The gene codifies for 2 proteins, Flvcr1a and Flvcr1b expressed at the cell membrane and on mitochondria respectively. Flvcr1a controls heme efflux out of the cell whereas Flvcr1b is involved in mitochondrial heme export.
Methods and Materials: We have already demonstrated that Flvcr1b isoform is fundamental for definitive erythropoiesis whereas Flvcr1a is dispensable (Chiabrando et al., JCI 2012). The aim of this work was to investigate the role of Flvcr1 isoforms in primitive erythropoiesis. To address this issue, we used the Zebrafish model, in which it is possible to down regulate exclusively Flvcr1a or both Flvcr1a and Flvcr1b by using specific antisense morpholino oligonucleotides. Starting from 24 hours post fertilization (hpf) the morphants in which only Flvcr1a was down regulated displayed an impairment of primitive erythropoiesis demonstrated by reduced O-dianisidine staining consistent with a significantly lower heme content compared to control embryos and reduced expression of embryonic globins. Interestingly, the adult globins were overexpressed in these morphants. Primitive erythropoiesis was rescued by injecting Flvcr1a .
Results: Morphants in which both Flvcr1a and Flvcr1b were down regulated showed an impairment of primitive erythropoiesis while the expression of adult globin genes was not affected. In agreement with data obtained in zebrafish, Flvcr1a-null mouse embryos showed a significant reduced expression of embryonic globin genes and of the primitive erythropoiesis specific transcription factor, KLF2 compared to wild-type embryos, at the embryonic stage E11,5 in the yolk sac. On the contrary the adult globin genes were correctly expressed, thus indicating that only the primitive, but not the definitive erythropoiesis was compromised.
Conclusion: Taken together, these data demonstrated a conserved and crucial role for Flvcr1a in the primitive erythropoiesis. The molecular mechanism through which the two Flvcr1 isoforms regulate distinct erythropoietic waves is under investigation.

Poster #175
HEME OXYGENASE 1 EXPRESSION REGULATES THE LEVEL OF HEME AVAILABLE FOR HEMOGLOBINIZATION OF ERYTHROID CELLS
Daniel Garcia-Santos, PhD[1], Matthias Schranzhofer, PhD[1], Monika Horváthova, PhD[2], Mehrad Mojtahed Jaberi Jaberi, Bsc[1], Jose Artur Bogo Chies, PhD[3], Alex Sheftel Sheftel, PhD[4] and Prem Ponka, PhD, MD[1]
[1]Lady Davis Institute for Medical Research, Jewish General Hospital; [2]Department of Biology, Palacky University; [3]Department of Genetics, Federal University of Rio Grande do Sul; [4]Heart Institute, University of Ottawa
(Presented By: Daniel Garcia-Santos)

Introduction: Red blood cells (RBC) are produced at a rate of 2.3 x 106 cells per second by a dynamic and exquisitely regulated process known as erythropoiesis. During this development, RBC precursors synthesize the highest amounts of total organismal heme (75-80%), which is a complex of iron with protoporphyrin IX. Heme is essential for the function of all aerobic cells, but if left unbound to protein, it can promote free radical formation and peroxidation reactions leading to cell damage and tissue injury. Therefore, in order to prevent the accumulation of 'free' heme, it is imperative that cells maintain a balance of heme biosynthesis and catabolism. Physiologically, the only enzyme capable of degrading heme are heme oxyganase 1 & 2 (HO). Red blood cells contain the majority of heme destined for catabolism; this process takes place in splenic and hepatic macrophages following erythrophagocytosis of senescent RBC. Heme oxygenase, in particular its heme-inducible isoform HO1, has been extensively studied in hepatocytes and many other non-erythroid cells. In contrast, virtually nothing is known

about the expression of HO1 in developing RBC. Likewise, it is unknown whether HO1 plays any role in erythroid cell development under physiological or pathophysiological conditions.
Methods and Materials: Using primary erythroid cells isolated from mouse fetal livers (FL), we have shown that HO1 mRNA and protein are expressed in undifferenetiated FL cells and that its levels, somewhat surprisingly, increase during erythropoietin-induced erythroid differentiation. This increase in HO1 can be prevented by succinylacetone (SA), an inhibitor of heme synthesis that blocks 5-aminolevulinic acid dehydratase, the second enzyme in the heme biosynthesis pathway.
Results: Moreover, we have found that downregulation of HO1 via siRNA increases globin protein levels in DMSO-induced murine erythroleukemic (MEL) cells. Similarly, compared to wild type mice, FL cells isolated from HO1 knockout mice (FL/HO1-/-) exhibited increased globin and transferrin receptor levels and a decrease in ferritin levels when induced for differentiation with erythropoietin. Following induction, compared to wild type cells, FL/HO1-/- cells showed increased iron uptake and its incorporation into heme.
Conclusion: We therefore conclude that the normal hemoglobinization rate appears to require HO1. On the other hand, MEL cells engineered to overexpress HO1 displayed reduced globin mRNA and protein levels when induced to differentiate. This finding suggests that HO1 could play a role in some pathophysiological conditions such as unbalanced globin synthesis in thalassemias.

Poster #176
INHIBITION OF ERYTHROPOIESIS BY IRON OVERLOAD IS MEDIATED THROUGH TGFβ SIGNALING
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[1]New York Blood Center; [2]Albert Einstein College of Medicine
(Presented By: Yelena Ginzburg)

Introduction: Several diseases of iron overload and dysfunction erythropoiesis are found in clinical practice. Multiple retrospective studies provide evidence suggesting that iron overload is associated with increased disease burden and shortened survival in patients. It is general consensus that iron overload has an inhibitory effect on erythroid differentiation, but direct evidence is lacking and the mechanisms underlying this phenomenon are incompletely understood. Excess iron is associated with the generation of reactive oxygen species (ROS) that result in cellular damage. We hypothesized that iron overload leads to the stimulation of myelosuppressive pathways that result in decreased erythropoiesis and resulting cytopenias.
Methods and Materials: We first evaluated the effect of exogenous iron on the growth of primary human CD34+ stem cells and demonstrated that iron overload leads to decreased erythroid colony formation in vitro. Furthermore, in our in vivo experiments, we administered a total of 130 mg iron dextran IP over 20 days to C57BL/6 mice and compared these mice with PBS injected age and gender matched controls (n=5/group). We demonstrate that iron injection results in fewer circulating reticulocytes (200 vs. 310 x 109 cells/L; P=0.001) and hemoglobin (13.8 vs. 15 g/dL; P=0.0004).
Results: Using anti-CD44 and TER119 as flow cytometric markers, our data reveals a reduction in bone marrow erythroid burder (total erythroid precursors 9.6 vs. 15.3% in controls; P=0.04) with a disproportionately greater effect on orthochromatophilic erythroblasts (4.4 vs. 6.9%; P=0.02). As expected, bone marrow-derived erythroid precursors from iron loaded mice have more ROS (MFI 1497 vs. 958; P=0.05) as measured by flow cytometry, again with a disproportionately greater effect on orthochromatophilic erythroblasts (MFI 671 vs. 403; P=0.01). No effect on cell cycle was observed. To evaluate the mechanism behind iron induced suppression of erythropoiesis, we conducted a functional screen with variety of cytokine inhibitors. We observed that a specific inhibitor of the TGFβ receptor I kinase led to reversal of iron induced suppression of erythroid colonies from primary CD34+ cells in vitro. The ability of TGFβ kinase inhibitor, LY-215, in reversing iron mediated suppression was confirmed in variety of hematopoietic cell lines. This effect was observed via the inhibition of iron induced apoptosis in these cells.
Conclusion: Taken together, our data demonstrates iron overload has direct suppressive effects on erythropoiesis. These effects are reversed by specific inhibitors of the TGFβ receptor I kinase and thus provide a preclinical rationale for these inhibitors in anemias associated with iron overload.

Poster #177
SOTATERCEPT, AN ACTIVIN RIIA LIGAND TRAP, INCREASES HEMOGLOBIN AND IRON AVAILABILITY IN HEPCIDIN TRANSGENIC MICE
Cindy Roy, PhD[1], Jacqueline Langdon, Alan Berger, PhD, Chris Cheadle, PhD, Victoria Sung, PhD[2] and Qian-Li Xue, PhD
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(Presented By: Cindy Roy)

Introduction: While erythropoietin (Epo) is required for survival of erythroid progenitors, hemoglobin production is largely independent of Epo and highly dependent on iron for heme biosynthesis. Hepcidin antimicrobial peptide (Hamp) is a potent regulator of iron available to the erythron. Over expression of Hamp is common in many types of anemia in humans. Adult mice engineered to over express Hamp (Tg-Hamp) demonstrate iron-restricted erythropoiesis and model some features of the anemia of inflammation. Sotatercept is comprised of the activin receptor type IIA fused to the Fc domain of IgG1 and acts by

trapping activin and other Transforming Growth Factor beta (TGFbeta) ligands. In clinical and non-clinical studies, sotatercept rapidly and dose-dependently stimulated hemoglobin and other red blood cell (RBC) parameters.
Methods and Materials: We investigated the erythroid response to Epo and RAP-011, a "murinized" version of sotatercept, in wild type C57BL/6 mice and Tg-Hamp mice. We assessed hemoglobin response and markers of systemic and erythroid-specific iron utilization.
Results: We found that both agents increased hemoglobin concentration in both genotypes. Epo increased hemoglobin 1.75 ± 0.89 g/dL in C57BL/6 mice ($p<0.001$) and 1.49 ± 0.80 g/dL in Tg-Hamp mice, ($p=0.027$) over 10 days. RAP-011 increased hemoglobin 2.11 ± 0.90 g/dL ($p<0.001$) and 2.67 ± 0.89 g/dL ($p<0.001$) in C57BL/6 and Tg-Hamp mice, respectively. Next, we assessed erythroid progenitor activity. We observed an increase in the percentage of early stage bone marrow erythroid progenitors (Ter+/CD44$^{high-mid}$, populations II, III, and IV) in mice of both genotypes treated with either agent ($p<0.05$). Furthermore, the percentage of committed splenic erythroid progenitors increased from 46 ± 7% in PBS-treated C57BL/6 mice to 67 ± 5% in RAP-treated C57BL/6 mice ($p<0.001$). The percentage of committed splenic erythroid progenitors also increased from 48 ± 6% in PBS-treated Tg-Hamp mice to 69 ± 5% in RAP-011-treated Tg-Hamp mice ($p<0.001$). Next, we assessed erythroid-specific iron utilization. Erythroid progenitors in the bone marrow of Tg-Hamp mice exhibited iron-restricted erythropoiesis, as indicated by increased mean fluorescence intensity of transferrin receptor immunostaining by flow cytometry ($p<0.01$ for Ter+/CD44$^{high-mid}$ populations II-IV). Bone marrow erythroid progenitors from Epo-treated C57BL/6 and Epo-treated Tg-Hamp mice also exhibited iron-restricted erythropoiesis ($p\leq 0.02$ for Ter+/CD44$^{high-mid}$ populations I-IV). In contrast, bone marrow erythroid progenitors from RAP-011-treated C57BL/6 mice did not exhibit iron-restricted erythropoiesis. Bone marrow erythroid progenitors from RAP-011-treated Tg-Hamp mice were predominantly protected from iron-restriction. To assess systemic iron utilization, we measured nonheme spleen iron. Epo treatment of C57BL/6 mice depleted splenic iron stores from 146 ± 41 mcg iron/g tissue to 82 ± 20 mcg iron/g tissue ($p<0.01$); however, splenic iron stores were unchanged in Epo-treated Tg-Hamp mice due to Hamp-mediated sequestration of iron in splenic macrophages. In contrast to Epo, RAP-011 treatment did not deplete splenic iron stores in mice of either genotype.
Conclusion: In conclusion, we have demonstrated that RAP-011 can improve hemoglobin concentration in Tg-Hamp mice. Our data support the hypothesis that RAP-011 has biologic effects, independent of Epo, which allow for sufficient iron acquisition by erythroid precursors. RAP-011 may, therefore, be an appropriate therapeutic for trials in human anemias characterized by increased expression of Hamp and iron-restricted erythropoiesis.

Poster #178
COMPLEX PATHOGENESIS OF ANEMIA IN PREGNANCY
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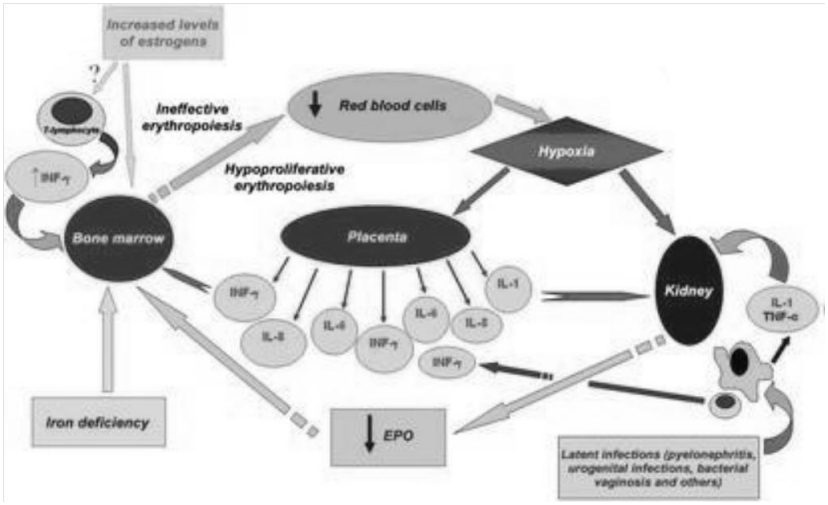
(Presented By: Valeriy Demikhov)

Introduction: It is reputed that pathogenesis of anemia in pregnancy (AP) is relation to simple iron deficiency and iron therapy is the basic treatment of anemic pregnant women all over the world. But own and other researchers data demonstrated that recombinant human erythropoietin (rHuEPO) therapy is very effective for the treatment of AP.
Methods and Materials: We have hypothesized AP has more complex pathogenesis than iron deficiency erythropoiesis, caused by iron deficiency. Erythropoiesis-stimulating agents (ESA) are very effective for the treatment of AP. If AP is iron deficiency anemia then ESA therapy of the anemia has not been such beneficial indeed. To show more complex pathogenesis an anemia in pregnancy we investigated the adequacy of the EPO production for the degree of anemia in pregnant women. A total 268 anemic pregnant women and 24 normal Hb level pregnants were tested. Control group consisted of 22 non-pregnant women with iron-deficiency anemia (IDA). EPO values were measured immunoenzymometrically by using ELISA-EPO kits (IBL, Germany).
Results: Serum levels of some proinflammatory cytokines (IL-8, INF-gamma) and estrogens were determinate in anemic pregnants and in 22 healthy non-pregnant women in addition. Majority anemic pregnant had iron deficiency but showed significantly lower serum EPO levels, than non-pregnant women with IDA. As compared with anemic non pregnants controls, the mean O/E (log EPO) ratio was significantly lower in these anemic pregnant women. But interestingly that inadequately low production of EPO was found in women who had Hb level loss 90 g/dL only. The significant elevated serum levels of IL-8 and INF-γ observed at all pregnant women versus healthy non-pregnant women. Highest INF-γ levels were found in pregnants with IDA as compared with normal iron status anemia. It is very interesting that anemia in pregnancy associated with increased levels of INF-gamma and estrogens only in aggregate, but not separately. We are suppose that development of IDA during second half of pregnancy can lead to hypoxia of placenta which increase production of proinflammatory cytokines (INF-γ). It is known that hyperestrogenemia increase lymphocytic production of INF-γ too. Excess of proinflammatory cytokines (INF-γ) inhibits erythropoiesis in IDA during pregnancy. That is why rHuEPO has high effectiveness in AP. Anemia in pregnants with normal serum ferritin levels may be caused by functional iron deficiency. It's known that increase erythropoiesis is typical feature of second and third trimesters of pregnancy.
Conclusion: In our opinion significant intensification of erythropoiesis during pregnancy may lead to functional iron deficiency and mild anemia in pregnants with low iron stores. At last, moderate anemia during pregnancy can associate with systemic inflammatory response which is typical for second half of normal pregnancy even. Thus, pathogenesis of anemia in pregnancy is complex, including iron deficiency erythropoiesis and hypoproliferative erythropoiesis (Figure). That is why complex

algorithm is necessary to provide optimal guidance in management of anemia in pregnancy. Main therapeutic options are oral iron, intravenously iron and ESA. Most effective therapy of anemia in pregnancy is combined use of ESA and intravenously iron.



Poster #179

LIVER IRON IS THE PRIMARY SIGNAL MODULATING HEPCIDIN EXPRESSION DURING CHRONICALLY ELEVATED ERYTHROPOIESIS

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(Presented By: Elena Gammella)

Introduction: Body iron balance is maintained through the coordination between the erythroid regulator (erythroid precursors are the major sites of iron utilization) and the store regulator (iron accumulation, primarily in the liver and reticuloendothelial system). The hepatic antimicrobial peptide hepcidin (HAMP), that negatively regulates the iron exporter ferroportin (Fpn) to reduce iron absorption and mobilization, is recognized as the main regulator of systemic iron homeostasis. HAMP expression, which is stimulated by iron and inflammation, is repressed in response to hypoxia, anemia, and erythropoiesis, in order to increase iron availability for the bone marrow. However, the signals at the basis of this response are not fully understood.
Methods and Materials: We have evaluated the interplay between the erythroid and the store regulator and investigated the effect of chronic elevated erythropoiesis on systemic iron homeostasis and HAMP expression. To this purpose, we have investigated iron homeostasis in wild type (Wt) animals and in a transgenic mouse line (Tg6) chronically overexpressing erythropoietin (Epo) (12-fold compared to Wt) and presenting a hematocrit ~80%. In addition, to modulate iron levels, Wt (Wt_DXT) and Tg6 (Tg6_DXT) animals were treated with iron dextran, and Tg6 mice were splenectomized to reduce erythropoiesis (Tg6_SPL), and thus iron consumption. HAMP mRNA levels were strongly repressed in Tg6. Iron treatment, which increased HAMP expression in Wt_DXT mice, also enhanced HAMP in Tg6_DXT animals to levels comparable to Wt mice. Moreover, HAMP expression was elevated in Tg6_SPL compared to Tg6.
Results: The variations in HAMP expression were paralleled by changes in the expression of BMP6, an iron-regulated inducer of HAMP transcription, ferritin and liver iron content. As opposed to animal models of β-thalassemia in which the erythroid regulator predominates over the storage regulator to set HAMP levels, these data suggest that liver iron overcomes the Epo signal in HAMP regulation and thus is the primary signal affecting iron homeostasis during chronically elevated erythropoiesis.
Conclusion: Moreover, evaluation of duodenal iron uptake and analysis of proteins involved in intestinal iron absorption, as well as iron deposition, acquisition and export from storage sites (liver, spleen, muscle) indicated that changes in duodenal Fpn, together with iron mobilization, were the major variations in iron homeostasis adopted by Tg6 mice to sustain such a high erythropoietic rate.

Poster #180

THE EFFECT OF BONE MARROW TRANSPLANTATION TO IRRADIATED MICE ON HEPCIDIN EXPRESSION

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(Presented By: Martin Vokurka)

Introduction: Hepcidin is a key regulator of systemic iron metabolism. It inhibits iron intestinal absorption and iron release from macrophages and thus prevents iron overload. However, hepcidin high concentration impairs iron delivery to the body and to the cells including red blood cell precursors in the bone marrow. Conversely, erythropoietin (EPO) and enhanced erythropoiesis belong to the crucial negative regulators of hepcidin expression. While the regulatory pathways of iron and inflammation have been described, the downregulation due to erythropoietic drive in normal and stress erythropoiesis has not been so far fully elucidated.
Methods and Materials: Transferrin saturation, soluble molecules like GDF15 and TWSG1 or direct effect of erythropoietin on hepatocyte have been proposed as possible regulators but some of them play role mainly in ineffective erythropoiesis in hematological diseases like thalassemia. To study the possible role of immature red blood cells in hepcidin regulation we transplanted syngeneic EPO-stimulated or normal bone marrow (BM) to sublethally irradiated mice. EPO (50 U) or saline were administered to mouse donors on four consecutive days and the bone marrow activation was confirmed by flow cytometry as well as by the increase of hemoglobin and decrease of hepcidin expression. Recipient mice were sublethally irradiated (6 Grays 60Co) two days prior to the transplantation of bone marrow cells (BMT) equivalent to two femurs. The mice were sacrificed 2, 6 and 24 hours after the BMT. Hematological parameters were determined and the livers were analyzed for iron content, expression of hepcidin and other iron-related genes by real-time PCR. The pSMADs were determined by Western blots.
Results: Irradiation resulted in the anemia in mice and in the increase of hepcidin expression as expected from our previous experiments. However, transplantation of both normal or Epo-stimulated BM to irradiated mice had no substantial effect on hepcidin expression in the studied intervals. This can be due to the fact that the bone marrow proliferation is needed to downregulate hepcidin while the presence of immature bone marrow erythroid precursors themselves had no effect or their amount was not sufficient. However, they represented a substantial part of the bone marrow cells present in mice recovering from sublethal irradiation. Irradiation is an inflammatory stimulus that can prevail over the erythropoietic stimuli. Levels of pSMADs decreased after EPO administration and slightly increased in 2 hour interval after irradiaton compared to the controls and 24 hour interval but no effect of BMT was observed.
Conclusion: In conclusion, hepcidin expression did not significantly change in irradiated mice after the BMT in the immediate posttransplantational intervals.
This work was supported by grants PRVOUK-P24/LF1/3, UNCE 204021 and SVV 264507/2012.

Poster #181

GENE COMPARISON STUDIES OF ERYTHROBLASTS DERIVED FROM DIFFERENT HAEMATOPOIETIC SOURCES

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(Presented By: Alison Merryweather-Clarke)

Introduction: There is a clinical need for alternative novel sources of red blood cells (RBCs), especially for diagnostic use, to provide panels of rare blood groups for use in antibody testing. Studying and manipulating in vitro models of erythropoiesis holds the prospect of developing methods to produce RBCs for diagnostic use. We have previously identified many novel patterns of gene expression during erythropoiesis using discrete populations representing four different stages of erythropoiesis.
Methods and Materials: We are now examining changes in gene expression in finer detail during erythropoiesis from different sources under different culture conditions. Previously published erythroid culture regimes include stromal cells (SCs) or foetal bovine serum (FBS). The latter has been shown to affect globin switching, promoting foetal globin expression in adult erythoid cultures. Prior to transcriptome analysis, we have established a modified erythroid culture regime in the absence of SCs and FBS. Erythroblast (EB) populations cultured from haematopoietic cells in cord blood (CB), adult peripheral blood (PB) and induced pluripotent stem cells (iPSC) have been collected.
Results: Erythroid precursors generated from fibroblast-derived iPSC express antigens found on mature RBCs. However the expansion, morphology and enucleation rates of CB- or PB-derived red cells are superior to red cells derived from iPSC grown under the same erythroid-promoting conditions. To identify the underlying mechanisms, we have undertaken a global approach to compare the transcriptome of EBs cultured from different sources. With these methods we aim to identify cellular processes absent in iPSC-derived EBs that are required for later stages of erythroid development.
Conclusion: These observations will be used to improve red cell production using iPSC generated from donors with rare red cell antigen phenotypes.
Funding: NIHR RP-PG-0310-1004

Poster #182
ANEMIA, HEMATOPOIESIS AND IRON METABOLISM IN TELEOST FISH
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(Presented By: João Neves)

Background: Anemia is one of the most common disorders of the blood, resulting from an abnormally low level of red blood cells or hemoglobin, mostly due to blood loss, excessive hemolysis or impaired erythropoiesis. Although there is a significant amount of information on the mechanisms of anemia development and the genes involved in hematopoiesis, it is mostly focused on mammals and there are still several gaps in the understanding of these processes in lower vertebrates, particularly fish.
Aims: The goal of this study was to clarify the link between anemia, the mechanisms of hematopoiesis and the regulation of iron metabolism in lower vertebrates, namely in teleost fish.
Results: Two different levels of experimental anemia were induced in healthy European sea bass (*Dicentrarchus labrax*), by withdrawal of 1% (low anemia) or 2% (high anemia) v/w of blood through the caudal vessels. The progress of both anaemias was then monitored after 1, 4, 7 and 14 days. Several blood parameters were measured and found to be reduced, namely RBC, hematocrit, serum iron and transferrin saturation. Liver iron content was also evaluated and found to be decreased in the higher anemia group. Expression of several genes, including genes involved in iron homeostasis and regulation (*hamp*, *tmprss6*, *hjv*, *bmp6*, *bmpr2*, *smad4*, *smad1/5/8*, *tfr1* and *tfr2*), hematopoiesis (*epo*, *epor*, *hgβ* and *gata2*), hypoxia (*hif1α*), iron uptake (*scl11a2α* and *slc11a2β*), iron storage (*fth*), iron export (*fpn*) and iron transport (*tf*), was evaluated by real-time PCR, in the liver, spleen, head kidney and intestine. Regarding the genes involved in iron metabolism, expression results show an overall down-regulation of hepcidin, as well as of its upstream regulators, with a subsequent increase in iron export by the liver (in line with the decreased liver iron levels), increased mobilization by transferrin, as well as increased iron uptake and release by the intestine. On the hematopoietic side, significant increases of *epo*, *epor*, *hgβ* and *tfr* were observed in the kidney and spleen, the main erythropoietic organs in teleost fish. A significant up-regulation of *epo*, but not of its receptors, was also observed in the liver.
Conclusions: Several of the players involved in response to anemia in mammals are also present in fish, with the head kidney and spleen apparently assuming a dominant role in hematopoiesis. However, variations in gene expression in the liver suggest that this organ may also be contributing to an increased epo-mediated signaling, leading to enhanced erythropoiesis by the kidney and spleen, somewhat reminiscent of the function of the mammalian liver. Expression changes in key regulators of the iron metabolism point towards an increase in iron absorption and release from storage, directing iron to the hematopoietic organs. Overall, the mechanisms of hematopoiesis seem to be conserved in sea bass, but the role of the liver still needs to be clarified.
Acknowledgements: FCT and COMPETE/QREN/UE project PTDC/CVT/100386/2008 for financial support.

Poster #183
IRP1-/- MICE EXHIBIT AN ABNORMAL ERYTHROPOIETIC PHENOTYPE
Nicole Wilkinson and Kostas Pantopoulos, PhD
Lady Davis Institute for Medical Research and McGill University
(Presented By: Nicole Wilkinson)

Introduction: Iron Regulatory Proteins, IRP1 and IRP2, are post-transcriptional regulators of cellular iron metabolism that control the translation or stability of several Iron Responsive Element (IRE)-containing mRNAs. While IRP2-/- mice misregulate iron homeostasis in tissues and develop microcytosis, IRP1-/- counterparts have not been reported to manifest any overt phenotypic abnormalities.
Methods and Results: We found that IRP1-/- mice have increased erythropoietin (EPO) mRNA levels in the kidneys, as compared to wild type littermates. Consistently, serum Epo levels of IRP1-/- mice were also increased ~4.8-fold higher ($p<0.01$). IRP1-/- mice exhibit splenomegly, associated with extramedullary hematopoiesis. FACS analysis of TER119 and CD71 demonstrate increased erythroid progenitor cells in the spleen without increases in terminally differentiated red blood cells (RBC). FACS analysis (thiazole orange) and blood smear staining confirms that serum reticulocytes are increased in IRP1-/- mice. This data uncovers previously unnoticed erythropoietic defects in IRP1-/- mice, which are apparently triggered by the absence of IRP1. EPO mRNA is transcriptionally regulated by Hypoxia Inducible Factor 2 alpha (HIF2α or EPAS1), a relatively recently discovered target of the IRE/IRP regulatory network.
Conclusion: We provide evidence that the abnormal erythropoietic phenotype of IRP1-/- mice is caused by misregulation of HIF2α expression. Our data suggest that HIF2α mRNA translation is preferentially regulated by IRP1 *in vivo*.

Poster #184

EDX-17, A SAFE PHYTOHORMONE THAT PROMISES A PHENOTYPIC CURE FOR SICKLE CELL DISEASE AND A BLOCKING TREATMENT FOR MALARIA – IN VIVO PROOF OF PRINCIPLE IN ANIMALS

Robert Broyles, PhD[1],[2],[3], Austin Roth, BA[1],[2], Robert Floyd, PhD[1],[2],[4] and Sunil Joshi, PhD, DVM[1],[5]
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(Presented By: Robert Broyles)

Objective: To test the phenotypic cure we have discovered for SCD and malaria in vivo in two transgenic mouse models.
Background: Gene regulation of developmental hemoglobin switching provides phenotypic cures for sickle cell disease and malaria since it is known that reactivation of fetal globin expression (HbF) alleviates these disorders. We discovered a protein that regulates this developmental switch. Ferritin heavy chain (FtH) represses adult beta-globin and activates gamma(fetal)-globin gene expression in embryonic/K562 erythroid cells (Broyles et al., PNAS 98: 9145, 2001; US Patents #7,517,669, #7,718,669, & #2009/0232783 A1; EU Patent # EP1354032B1; Australia patent #2002217964), leading us to propose FtH as a therapeutic agent. We have recently discovered a plant compound, EdX-17, that activates the human ferritin-H gene and shows promise as an oral agent for inducing HbF.
Methods: Normal C57BL/6 mice, transgenics carrying the complete human beta-globin gene cluster (Beta-YAC Tg), and transgenic sickle cell (Townes) mice that express only human globin genes with the adult beta-gene being the S-mutant, have been used under IACUC-approved protocols to show the safety and efficacy of human FtH and our phytotherapeutic EdX-17 in vivo. EdX-17 has been administered by either i.v. injection or orally in drinking water. Hemoglobins were identified and quantified by HPLC; fetal Hb was confirmed by immunofluorescence.
Results and Discussion: Initial results with normal mice and Beta-YacTg mice show that FtH protein and our phytotherapeutic EdX-17 are both well tolerated in vivo. We have used numbers of target cells (found in the FtH transgenics) as an initial screen for determining an effective dosing regimens. I.v. as well as oral adminstration of EdX-17 to beta-YAC Tg mice results in elevated human fetal globin, such that fetal globin is approximately 30% (oral) to 50% (i.v.) of human globin expression in these mice, levels which correspond to therapeutic targets in human that are known to completely suppress the SCD phenotype as well as block the chronic RBC-phase of malaria. Initial results with sickle cell transgenic mice confirm the safety and efficacy of EdX-17 and indicate response similar to beta-YAC mice. Townes mice will be followed to see how well EdX-17 prevents the expression of the SCD pathology as these mice age.
Supported in part by donations to The Sickle Cell Cure Foundation, by a Grand Challenges in Global Health grant from the Bill & Melinda Gates Foundation, and funding through EpimedX, LLC. We thank Marie Trudel of the IRCM for the Beta-YAC Tg mice, and Dr. Gary White and colleagues for excellent animal care.

Poster #185

ERYTHROPOIESIS AND IRON HOMEOSTASIS IN MICE AND HUMANS WITH MUTATED DMT1

Zuzana Zidova, Katarina Kapralova, Dalibor Dolezal, Pavla Luzna, Vladimir Divoky and Monika Horvathova
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(Presented By: Zuzana Zidova)

Introduction: Divalent metal transporter 1 (DMT1; also known as NRAMP2 and SLC11A2) is an intestinal and endosomal iron (Fe2+) transporter. Homozygous or compound heterozygous mutations in DMT1 are associated with moderate to severe hypochromic microcytic anemia in human patients and a mouse model - *mk/mk* mice.
Methods and Materials: We showed that in contrast to the original *mk/mk* mice (MK/ReJ) increased serum iron is present not only in the DMT1-mutant patients but also in a 129S6/SvEvTac strain of *mk/mk* mice. Reduced hepcidin levels detected in DMT1-mutant patients and *mk/mk* mice confirm predominant effect of accelerated erythropoiesis on hepcidin regulation. The negative effect of DMT1 deficiency on the erythroid lineage is documented by the abnormal morphology and reduced number of 129S6/SvEvTac-*mk/mk* erythroid colonies in comparison to colonies of wild-type mice, by increased apoptosis of *mk/mk* erythroid precursors, and by accelerated *in vivo* clearance of fluorescently labeled 129S6/SvEvTac-*mk/mk* erythrocytes when compared to wild-type erythrocytes.
Results: In parallel with the patients' data, high doses of erythropoietin improved anemia of 129S6/SvEvTac-*mk/mk* mice. On the other hand hepatic iron overload documented in some DMT1-mutant patients distinguishes them from 129S6/SvEvTac-*mk/mk* mice. In order to address whether it may reflect increased intestinal iron (heme or non-heme) absorption in the human subjects, mice were fed iron-rich and heme iron containing diet. Our preliminary results show that this modification of the diet has no impact on the degree of anemia, but increases serum iron levels in both 129S6/SvEvTac-*mk/mk* mice and wild-type littermates. Concomitantly tissue iron stores markedly increase in wild-type mice; however the tissue non-heme iron content in 129S6/SvEvTac-*mk/mk* mice seems to be unchanged.
Conclusion: Further experiments are undergoing to clearly define the impact of iron-rich and heme iron diet on iron homeostasis in mice with mutated DMT1.
Grant support: Czech Grant Agency, grants No. P305/10/P210 and P305/11/1745; Internal Grant of Palacky University Olomouc (LF_2012_016).

Poster #186

HOLOTRANSFERRIN REGULATES TRANSFERRIN RECEPTOR 2 IN THE ERYTHROID LINEAGE

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(Presented By: Maud Vieillevoye)

Introduction: Transferrin receptor 2 (TfR2) is a type II membrane glycoprotein. TfR2 is formed of a large extracellular domain which contains 2 cysteines that allow the formation of homodimers. TfR2 has a unique transmembrane domain and a short cytoplasmic tail that encloses an YQRV motif which is important for receptor internalization. TfR2 protein expression is restricted to the hepatic, erythroid and enterocytic lineage and its role seems to be lineage−dependent. In erythroblasts, TfR2 interacts with the erythropoietin receptor (EpoR) and is required for efficient erythropoiesis (Forejtnikova et al. Blood 2010).
Methods and Materials: We determined the interaction domains between the two receptors using EpoR/ Prolactin Receptor chimeric constructs. Co-immunoprecipitation experiments demonstrate that the extracellular part of both receptors is interacting. We compared TfR2 protein expression in the hepatic and in the erythroid lineages by western blotting.
Results: TfR2 appears to be expressed as two 92 kDa and 102 kDa isoforms in the erythroleukemic UT−7 cell line, in human primary erythroblasts as well as in human primary hepatocytes. We demonstrated that the 92 kDa isoform is due to a short deletion of the intracellular part of TfR2 and is able to heterodimerize with the 102 kDa isoform. Like in hepatocytes, TfR2 is stabilized by holotransferrin in the erythroid lineage at the post−transcriptional level. However, EpoR expression is not modified by transferrin saturation, thereby indicating that the entire pool of TfR2 is not associated with EpoR and can be modulated independently of EpoR expression.
Conclusion: In conclusion our results show that TFR2 and EpoR interact through their extracellular domains, and that TfR2 is expressed under 2 different forms in the erythroid lineage; the specific role of the membrane 92 and 102 kDa isoforms is currently under investigation.

Poster #187

ABO AS POTENTIAL NOVEL LOCUS FOR HAEMATOLGICAL TRAITS IN EUROPEANS

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(Presented By: Stela McLachlan)

Introduction: Genome wide association studies have identified several loci associated with haematological traits in Europeans, among them most notably *HFE*, *HBS1L/MYB* and *TMPRSS6* for haemoglobin (Hb), haematocrit (HCT), mean corpuscular haemoglobin (MCH), mean corpuscular volume (MCV), and red blood cell count (RBC). *ABO* has been reported as a genetic factor associated with haematological traits in the Korean population, but there have been no previous reports of its association in Europeans.
Methods and Materials: The UCL-LSHTM-Edinburgh-Bristol (UCLEB) Consortium has been established to allow interrogation of genetic associations using the Metabochip by pooling individual-level data on a number of carefully harmonised phenotypes. The Metabochip is a custom Illumina iSelect genotyping array designed to test~200,000 SNPs of interest for metabolic and atherosclerotic/cardiovascular disease traits. The consortium consists of 12 well-established prospective observational studies comprising over 30,000 participants, of which 21,000 are genotyped with Metabochip. For five haematological traits, Hb, HCT, MCH, MCV and RBC, number of participants with both genetic and phenotypic information varied from 4,348 in 2 studies for MCH to 10,980 in 6 studies. Quality control of genotypes was done on study-by-study basis using the same parameters (sample and SNP call rate>0,95, HWE>10-4, IBD>0.4). Analyses were restricted to White/European groups.
Results: Association analysis was performed using linear regression, with age and sex as covariates, in each study separately and then results were combined by meta-analysis. Beta estimates with p-values below 1x10-5 were considered suggestive, and below 1x10-7 were considered significant. Association analysis confirmed previously identified loci with effects on haematological traits: *HFE* was significantly associated with Hb, MCH and MCV, *HBS1L/MYB* was significantly associated MCH, MCV and RBC,*TMPRSS6* was significantly associated with MCH and MCV. Additionally, Hb and HCT showed suggestive association with *ABO* locus (p= 5.23x10-7 and p= 9.77x10-7 at the most significant SNP rs507666, respectively). *ABO* locus has been associated with a wide range of traits and diseases varying from cholesterol, inflammatory markers and liver enzyme levels to malaria, duodenal ulcer and coronary heart disease in individuals of European, Asian and African American ancestry. It was also recently reported to be associated with haematological traits (RBC and MCV) in a Korean population, which are frequently used in practice as markers of haematological disorders and general health. The mechanisms by which *ABO* influences these traits are still not clear. Some possibilities include through the relationship between ABO blood phenotype and cholesterol levels (with type A associated with higher levels of total cholesterol and LDL-cholesterol compared to other types), through a role in clotting (associated with O type) and links to inflammation. These factors play a significant role in the development of some diseases, particularly cardiovascular disease. However, further studies are necessary to robustly confirm the role of *ABO* as a risk factor in the development of different diseases and reveal underlying mechanism of action.
Conclusion: In conclusion, *ABO* is a potential novel locus influencing haemoglobin and haematocrit levels in Europeans.

Poster #188

NUCLEAR FERRITIN: FROM DISCOVERY TO PROMISING TREATMENTS TO STOP SICKLE CELL DISEASE AND MALARIA

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(Presented By: Austin Roth)

Introduction: Approximately 37 years ago, a group of people in Saudi Arabia were discovered that have homozygous sickle cell but no disease symptoms; it was found that these people have up 45% fetal hemoglobin (HbF) as adults, suggesting that elevation of HbF in adults can block expression of sickle cell disease, SCD (Arch Dis Childhood 56:187-192, 1981). Subsequent laboratory tests have confirmed that 25-30% HbF will prevent sickling and all manifestations of SCD, and that HbF in any amount is not harmful in adults (Blood 120: 2945-2953, 2012). It has also been shown that 30% HbF blocks the chronic red blood cell phase of the malaria life cycle, reducing malaria to a transient, self-limiting event in humans (PloS ONE 6(4):e14798, April 2011; British J Haematology 141:276-286, 2008). The story told below relates how we discovered that a nuclear form of ferritin heavy chain, FtH, can induce HbF in adults, and how, in turn, a plant-derived compound that can be delivered orally and is inexpensive to manufacture induces HbF via its induction of FtH.
Methods and Results: Nuclear ferritin (FtH) was discovered almost 20 years ago in nuclear extracts of K562 cells (Colloque INSERM 234, 43-51,1995) and found to repress adult hemoglobin (Hb) beta-chain expression in co-transfection experiments by specific binding to the beta-globin promoter (PNAS 98: 9145-9150, 2001). Other results had shown that FtH clones up-regulate fetal/gamma globin expression in co-transfections (JBC 266: 17566–17572, 1991). On the basis of these results and early papers from Elizabeth Theil's group that showed that embryonic/fetal red cells contain high amounts of ferritin and FtH whereas maturing adult red cells are virtually devoid of ferritin, we postulated that FtH is developmentally regulated in erythroid cells and is the long-sought developmental Hb-switching factor. The beta-promoter binding motif for FtH is also found in the gamma-globin promoter, but in different contexts. An antisense to FtH knocks down FtH 90% in K562 cells, relieves betarepression, and knocks down gamma expression 90%, confirming that FtH is an activator of gamma-globin. The ability of FtH to mediate a switch from adult Hb back to fetal Hb has been confirmed in cultured red blood cell precursors from sickle cell disease (SCD) patients (Blood 108: 790a, 2006). We have recently found that a phytohormone reported to induce a plant ferritin (Eur J Biochem 231:609-619,1995; Biochem J 359:575-582, 2001) induces FtH expression in human cells. This compound, which we call EdX-17, is a strong inducer of HbF in human SCD-patient erythroid precursors as well as in transgenic mice carrying human globin genes.
Conclusion: Recent experiments confirm the strong safety record of EdX-17 and show that this compound can be delivered orally as an effective treatment for SCD and, most probably as a preventative for malaria.
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Poster #189

CONTRASTING RELATIONSHIPS OF SERUM NON-TRANSFERRIN BOUND IRON AND TRANSFERRIN SATURATION TO IRON OVERLOAD, INEFFECTIVE ERYTHROPOIESIS AND SERUM HEPCIDIN IN SICKLE CELL AND HALASSAEMIA DISORDERS

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(Presented By: Patricia Evans)

Background: Serum Non-Transferrin Bound Iron (NTBI) is implicated in iron accumulation in tissues susceptible to iron overload. NTBI is low in sickle cell disease (SCD) relative to thalassaemias and may account for the low propensity to extra-hepatic iron distribution in multi-transfused SCD (1). We have previously hypothesized that because of the chronic inflammatory state in SCD, high levels of serum hepcidin relative to thalassaemia disorders account for this difference. Here we examine how NTBI and transferrin saturation differ between Sickle Cell Disease (SCD) and both transfusion-dependent thalassaemia (TM) and non-transfusion-dependent thalassaemia (NTDT) in relation to iron load, serum hepcidin and to markers of ineffective erythropoiesis (IE) or hypoxia. A key objective of this work was to understand the dominant factors that determine NTBI levels in SCD versus thalassaemia disorders.
Patients and Methods: Sickle cell disease patients (SCD, n=67) had all received blood transfusions. Thalassaemia patients included both transfusion dependent Thal Major patients (TM, n=47) and non-transfusion dependent thalassaemia patients, (NTDT, n=28) that were analysed both together (Thal) and separately. All patients were attending followup at University College Hospital London and gave written consent to blood sampling. Blood samples were drawn pre-transfusion for those receiving ongoing transfusion therapy. All SCD patients were in steady state and all patients withheld chelation for at least 48h

prior to sampling. Serum hepcidin (2) and serum NTBI (3) were measured as previously described. Serum erythropoietin (Epo), soluble transferrin receptor (sTr) and GDF15 were measured by ELISA.
Results: NTBI and transferrin saturation were significantly higher in Thal than SCD when matched for iron load and correlated with LIC and serum ferritin (SF) in both SCD and Thal. NTBI levels correlated with markers of ineffective erythropoiesis (sTr and GDF-15) and hypoxia (Epo) in NTDT where these markers were most elevated, but not in SCD. When serum hepcidin was corrected for iron overload (hepcidin/ferritin ratio), this was similar in SCD, TM and NTDT. This hepcidin/ferritin ratio correlated strongly with NTBI for both SCD and Thal but NTBI levels were higher in Thal for any given hepcidin/ferritin ratio. Serum hepcidin showed significant and similar relationships to SF (positive), or to ineffective erythropoiesis and hypoxia markers (inverse) in SCD and Thal.
Conclusions: In both SCD and Thal, NTBI levels are increased by iron overload, ineffective erythropoiesis and hypoxia, and fall with increasing hepcidin/ferritin ratios. However in Thal, particularly NTDT, high levels of ineffective erythropoiesis, relatively lacking in SCD, are an additional factor associated with NTBI increments. Thus increased ineffective erythropoiesis in Thal, especially in NTDT, rather than increased hepcidin in SCD, appears to be the dominant factor accounting for lower NTBI and Tf saturation in SCD relative to Thal.

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Poster #190

HEMOPEXIN THERAPY IMPROVES CARDIOVASCULAR FUNCTION IN MOUSE MODELS OF HEMOLYTIC DISORDERS BY CHELATING PLASMA FREE HEME AND REDISTRIBUTING HEME-IRON TO THE LIVER

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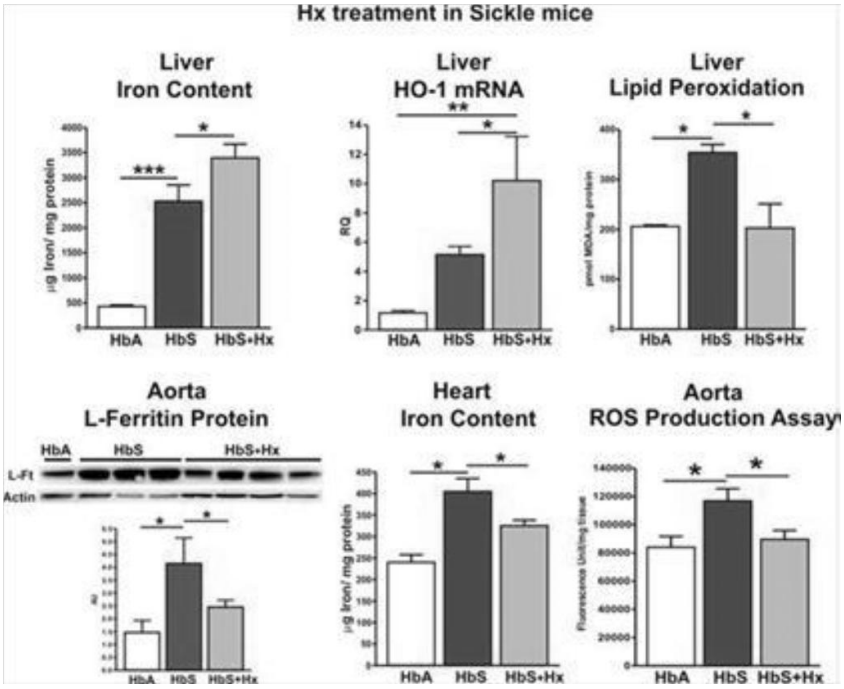
(Presented By: Francesca Vinchi)

Background: Hemolytic diseases are characterized by enhanced intravascular hemolysis resulting in heme-catalyzed reactive oxygen species (ROS) generation, that leads to endothelial dysfunction and tissue oxidative damage. Hemopexin (Hx) is a potent plasma heme scavenger able to prevent endothelial damage and tissue congestion in a model of heme overload. In vivo studies on wild-type and Hx-null mice showed that Hemopexin favours hepatic heme metabolism by enhancing HO-1 expression/activity in hepatocytes and promoting iron storage in Ferritins. This results in heme-iron accumulation in the liver and in the protection of the endothelium from heme-iron loading. Here, we tested whether Hx could be used as a therapeutical tool to counteract heme toxic effects on the cardiovascular system in hemolytic diseases.
Methods and Results: Sickle Cell Disease (SCD) and β-thalassemia mice were employed as models of hemolytic diseases and treated with purified human Hx twice a week for 1 month. We demonstrated that in hemolytic mice Hx administration (i) promoted heme recovery and detoxification by the liver mainly through the induction of Heme Oxygenase (HO) mRNA/activity; (ii) prevented heme-iron loading in endothelial and heart cells, thus limiting the induction of HO-1 and adhesion molecules, the production of ROS and the oxidative inactivation of NOS/NO; (iii) counteracts heme-driven oxidative stress and inflammation, thus preventing tissue damage. Finally, we observed that in SCD mice Hx therapy reduced blood pressure and restored cardiac output, highlighting a key role for Hx in the protection against heme-induced cardiovascular dysfunction.
Conclusions. Here, we demonstrated that Hemopexin administration replenishes the endogenous Hx pool and chelates heme-iron, thus reducing circulating free heme, and promotes heme-iron recovery to the liver in anemic animals. This resulted in heme-iron redistribution from extra-hepatic tissues to the liver and in a significant enhancement of the liver capacity to detoxify heme excess. This redistribution accounts for a significant reduction in heme-mediated oxidative injury in the liver, vascular endothelium, kidney and heart and a marked improvement of the health status of both sickle and β-thalassemic mice. Here, Hemopexin treatment is proposed as a promising novel heme chelation therapy to deliver heme-iron to the liver and counteract heme-driven oxidative damage and inflammation, leading to vasculopathy in hemolytic disorders.



Poster #191

CERULOPLASMIN OXIDATION, A FEATURE OF PARKINSON´S AND ALZHEIMER'S DISEASES CSF, INHIBITS FERROXIDASE ACTIVITY AND PROMOTES CELLULAR IRON RETENTION

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Ospedale San Raffaele
(Presented By: Marco Barbariga)

Introduction: Parkinson´s (PD) and Alzheimer's (AD) are diseases characterized by oxidative stress and deposition of toxic ferrous iron (Fe++) in specific areas of the central nervous system. Ceruloplasmin (Cp) is an extracellular ferroxidase that regulates cellular iron loading and export, and protects tissues from oxidative damage.
Methods and Materials: With 2D-electorphoresis technique we found that Cp profile was modified in the CSF of PD and AD patients, showing a shift to a more acidic isoform. This shift allowed us to significantly discriminate PD patients versus AD patients and versus healthy subjects.
Results: We then investigated the cause of the modification and the effects of this changes on Cp function. Using an in vitro hydrogen peroxide treatment we simulated the oxidative environment present in the patients and we discovered that CSF oxidation induced, in 2D electrophoresis, a Cp shift resembling that observed in PD and AD, and co-occurred with an increase in protein carbonylation. Moreover, increased protein carbonylation was observed in Parkinson´s disease patients CSF. Cp enzimatic activity analysis showed that Cp oxidation caused a decrease in its ferroxidasic activity. Finally, cell cultures were used to investigate the role of Cp in the cellular iron export: we demonstrated that, as consequence of protein oxidation, Cp loss of ferroxidase activity promotes intracellular iron retention in neuronal cell lines and primary neurons.
Conclusion: Concluding, Cp oxidation is responsible for the modification observed in PD's and AD's CSF, and might be used as a marker for oxidative damage. Functional impairment and cellular iron retention might provide new insights into the underlying pathological mechanisms. Among oxidative modifications, the asparagine deamidation is of interest because Cp's aminoacidsequence contains motifs that upon deamidation may acquire binding properties to membrane receptors. If Cp deamidation occurs in the oxidative milieu of the neurodegenerative disease, it's conceivable that out of to lose its physiological function, Cp might gains new pro-adhesive function.

Poster #192

A LOW MOLECULAR WEIGHT FERROXIDASE IS INCREASED IN THE CSF OF SCJD CASES: CSF FERROXIDASE AND TRANSFERRIN AS DIAGNOSTIC BIOMARKERS FOR SCJD

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(Presented By: Neena Singh)

Aims: Most biomarkers used for the pre-mortem diagnosis of sporadic Creutzfeldt-Jakob disease (CJD) are surrogate in nature, and provide suboptimal sensitivity and specificity.
Results: We report that CJD-associated brain iron dyshomeostasis is reflected in the cerebrospinal-fluid (CSF), providing disease-specific diagnostic biomarkers. Analysis of 290 pre-mortem CSF samples from confirmed cases of CJD, Alzheimer's disease, and other dementias, and 52 non-dementia controls revealed a significant difference in ferroxidase (Frx) activity and transferrin (Tf) levels in sCJD relative to other dementia and non-dementia controls. A combination of CSF Frx and Tf discriminated sCJD from other dementias with a sensitivity of 86.8%, specificity of 92.5%, accuracy of 88.9%, and area under the receiver-operatingcharacteristic (ROC) curve of 0.94. This combination provided a similar diagnostic accuracy in discriminating CJD from rapidly-progressing cases that died within 6 months of sample collection. Surprisingly, ceruloplasmin and amyloid-precursor-protein, major brain ferroxidases, displayed minimal activity in the CSF. Most of the Frx activity was concentrated in <3 kDa fraction in normal and disease CSF, and resisted heat and proteinase-K treatment.
Discussion: 1) A combination of CSF Frx and Tf provide disease-specific pre-mortem diagnostic biomarkers for sCJD. 2) A novel, non-protein Frx predominates in human CSF that is distinct from the currently known CSF ferroxidases.
Conclusion: The underlying cause of iron imbalance is distinct in sCJD relative to other dementias associated with brain iron imbalance. Thus, change in CSF levels of iron-management proteins can provide disease-specific biomarkers and insight into the cause of iron-imbalance in neurodegenerative conditions.

Poster #193

IRON: KEY TO INDIVIDUAL DIFFERENCES IN SUSCEPTIBILITY TO PESTICIDE NEUROTOXICITY?

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(Presented By: Byron Jones)

Introduction: Chemical pesticides, especially those applied in agriculture, are suspected to be risk factors for neurodegenerative diseases such as Parkinson's disease. Epidemiological studies, however, report inconsistencies in rates of relative risk for those exposed to pesticides such as Rotenone, Maneb. and Paraquat (PQ). PQ is an herbicide used world-wide, except for the European Union. In addition to its herbicidal effects, PQ is highly toxic to mammalian lung, and in animals PQ has been shown to be neurotoxic. A major target of PQ neurotoxicity is the substantia nigra pars compacta (SNc), and this region's population of dopaminergic neurons. Degeneration of these cells with subsequent loss of dopamine in the caudate-putamen is the primary pathophysiological feature of idiopathic Parkinson's disease.
Methods and Materials: We recently showed that differential susceptibility to PQ neurotoxicity in four inbred mouse strains from the family of BXD recombinant inbred strains is associated with the increase in iron concentration in the ventral midbrain – the area containing both the SNc and ventral tegmentum. In comparing the effect of PQ on gene expression in the ventral midbrain, one PQ-susceptible strain showed changes in more than 300 transcripts whereas one resistant strain showed changes in fewer than one dozen transcripts.
Results: In the sensitive strain, the preponderance of genes with altered expression is iron binding protein genes; whereas in the resistant strain, the few altered genes are related to intermediate metabolisms. While we have shown the likely involvement of iron in PQ-related dopamine neurotoxicity, the question remains whether the relationship between iron and PQ is unique to PQ. We also studied genetic differences among 10 BXD recombinant inbred mouse strains to MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), a proneurotoxicant (the active agent is the metabolite, MPP+ produced in astrocytes) used to model the pathophysiology of Parkinson's disease. Again, we detected large genetic differences in neurotoxicity produced by this agent. In the strain most susceptible to MPTP toxicity we observed a significant increase in iron concentration in the ventral midbrain.
Conclusion: Our research is not the first to show a relationship between PQ and iron in neurotoxicity. It is the first however, to show that individual differences in susceptibility to PQ and probably other neurotoxicants are related to genetic differences in toxicant-based influx of iron into the ventral midbrain, especially in the SNc.

Poster #194

A NOVEL MODEL FOR BRAIN IRON UPTAKE: INTRODUCING THE CONCEPT OF REGULATION

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(Presented By: James Connor)

Introduction: Many common neurological disorders such as Alzheimer's, Parkinson's disease and Restless Legs Syndrome involve a loss of brain iron homeostasis. Moreover, iron deficiency is the most prevalent nutritional concern worldwide with many associated cognitive and neural ramifications.
Methods: Therefore, understanding the mechanisms by which iron enters the brain and how those processes are regulated may help to address significant global health issues. The existing paradigm assumes that the endothelial cells forming the blood-brain-barrier (BBB) serve as a passive conduit for transport of transferrin-bound iron. This concept is a significant oversimplification. Most notably, it fails to account for the iron requirements of the endothelial cells and for mechanisms regulating brain iron uptake.
Conclusion: Herein we propose a data-based model where endothelial cells of the BBB, far from serving as a simple conduit, are a key focal point for the regulation of cerebral iron metabolism.

Poster #195

HEPCIDIN AND FERROPORTIN PARTICIPATE IN IRON CLEARANCE FROM BRAIN ENDOTHELIUM: FAILURE OF THIS PROCESS LEADS TO IRON ACCUMULATION IN ALZHEIMER'S DISEASE

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(Presented By: Ruma Raha-Chowdhury)

Introduction: Alzheimer's disease (AD) is characterized by cerebrovascular and neuronal dysfunction leading to a progressive decline in cognitive functions and the development of dementia. Pathological hallmarks of AD include neurofibrillary tangles consisting of hyper-phosphorylated microtubule-associated protein tau and extracellular amyloid plaques derived from amyloid precursor protein (APP), a widely expressed trans-membrane metalloprotein essential for neuronal growth, survival and post injury and repair.
Methods and Materials: The main component of amyloid plaques is amyloid β (Aβ) peptide, (38–43 amino acids), generated by sequential cleavage of APP by β- and γ- secretase. Apolipoprotein E (ApoE) is an Aβ chaperone, critical for lipid transport, neuronal integrity and repair. It is reported that there is a link between the ageassociated increase in iron stores and AD. APP possesses ferroxidase activity and facilitates neuronal iron export by binding to the iron exporter, ferroportin. Hepcidin controls systemic iron homeostasis by regulating iron uptake into the plasma through inhibition of cellular iron efflux by binding to ferroportin. We investigated the levels of expression for ApoE and Aβ42 protein, together with hepcidin and ferroportin in human AD, age matched control brains and APP transgenic (APP-Tg) mouse brain. Additionally, primary neurons were cultured from embryonic rat brains to evaluate the toxic effects of cellular iron accumulation. Ferroportin and hepcidin were identified solely in the choroid plexus, capillary endothelium and in astrocytes in close proximity to ventricles in normal human brain and were markedly decreased in AD brain, visible only in fibrillary neurons and in periphery of plaques.
Results: Using western blotting analysis, Aβ42 protein was increased ($P < 0.005$) while hepcidin and ferroportin were significantly decreased in AD brain compared to controls ($P<0.005$ and $P<0.001$). Using immunofluorescent confocal microscopy, extensive blood vessel damage was visible throughout the cortex. Hepcidin and ferroportin levels were decreased whereas ApoE andAβ42 protein were increased in plaques. Hepcidin was however observed in the periphery of plaques in some surviving neurons. Similar observations were also seen in APP-Tg mice from 6 months of age.
Conclusion: Our data support the hypothesis that impaired ferroportin-mediated iron efflux leads to iron accumulation in AD.

Poster #196

ANIMAL MODELS OF NEUROFERRITINOPATHY

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(Presented By: Federica Maccarinelli)

Introduction: Neuroferritinopathies are rare genetic diseases with a dominant autosomal transmission caused by nucleotide insertions in the fourth exon of ferritin L−chain (FTL). They belong to the Neurodegeneration with Brain Iron Accumulation (NBIA) group of pathology.

Methods and Materials: We have studied the aging of animal models that express the pathogenic human FTL mutant 498InsTC (named LN2) under the PGK promoter. Tg mice in the FVB background showed: i) strong accumulation of LN2 in all tissues, particularly in the brain, increasing with age; ii) iron deposition detected by brain MRI imaging; iii) accumulation of ferritin-iron bodies revealed by Perl's stain; and iv) signs of oxidative damage. Interestingly, the number of the iron bodies decreased after 3 weeks treatment with the oral iron chelator deferiprone. The mice were then backcrossed into the C57/BL6J background for 7 generations. Transgene ferritin accumulation was much lower in this background than in the FVB, however, tg-LN2 mice showed sign of oxidative damage in the brain.
Results: Electron microscopy analysis revealed iron deposits associated with granular pigment in areas of cerebellum and striatum of a C57-LN2 mouse. These intracellular ultrastructures, never observed within nuclei, had a density one order of magnitude higher than in controls. Post-natal hippocampal neurons, obtained from C57-LN2 mice showed significant higher percentage of death in response to chronic iron overload and/or acute administration of H2O2, compared to control cells. Preliminary studies performed on C57-LN2 mice tested at 2 and 7 months of age showed a defect in motor coordination that increases with age (as measured through Beam Walking and Rotarod). Furthermore, we analyzed behavior in 2 years old LN2 mice (FVB background) treated with herbicides (Paraquat and Maneb) known to cause oxidative stress and neurodegeneration. No major motor deficit emerged and the tg-mice showed a paradoxical behavioural activation. Finally, we prepared vectors to generate two new tg-mice expressing human L ferritin (FTL-wt) or another pathogenic mutant (LN4). The sequences were optimized for expression in mouse, cloned in a plasmid under the control of mouse PGK promoter and the constructs verified by sequencing. Experiments of transient transfection showed that the expression of the human ferritins in murine cells is considerably limited with respect to human cells.
Conclusion: In conclusion, the transgenic mice appear to recapitulate some of the pathological signs of human neuroferritinopathy, and represent useful models for testing new pharmacological strategies to reduce brain ferritin/iron accumulation.

Poster #197

DOES IRON HAVE CAUSAL ROLES IN NEURODEGENERATION WITH BRAIN IRON ACCUMULATION? - A NEW VIEW FROM A MOUSE MODEL OF HEMOCHROMATOSIS

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(Presented By: Moones Heidari)

Introduction: It is unclear how the brain is affected in the iron loading disorder hereditary hemochromatosis, which can be caused by mutations in the HFE gene or the transferrin receptor 2 (*TFR2*) gene. We have investigated brain iron measures and gene transcript levels in a 'double mutant' mouse model of hemochromatosis, the *Hfe*$^{-/-}$x*Tfr2*^{Y245X} mouse, on short-term dietary iron supplementation.
Methods and Materials: All mice were male and on an AKR background. *Hfe*$^{-/-}$x*Tfr2*^{Y245X} double mutant mice received a high iron diet (2% carbonyl iron) for 3 weeks before sacrifice at 12 weeks. Brain iron was assessed by inductively coupled-atomic emission spectroscopy and non-heme iron assay. Ferritin levels were assessed by Western immunoblotting. Iron-supplemented *Hfe*$^{-/-}$x*Tfr2*^{Y245X} double mutant mice had significantly higher levels of brain iron (> 1.7-fold increase, $p < 0.025$, $n \geq 5$/group) and ferritin (2.3-fold increase, $p < 0.001$, $n \geq 5$/group) than age-matched, gender-matched wild-type mice on normal chow.
Results: Brain mRNA transcripts were assessed by microarray and real-time reverse transcription-polymerase chain reaction (RT-PCR). Array data were normalized by average and cubic spline methods (Illumina Genome Studio). In total, 760 genes were differentially expressed in the *Hfe*$^{-/-}$x*Tfr2*^{Y245X} brain compared to wild-type brains ($p < 0.05$, $n = 4$/group). Analysis for pathway enrichment using the Database for Annotation, Visualization and Integrated Discovery (DAVID v6.7) revealed significant over-representation (Benjamini corrected $p = 0.0073$) of genes important in the mitogen-activated protein kinase (MAPK) signaling pathway.
One gene in the MAPK signaling pathway showing transcript changes was phospholipase A2, group VI (Pla2g6, 1.7-fold decrease, p=0.01). This gene is causatively linked to the severe family of neurogenetic diseases Neurodegeneration with Brain Iron Accumulation (NBIA). Three other NBIA-linked genes also had significantly decreased transcripts: fatty acid 2-hydroxylase (Fa2h, 1.4-fold decrease, p=0.002), ATPase type 13A2 (Atp13a2, 1.1-fold decrease, p=0.04) and chromosome 19 open reading frame 12 (C19orf12, 1.2-fold decrease, p=0.02). This was also validated by real-time RT-PCR.
The NBIAs are rare neurodegenerative disorders with childhood, adolescent or adult onset of severe movement, cognitive or behavioral problems. The term NBIA can be a misnomer as not all patients diagnosed with NBIA based on mutation in an NBIA-associated gene show brain iron accumulation. Also most of the causal genes so far identified have no known relationship with iron, leading some authors to propose that iron is not a direct player in the neuropathology.
Conclusion: The finding that brain transcript levels of at least 4 NBIA genes are altered in the hemochromatosis model puts iron back at centre stage as a primary causal factor in NBIA diseases and hints at close relationships between these genes and iron. It also suggests hemochromatosis patients may experience perturbations in brain molecular systems involved in severe neurodegenerative disease. This may contribute to movement impairment and other neurological problems in some patients.

Poster #198

IRON UPTAKE IN RESTING AND REACTIVE ASTROCYTES: A POTENTIALLY NEUROPROTECTIVE CONTROL OF IRON BURDEN

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(Presented By: Franca Codazzi)

Introduction: Astrocytes play a crucial role in the iron handling within the central nervous system since they: regulate brain iron uptake through the blood brain barrier; are responsible for iron redistribution to neuronal cells; and buffer its concentration in extracellular fluids. This competence can be fundamental, particularly during neuroinflammation, and neurodegenerative processes, where an increase in iron content can favour oxidative stress, thereby worsening disease progression. Under these pathological conditions, astrocytes undergo a process of activation that confers them either a beneficial or a detrimental role on neuronal survival.
Methods: Primary rat hippocampal astrocytes were analyzed by fluorescent probe-based videomicroscopy approaches (conventional epifluorescence setup, total internal reflection microscopy and high throughput microscopy) under different pharmacological treatments. Single-cell analyses were combined with radiolabeled iron measurements, RT-qPCR, western blotting and over-expression experiments, in order to characterize iron entry pathways and iron content both in resting condition and after activation (24 h exposure to 10 nM IL1-β and 30 nM TNFα).
Results: This study investigates the mechanisms of iron entry in cultures of resting and cytokine-activated hippocampal astrocytes. Our data confirm that the major source of iron is represented by the non-transferrin-bound iron (NTBI) and show the involvement of two different routes for its entry. The opening of resident transient receptor potential canonical (TRPC) channels accounts for iron uptake in quiescent astrocytes, with a potentially relevance at the synaptic level, where glutamate spill over can favour TRPC activation. On the other hand, the cytokine-mediated astrocyte activation promotes a raise of divalent metal transporter 1 (DMT1) expression, both at transcript and protein level, with the ensuing potentiation of iron entry and accumulation. Our results also indicate that the DMT1-1A/IRE(+), highly localized at the plasma membrane level, represents the main DMT1 isoform involved in this process.
Discussion and Conclusions: Overall, our data suggest that at rest, but even more after activation, astrocytes have the potential to buffer the excess of iron, thereby protecting neurons from iron overload. These findings further extend our understanding of the protective role of astrocytes under the conditions of iron-mediated oxidative stress observed in several neurodegenerative conditions.

Poster #199

DIVALENT METAL TRANSPORTER 1-DEPENDENT ISCHEMIC NEURODEGENERATION: STRATEGIES TO KNOCKDOWN FERROUS IRON UPTAKE

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(Presented By: Rosaria Ingrassia)

Introduction: The molecular mechanisms responsible for increasing iron and neurodegeneration in brain ischemia are an interesting area of research which could open new therapeutic approaches. We already addressed that 1B/(-)IRE DMT1 isoform is a target gene for NF-kB RelA activation through Lys310-acetylation during the pathogenesis of the early post-ischemic injury.
Methods and Materials: We showed that either oxygen-glucose-deprivation (OGD) or over-expression of 1B/(-)IRE DMT1 isoform significantly increased iron uptake, as detected by total reflection X-ray fluorescence, and iron-dependent cell death in neuronally differentiated SK-N-SH cells. Iron chelation by deferoxamine treatment or (-)IRE DMT1 RNA silencing displayed significant neuroprotection against OGD with concomitantly decreased intracellular iron levels.
Results: It has been demonstrated that administration of the adipose hormone leptin exerts NF-kB/c-Rel-dependent protection against ischemic neurodegeneration both in vitro and in vivo (Valerio et al, 2009, Stroke, 40:616-617). More recently, the ß cell-conditional DMT1 knockout was shown to reduce blood glucose with concomitant increase of insulin secretion and to protect ß cell from cytokine-induced apoptosis in mice subjected to high-fat diet (Hansen et al, 2012, Cell Metabolism, 16,449-461).
Conclusion: Since leptin and insulin partly share downstream signaling pathways, we performed experiments showing that leptin and insulin promote neuroprotection during both OGD and acute ferrous iron overload in neuronally differentiated SK-N-SH cells. Further experiments are in progress to establish the influence of leptin and insulin on DMT1 transporter during neuronal ischemia.

Poster #200

IRON RELATED GENE EXPRESSION AND BIOCHEMICAL PHENOTYPE SUPPORT IRON HOMEOSTASIS DYSREGULATION IN ALZHEIMER'S DISEASE

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(Presented By: Bruno Silva)

Introduction: The distinction between normal aging and Alzheimer's disease (AD) is a relevant step to combat this disease efficiently. Thus, the identification of biomarkers and genetic factors underlying AD pathology is extremely important. Oxidative injury in the brain, mediated by the imbalance of redox-active metals as iron (Fe) has been recognized to contribute to the pathology of AD. In this context, we explored this hypothesis by: (i) screening a set of SNPs in Fe metabolismrelated genes and *APOE* in a sample of 116 AD patients and 98 healthy controls; (ii) comparing serum biomarkers of Fe metabolism in the same samples and (iii) analyzing the expression level of several Fe metabolism genes in the peripheral blood mononuclear cells (PBMCs).

Methods: Genetic analysis was performed through high density SNP genotyping of the candidate genes *CYBRD1*, *HAMP*, *HFE*, *ACO1*, *IREB2*, *SLC11A2*, *SLC40A1*, *TF*, *TFR2*, and *APOE*. Biochemical analysis was assessed for: serum Fe, transferrin (Tf), ferritin (Ft) and Tf saturation. The expression of *TFR1*, *TFR2*, *SLC40A1*, *HAMP* and *SLC11A2* genes were determined by quantitative Real-Time PCR in PBMCs.

Results: Several significant SNP associations with AD were found in this study. Besides the previously reported association with *APOE* (P=0.0007), we also showed association with three SNPs in *TF* (0.0147<P<0.0537) and one SNP in *TFR2* (P=0.0055), *ACO1* (P=0.0258) and SLC40A1 (P=0.0210) genes. Also, significant differences have been found in the biochemical markers of Fe metabolism (P=0.003, overall MANCOVA). These are mainly driven by the significant decrease of serum Fe concentration measured in AD patients compared to controls. Finally, the mRNA levels of *TFR1*, *TFR2* and *SLC40A1* were significantly decreased in PBMCs of AD patients (P<0.001), while no significant differences have been found in the *HAMP* and *SLC11A2* gene expression.

Conclusions: The results obtained in this study support Fe homeostasis dysregulation in AD. Importantly, our findings integrate Fe metabolism alterations in genotype and phenotype at both transcriptional and biochemical levels that suggest the involvement of specific biological pathways in this disease. In particular, we hypothesize that the low systemic Fe status profile observed in AD patients could be due to impaired regulation of cellular Fe efflux. The intracellular accumulation of Fe, particularly in the brain would lead to a rise in oxidative damage, contributing to the AD physiopathology.

Poster #201

NEW HUMAN NEURONAL MODELS OF PANTOTHENATE KINASE ASSOCIATED NEURODEGENERATION FOR THE STUDY OF OXIDATIVE STATUS AND MITOCHONDRIA FUNCTIONALITY

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(Presented By: Sonia Levi)

Introduction: Pantothenate Kinase-Associated Neurodegeneration (PKAN) is an early onset autosomal recessive movement disorder. The disease is caused by mutations in the Pantothenate Kinase-2 (PANK2) gene that encodes a mitochondrial protein involved in Coenzyme A synthesis and it is hallmarked by severe iron accumulation in the brain.

Methods and Materials: Analysis of iron homeostasis parameters on patient's fibroblasts suggested that Pank2 deficiency promotes an increased oxidative status that is further enhanced by the addition of iron. This is also corroborated by the glutathione quantification, which resulted about 20% lower in PKAN fibroblasts respect to the controls cells. To clarify the molecular mechanism leading to iron homeostasis dysfunction in more suitable models of disease, we are developing and characterizing new human neuronal models obtained by patients fibroblast's direct reprogramming. Primary skin fibroblasts from three PKAN patients and three unaffected subjects were infected with lentivirus carrying the three-transcription factors-Mash1, Nurr1 and Lmx1a to obtain dopaminergic neurons (iDANs).

Results: The efficiency of fibroblasts reprogramming is around 5%, as identified by the expression of TuJ1, Tyrosine hydroxylase and N-CAM neuronal markers. Radical oxygen species (ROS) and mitochondrial functionality were evaluated by specific fluorescence probes at single cell level, utilizing In Cell Analyzer technology. In basal condition, PKAN iDANs showed an increase in ROS level, about 50% higher respect to the iDANs from healthy subjects. Evaluation of TMRM signal indicated

that also the mitochondrial membrane potential is affected in PKAN iDANs, resulting in a decrease of about 15% in PKAN neurons respect to controls.
Conclusion: The data indicated that dopaminergic neurons can be reprogrammed from PKAN fibroblasts. They confirmed the results obtained in fibroblasts showing an altered oxidative status and sign of mitochondrial dysfunction, probably due to iron mishandling.
The financial support of Telethon (Grant n°: GGP11088) is gratefully acknowledged.

Poster #202
MODELING HEME, IRON AND COPPER HOMEOSTASIS IN BRAIN INJURY
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(Presented By: Ann Smith)

Introduction: The metabolism of redox active metals including heme, iron and copper is perturbed in several different neurodegenerative conditions but the mechanisms remain essentially unknown. The hemopexin heme transport system coordinately regulates heme, iron and copper homeostasis in non-neuronal cells. Furthermore, the heme transporting protein, hemopexin provides neuroprotection in mouse models of stroke and intracerebral hemorrhage showing the heme is one toxic molecule for the brain. Hemopexin protects primary neurons in vitro against heme or reactive oxygen species (ROS) toxicity via heme oxygenase-1 (HO1) activity. There is an increased incidence of microbleeds in the brain upon aging that releases blood components including heme. Brain iron levels are increased in neurodegenerative diseases and contribute to the pathology. As hemopexin null mice mature, brain iron increases and basic myelin protein production is deficient diminishing oligodendrocyte function.
Methods and Materials: Here, we model human brain neurons experiencing hemorrhages and inflammation using human SH-SY5Y neuroblastoma cells, heme-hemopexin complexes and physiologically-relevant ROS, e.g. H2O2 and hypochlorous acid (HOCl), to provide novel insights into the underlying mechanism whereby hemopexin safely maintains heme and iron homeostasis in the brain. Human amyloid precursor protein (hAPP), needed for iron export from neurons, is induced ~2-fold after hemehemopexin endocytosis by iron from heme catabolism via the iron regulatory element of hAPP mRNA.
Results: Heme-hemopexin is relatively resistant to damage by ROS and retains its ability to induce the cytoprotective HO1 after exposure to peroxides. Apo-hemopexin, which predominates in non-hemolytic states, resists damage by H2O2 and HOCl except for the highest concentrations likely in vivo. In contrast to hemopexin, heme-albumin and albumin are preferential targets for ROS; thus, albumin protects hemopexin in biological fluids like CSF and plasma where it is abundant. Upon endocytosis of heme-hemopexin the intracellular distribution of coppersensitive proteins including hAPP is regulated in a copper-dependent manner.
Conclusion: In conclusion, our data provide strong evidence that hemopexin will be neuroprotective immediately after traumatic brain injury with heme release in the CNS and also during the ensuing inflammation. Hemopexin sequesters heme thus preventing unregulated heme uptake that leads to toxicity; it safely delivers heme to neuronal cells; and uptake of heme-hemopexin complexes regulates heme, iron and copper in neuronal cells activates the induction of proteins including HO1 and hAPP that help keep heme and iron at safe levels in neurons. Supported by the UMKC-RIF (A.S.) and an Alzheimer's Zenith Award (J.T.R.)

Poster #203
DELIVERY OF IRON CHELATORS ACROSS THE BLOOD-BRAIN BARRIER
Ferdinand Fuchs, Anna Preisner, Dr. Sourav Roy, Katharina Brandner, Ana Georgian, Dr. Gerd Wagner, Dr. Jane Preston and Robert Hider, Prof.
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(Presented By: Ferdinand Fuchs)

Introduction: The healthcare burden of neurodegenerative diseases is ever growing, as is the need for treatments of the underlying causes – rather than symptomatic treatments. Increased iron levels, which are a common feature in Parkinson's Disease and other neurodegenerative diseases, cause elevated oxidative stress and protein aggregation, promoting neuronal death and cognitive decline. Iron chelators can bind this iron and have been shown to reduce the concentration of free iron in the brain.
Methods and Results: The administration of iron chelators in rodent models of Parkinson's Disease had neuroprotective effects. As a result, there is increasing interest to treat neurodegenerative diseases with iron chelators, culminating in an ongoing clinical trial of deferiprone (3-hydroxy-1,2-dimethylpyridin-4(1H)-one) for the treatment of Parkinson's patients.
The ultimate obstacle for CNS active drugs remains the delivery across the blood-brain barrier (BBB), however, which acts as a mechanical, metabolic and transport barrier. For deferiprone, only a small fraction of the given dose enters the brain. Strategies to increase the BBB permeability of iron chelators, and deferiprone in particular, are highly sought after. A number of more lipophilic 3-hydroxypyridin-4-ones (HPOs) showed a good correlation between brain uptake and logP (lipophilicity),

albeit at the expense of increased first pass metabolism and excretion. In line with a longstanding interest in deferiprone and related iron chelators, a range of novel, metabolically more stable (fluorinated) HPOs were tested in guinea pigs, showing a good correlation between lipophilicity and brain uptake. In order to establish a cheaper and less labour intensive model, porcine brain endothelial cells (PBECs) were investigated for their suitability to measure the BBB permeability of HPOs and replace *in vivo* experiments. Different culture conditions were evaluated, of which co-culture with C6 astrocytes was found to have the best conformity with the *in vivo* data. Another important aspect of the BBB is the high expression of nutrient transporters, which might be utilised for the facilitated transport of drugs. We are therefore looking at HPO-sugar conjugates as potential substrates of the facilitative glucose transporter GLUT-1, which should greatly enhance BBB permeability and selectivity to the brain.
Conclusion: We are looking at previously described and novel HPO-sugar conjugates, and currently testing their BBB permeability in the PBEC monoculture model. Preliminary results indicate an interaction with the GLUT-1 transporter, but additional experiments are required to establish the exact structural prerequisites for substrate recognition and transport.

Poster #204
DEXRAS1, A SMALL GTPASE, IS REQUIRED FOR GLUTAMATE-NMDA NEUROTOXICITY IN VIVO
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(Presented By: Sangwon Kim)

Introduction: Dexras1 displays about 35% homology with the Ras family of proteins but differs in incorporating a 7 kDa C-terminal extension which it shares with Rhes (Ras Homologue Enriched in Striatum), a G protein highly enriched in the corpus striatum and involved in the neurotoxicity associated with Huntington's Disease.
Methods and Results: Dexras1 plays a role in synchronizing circadian rhythms, as its deletion impairs circadian entrainment to light cycles and alters phase shifts to light. A variety of influences upon adenylyl cyclase and G protein linked neurotransmitter influences have been reported for Dexras1 NMDA receptor-mediated neurotransmission, via stimulation of nNOS, enhances Dexras1 activity. Thus, NMDA transmission leads to the binding of nNOS to CAPON, which in turn binds to Dexras1 with the ternary complex of proteins facilitating the S-nitrosylation of Dexras1 to activate its GTP binding activity. Recently, we discovered a signaling cascade wherein Dexras1 binds to the peripheral benzodiazepine receptor-associated protein (PAP7), which in turn binds to the divalent metal transporter (DMT1), an iron import channel. Stimulation of NMDA receptors activates nNOS leading to nitrosylation and activation of Dexras1, which through linkage to PAP7 and DMT1, physiologically enhances iron uptake.
Conclusion: We here report that iron influx is elicited by nitric oxide but not by other pro-apoptotic stimuli such as H2O2 or staurosporine. Deletion of Dexras1 attenuates NO-mediated cell death in dissociated primary cortical neurons and retinal ganglion cells in vivo. Thus Dexras1 appears to mediate NMDA-elicited neurotoxicity via NO and iron influx.

Poster #205
INTRACELLULAR NEURONAL IRON PLAYS A CRUCIAL ROLE IN REGULATING NMDA EXCITABILITY IN AREA CA1 OF THE HIPPOCAMPUS
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(Presented By: Sangwon Kim)

Introduction: Iron is the most abundant metal in our body participating in a wide variety of metabolic processes (oxygen transport, electron transfer, DNA synthesis, etc.) but also having the potential for deleterious effects. We have recently identified a novel signaling cascade in neurons whereby stimulation of glutamate-NMDA receptors activates neuronal Nitric Oxide Synthase (nNOS), leading to S-nitrosylation and activation of Dexras1 which, via Peripheral Benzodiazepine Receptor Associated Protein (PAP7) and Divalent Metal Transporter (DMT1), physiologically induces iron uptake. This led us to demonstrate that Dexras1-mediated iron influx plays a crucial role in NMDA excitotoxicity. However, the effect of NMDA-mediated iron trafficking on neural function under physiological conditions is not understood.
Methods and Materials: We have utilized voltage sensitive dye imaging (VSDI) from mouse hippocampal slices to investigate the role of iron on net evoked neural circuit activity.
Results: Here, we report that membrane permeable iron chelator, PIH increased the synaptic excitatory activity in area CA1, with little change in the kinetics of voltage responses in other hippocampal areas. This effect was attenuated by the pre-incubating a slice with either NOS inhibitor, NMDA antagonist or in slices from Dexras-/- mice (n=4, and $P<0.005$). This spatial and temporally specific action of PIH, suggests in turn that intracellular iron modulates synaptic activity to change circuit responses to afferent input.
Conclusion: As abnormal evoked activity is associated with a number of neuropsychiatric disorders, these data provide a potential mechanism linking changes in Iron signaling to disease etiology.

Poster #206

A DECREASE IN NEURONAL FERROPORTIN ACCOMPANIES CHRONIC BRAIN INFLAMMATION WITH IRON DEPOSITION

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(Presented By: Torben Moos)

Introduction: Conditions with neurodegeneration lead to significant accumulation of iron in the brain. The mechanisms for this accumulation of iron remain unresolved but two possible causes are denoted by: 1) the incapability of affected neurons to excrete iron mediated by ferroportin and 2) iron-containing inflammatory cells accumulating in brain areas with neurodegeneration. An experimental model of neurodegeneration can be generated by denervation of GABAergic striatal inputs to the substantia nigra via stereotactic injection of the glutamatergic receptor ligand ibotenic acid, which leads to deprivation in inhibitory inputs to the neurons of the substantia nigra pars reticulate due to a relative abundance of glutamate originating from excitatory projections from the subthalamic nucleus.
Methods and Materials: Accordingly, adult male Sprague-Dawley rats were injected unilaterally with ibotenic acid in the striatum, and brain stems were examined at one, four or thirteen weeks following surgery. In spite of being anatomically localized in distance from the striatum, the substantia nigra pars reticulate was clearly affected, indicating that remote surgery leads to an excitotoxic lesion. The substantia nigra pars reticulate contained numerous inflammatory cells expressing the clone-markers ED1 and CD11b suggesting these cells to be derived from the myelo-monocytic cell lineage. Notably, these cells also contained ferritin, and it was found that the substantia nigra pars reticulate continued to express these inflammatory markers over time together with an increase in ferritin. Neurons of the substantia nigra pars reticulate decreased their density with time, and surviving neurons contained less ferroportin with time. Supporting the latter observation, ferroportin mRNA was also less abundant with increasing time in the substantia nigra pars reticulate, whereas ferritin mRNA increased with time in the substantia nigra pars reticulate.
Results: The results show that chronic neurodegeneration can be induced in the brain with an accompanying inflammatory process that leads to deposition of iron in the affected area. The iron deposition was followed by an increase in ferritin mRNA and protein. In contrast the quantity of ferroportin mRNA was lower, an observation that was adjoined by lower ferroportin content in surviving neurons. The latter observation indicates that ferroportin is not upregulated in surviving neurons when iron increases as a mechanism to scavenge iron excess.
Conclusion: In additional explanation to the fall in ferroportin expression, the inflammatory process leading to migration of monocytes and macrophages to the brain leads to that these cells probably downregulate ferroportin via their release of hepcidin. In conclusion, experimentally induced chronic neurodegeneration leads to iron accumulation, upregulation of ferritin mRNA and protein, but a decrease in ferroportin mRNA and protein.

Poster #207

EVALUATION OF IRON LEVELS IN ALZHEIMER'S DISEASE USING MRI RELAXOMETRY

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(Presented By: Christos Michaelides)

Introduction: Alzheimer's disease (AD) is a neurodegenerative disease involving the gradual loss of neuronal cells within the central nervous system. Therapeutic strategies remain anti−symptomatic and methods for diagnosis and monitoring disease progression remain poorly validated. Iron dysregulation is being increasingly identified as a supporting mechanism for oxidative stress and cell death of neurons[1]. Increased levels of iron have been identified in brain regions most associated with AD[2] and iron has been found associated with β−amyloid plaques and neurofibrillary tangles, two major hallmarks of AD[3]. This study was performed to evaluate whether changes in iron can be detected between AD and control brain samples using MRI relaxometry.
Methods: Formalin−fixed post−mortem medial temporal gyri from AD (n=5) and control (n=5) human subjects were immersed in perfluoropolyether and positioned within a 7T Agilent Technologies VNMRS scanner. R1 and R2 relaxometry were performed using spin−echo sequences with varying TR and TE, respectively. R2* relaxometry was performed using a gradient−echo sequence with varying TE. Voxel sizes were 0.11x0.15x0.50mm. Elemental iron maps were obtained from a 10μm thick section of each sample using synchrotron radiation X−ray fluorescence, at 100μm resolution. Regions of interest were drawn around grey and white matter in R1, R2, R2* and iron elemental maps to compare mean values between control and AD samples.
Results and Discussion: Mean white and grey matter relaxometry values obtained using all five samples within each group were highly variable due to fixation time effects. R1 increased, whilst R2 and R2* values decreased with longer fixation times. Whilst changes in relaxometry during the fixation process have been characterised previously[4], our data highlights potential long term effects of fixation on human brain tissue, consistent with previous findings[5]. Thus, samples fixed for longer than 18 months were removed from statistical analysis and remaining control and AD samples (n=3/group) were paired according to

fixation time. Levels of iron appear to be increased in AD grey matter (152.9±20.2 ppm, Mean±SEM) compared to controls (118.2±15.4 ppm, P=0.062), with a concomitant increase in R2 (AD 36.7±2.7 sec^{-1}, Control 32.0±2.5 sec^{-1}, P=0.006) and R2* (AD 46.9±4.9 sec^{-1}, Control 38.5±4.1 sec^{-1}, P=0.036) relaxometry values. R1 were similar between control and AD. Our findings are consistent with iron−enhancement of T2 relaxation and lengthening of R2 and R2* values[6], and imply R2 and R2* relaxometry may provide information on the iron content in tissue. White matter showed similar values for iron levels, R1, R2 and R2* between AD and control tissue.
Conclusion: These results highlight a potential role for iron during AD within the medial temporal gyrus, and outline methods for non−invasive assessment using MR relaxometry. This may also substantiate the application of novel therapeutic strategies such as iron chelation therapies against AD.
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Poster #208
ALTERED BRAIN IRON HOMEOSTASIS IN PARKINSON'S DISEASE AND THE POTENTIAL FOR IRON CHELATION THERAPY
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(Presented By: David Dexter)

Introduction: There is ample evidence which shows that excessive accumulation of iron occurs in specific brain regions in many neurodegenerative diseases, including Parkinson's disease, which may exacerbate the progression of the disease. A common feature in many neurodegenerative diseases is the occurrence of neuro-inflammation which is associated with the neurodegenerative process. The etiology of iron accumulation remains undefined although it maybe as a result of the inflammatory process triggering iron sequestration into neurons, within certain brain regions.
Methods and Materials: In our cell culture studies, the supernatant from cultured N9 microglia, activated with lipopolysaccharide (LPS) for 24h, was incubated with either dopaminergic neurons (N27) or astrocytic (C6) cell lines for time periods up to 24h. The expression of ferritin, transferrin receptors, DMT1 and ferroportin was assayed in the cells at the different time periods. Intracellular iron uptake in the N27 neurons was assessed by the ferrocene assay. The results showed that genes, which are involved in systemic iron homeostasis, transferrin receptors, DMT1 and ferroportin, were expressed on both astrocytes and neurons.
Results: Exposure of N27 neurons to supernatant from activated microglia stimulated a persistent increased neuronal expression of DMT1 and transferrin receptor, whilst the expression of ferroportin and ferritin were unchanged over the 24 hour period. This was mirrored by an increase in neuronal iron uptake. In contrast in the C6 astrocytes exposed to supernatants from activated microglia there was a transient increase in DMT1 expression which declined over 24 hours and there was a reduction in transferrin receptor expression at 12 and 24 hours. Ferroportin expression increased over the 24 hour period in the astrocytic cells. Hepcidin expression was evident in both microglia and astrocytes; and LPS activation of microglia was associated with a time dependent increase in hepcidin expression. The removal of such excesses of iron, via various iron chelators has been extolled in various publication although caution is needed because of the possible adverse effects of the administration of iron chelators to patients with otherwise normal iron homeostasis. A clinical trial to investigate the possibility of iron chelation in Parkinson's patients has commenced. Parkinson's patients received the oral ion chelator deferiprone at low doses, of either 20 or 30 mg/kg/day, for a period of 6 months. Such low doses of the oral iron chelator were well tolerated by the patients, showed minimal toxic effects, and induced decreases in iron in specific brain regions assayed by MRI T2*
Such iron chelation may represent a new therapeutic tool to slow the progression of PD.
Conclusion: Furthermore by increasing our knowledge of brain homeostasis, it should become possible to more closely control the expression of genes which are responsible for iron accumulation in specific glial and neuronal cells.

Poster #209
ANALYSIS OF IRON METABOLISM IN TFR2 TARGETED ANIMALS BRAIN
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(Presented By: Antonella Roetto)

Introduction: It is well known that iron is an essential element for all cells and in brain also it is involved in important functions as neurotransmission and myelination as well as in neuronal cells division (Moos et al, 2007). Iron metabolism in the brain must be tightly regulated since iron overload has been evidenced in quite common neurodegenerative disorders like Parkinson and Alzheimer diseases. Hepcidin (Hamp), a key iron regulator, resulted to be present in the brain (Hanninen et al 2009) but it is not clear how Hamp and its regulating proteins modify iron availability in the nervous tissues.
Methods and Materials: Transferrin receptor 2 (TFR2) is one of the proteins involved Hamp regulation. The TFR2 gene is transcribed in two main isoforms, tfr2 alpha, an iron sensor that contributes to hepatic regulation of Hamp, and Tfr2 beta that seems to be involved in transcriptional regulation of iron exporter Ferroportin 1 (Fpn1). To get inside on Tfr2 isoforms functions

in the brain we employed Tfr2 alpha and /or beta targeted mice (Tfr2 KI, KO and LCKO-KI) (Roetto et al 2010) compared to wild type (WT) sib pairs. Main brain compartments, cerebellum (CRBL), cortex (CRTX), striatum (STR), spinal cord (SC) and hippocampus (HIPP) were isolated and in these tissues we analyzed: a) iron amount; b) Tfr2 isoforms transcription and production in WT animals and c) expression of the main iron genes (Hamp, Fpn1). Iron amount in WT 13 months old mice brain compartments is quite constant except for CRBL, that has the higher iron amount and HIPP, in which iron is lower than in the other compartments.
Results: Tfr2 isoforms null mice (KO) have an increased iron amount vs WT only in HIPP. Notably, in WT animals a significant Tfr2 alpha transcription is evidenced through quantitative real time PCR. This result was confirmed at the protein level by a strong immunofluorescent labeling of the Tfr2 alpha isoform specific antibody in the hippocampal hilus, suggesting that iron has a peculiar role in the functions of this brain compartment. Since the hippocampus has a well-known role in learning and memory, we performed behavioral studies on adult Tfr2 KO vs WT mice. Interestingly, preliminary data indicate an alteration in learning paradigms in mutant animals. Tfr2 beta resulted to be well expressed in all brain compartments, but no variation in iron amount are present in tfr2 beta null mice (KI) even if a significant decrease in Fpn1 transcription has been evidenced ($p<0.01$). Tfr2 LCKO-KI mice, Tfr2 beta null animals with liver conditioned Tfr2 alpha inactivation, present a severe systemic iron overload and the same situation is found in all brain compartments. We analyzed Hamp and Fpn1 transcription in these animals total brain vs WT. Hamp transcription seems to be increased while Tfr2 alpha and Fpn1 mRNA are decreased.
Conclusion: All together these evidences support the hypothesis of a functional activity of Tfr2 isoforms in brain iron metabolism, being Tfr2 alpha involved in iron homeostasis in hippocampus and Tfr2 beta in more generalized function on Fpn1 regulation.

Poster #210
C19ORF12 SILENCING IN NEUROBLASTOMA SH-SY5Y CELLS PERTURBS IRON HOMEOSTASIS
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(Presented By: Paola Ruzzenenti)

Introduction: Increased iron deposition is a hallmark of many neurodegenerative diseases, but it's pathogenic role is unclear yet. A strong link between iron and neurodegeneration is evident in a set of heterogeneous neurological syndromes known as Neurodegeneration with Brain Iron Accumulation (NBIA). The most common form of inherited NBIA is due to mutations in PANK2 gene (PKAN). Recently a new subtype (MPAN) has been identified with variations of the orphan gene C19orf12. While we know that Pank2 is involved in CoA biosynthesis, very little is known about C19orf12 function. Interestingly both proteins localize in the mitochondria and are putatively involved in lipid metabolism. Downregulation of PANK2 expression perturbs cellular iron homeostasis, with reduced levels of ferritin, the iron storage, and increase of Transferrin Receptor 1 (TfR1) and of ferroportin, the sole cellular iron exporter (Poli et al 2010).
Methods and Materials: We are now evaluating possible connection between C19orf12 defects and cellular iron maintenance. C19orf12 expression was first studied upon exposure of SH-SY5Y cells to stimuli such as iron chelation, iron supplementation and H_2O_2 for 16 h. Then different iron-related parameter were investigated upon specific C19orf12 silencing.
Results: While iron chelation and supplementation did not alter mRNA and protein levels of C19orf12, we observed an increased expression upon H_2O_2 exposure. When cells were treated with 10nM of specific siRNA, we observed a significant reduction (>80%) of C19orf12 mRNA and protein, 48 and 72 h after the transfection; this was associated to high levels of cell death (50%). In this experimental setting, downregulation of C19orf12 expression leads to reduced level of FTL protein (<50%) and major increase of TfR1 and ferroportin proteins. The phenotype is highly reminiscent of that observed in PANK2 depleted cells, thus suggesting a possible functional link among the two proteins and with iron regulation.
Conclusion: We are exploring this hypothesis and further study their biological function in different cell lines and in fibroblasts from MPAN patients. Given that the accumulation of iron is often an early event in PKAN and MPAN, understanding the pathophysiological underpinnings may provide new therapeutic avenues and enhance our comprehension of brain iron homeostasis.

Poster #211
THE H63D HFE GENE VARIANT ACCELERATES DISEASE PROGRESSION IN AMYOTROPHIC LATERAL SCLEROSIS (ALS)
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(Presented By: James Connor)

Introduction: There is considerable interest in identifying a genetic basis for amyotrophic lateral sclerosis (ALS). The most common HFE gene variant, H63D HFE, is present at higher frequency in ALS patients and its presence increases the risk of developing ALS 4-fold. The H63D HFE is associated with iron accumulation, oxidative stress, abnormal glutamatergic secretion and prolonged endoplasmic reticulum stress, each of which is proposed as a contributing factor to ALS pathogenesis.

Methods and Materials: We propose that H63D HFE increases the risk of disease by creating an environment that promotes the convergence of disease processes in ALS. In the present study, we determined the role of H63D HFE in ALS pathogenesis using a double transgenic mouse line that carries H63D HFE and SOD1(G93A) mutation. We crossed SOD1 mouse model of ALS (SOD1G93A) with H67D mice to generate a double transgenic mouse line (SOD1/H67D). Disease onset was determined by monitoring the motor performance on a rotarod. A gripstrength meter was used to measure forelimb and hindlimb strength, which represents the disease progression. End-stage was defined as the inability of the animal to right itself within 30 seconds after being placed on its side. Disease duration was the mean time from onset to end-stage.
Results: Although age of disease onset was not different between double transgenic mice (SOD1/H67D) and SOD1(G93A) mice, SOD1/H67D mice exhibited decreased forelimb and hindlimb strength than SOD1(G93A) mice suggesting an accelerated disease progression. Survival and disease duration of female SOD1/H67D mice was significantly reduced when compared with female SOD1(G93A). However, the survival and disease duration of SOD1/H67D males was not different from male SOD1(G93A) mice. To evaluate how H63D HFE contributes to accelerated disease progression and reduced survival in SOD1/H67D mice, we determined the expression of proteins involved in iron metabolism and oxidative stress in the lumbar spinal cord of SOD1/H67D mice at 90- (presymptomatic) and 110-days (symptomatic). Although SOD1(G93A) and SOD1/H67D mice had decreased transferrin receptor expression at both ages compared to the wild-type, SOD1/H67D mice had significantly higher L-ferritin expressions than SOD1(G93A) mice suggesting further disruption of iron homeostasis in SOD1/H67D mice and increased microglial activation. There was also increased hemeoxygenase-1 expression in SOD1/H67D mice decreased nuclear factor E2-related factor 2 (Nrf2) expression.
Conclusion: Together, these data suggest that H63D HFE accelerated disease progression in ALS by enhancing loss of iron homeostasis and exacerbating oxidative stress. Because H63D HFE occurs in as many as 30% of ALS patients, the mouse model presented here has meaningful implications for human disease.

Poster #212
THE H63D HFE GENE VARIANT IMPACTS AMYOTROPHIC LATERAL SCLEROSIS VIA MITOCHONDRIAL MECHANISMS
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(Presented By: James Connor)

Introduction: Although many genetic and environmental risk factors for amyotrophic lateral sclerosis (ALS) have been identified, the etiology of sporadic ALS (sALS), representing approximately 90% of cases, remains unknown. The H63D HFE gene variant is found at increased frequency in sALS, occurring in up to 30% of sALS patients, and is associated with a roughly 4-fold increase in disease risk. Separately, mitochondrial dysfunction is implicated in ALS pathophysiology. Perturbed mitochondrial morphology, trafficking, fusion/fusion, signaling pathways, and mitophagy are reported in *in vitro* and *in vivo* animal models of ALS. Because mitochondria are dependent on iron homeostasis due to the essential role of iron in the electron transport chain (ETC), we hypothesized H63D HFE-induced iron dysregulation causes mitochondrial dysfunction, contributing to ALS pathophysiology. Here we use *in vitro* and *in vivo* models to determine if HFE-gene variant induces mitochondrial dysfunction. Because statins exert adverse effects partly via mitochondrial mechanisms, and statins may accelerate functional decline in ALS patients, we also analyzed the effect of statin treatment in ALS animal models, with an emphasis on HFE genotype.
Methods: Mitochondria were isolated from human SH-SY5Y neuroblastoma cells stably expressing wild-type (WT) or H63D HFE, and levels of complex IV, an essential member of the ETC, were determined. Gastrocnemius muscle was isolated from 6-mo transgenic mice hetero- or homozygous for H67D HFE (equivalent to human H63D HFE) or their WT HFE littermates, and levels of complex IV were determined from isolated mitochondria. Gastrocnemius muscle was isolated from presymptomatic, 90-day double transgenic animals harboring both H67D HFE and G93A SOD1 (mutant ALS model mice), as well as their WT, H67D HFE and G93A SOD1 littermates, and levels of complex IV were determined from isolated mitochondria. Separately, double transgenic mice were treated with simvastatin, which crosses the blood brain barrier, and disease onset and progression were measured by gripstrength and rotarod. Kaplan-Meier survival analysis was conducted to determine the effect of simvastatin treatment.
Results: Neuroblastoma cells expressing H63D HFE had decreased levels of complex IV versus cells expressing WT HFE. Six-mo single transgenic mice hetero- or homozygous for H67D HFE had decreased levels of muscle complex IV versus WT mice. Presymptomatic 90-day double transgenic mice harboring H67D HFE and G93A SOD1 had a trend towards decreased muscle complex IV levels, with greater effects in males. Simvastatin treatment caused a trend towards accelerated disease progression in both G93A SOD1 and H67D HFE / H67D HFE ALS mice.
Discussion: The HFE gene variant induces mitochondrial dysfunction as evidenced by decreased levels of complex IV of the mitochondrial ETC in *in vivo* and *in vitro* models of this gene variant. Additionally, statins, which are believed to perturb mitochondria, cause a trend towards accelerated ALS progression in animal models. This may be particularly relevant to the subset of patients harboring H63D HFE.
Conclusion: The H63D HFE gene variant induces mitochondrial dysfunction, contributing to ALS pathophysiology. Statins may adversely impact the subset of ALS patients harboring H63D HFE, suggesting a need for genotype-based treatment decisions.

Poster #213

PANK2 -/- MICE TISSUES SHOW SIGN OF OXIDATIVE DAMAGE

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(Presented By: Michela Guaraldo)

Introduction: Pantothenate Kinase2 Associated Neurodegeneration (PKAN) is a genetic movement disease characterized by abnormal iron accumulation and degeneration in the brain basal ganglia. The Pantothenate Kinase2 (Pank2) gene responsible of PKAN encodes a mitochondrial key regulatory enzyme in Coenzyme A biosynthesis. The PANK2-/- mouse model showed growth reduction, retinal degeneration and male infertility due to azoospermia, but no movement disorders or brain iron accumulation.
Methods and Materials: We analyzed the expression of antioxidant/iron proteins in PANK2-/- and wild type mice tissues to verify if iron-dependent oxidative damage can occur in mice tissues even in absence of iron overload. We started to evaluate SOD1, catalase and ferritins in testis and brain homogenates from 6 months aged mice. Quantitative RT-PCR, ELISA and immunoblotting were performed to quantify the transcription and translation levels of the proteins.
Results: In testis, results indicated that mitochondrial ferritin expression was reduced both at mRNA (about 2,7 fold) and at protein level (about 7 fold). As expected in altered oxidative status, the transcription of cytosolic H-ferritin expression was enhanced (about 2 fold), on the contrary its protein level was reduced of about 1,5 fold, probably due to an increased oxidized ferritin degradation. A similar behavior was observed for the cytosolic scavenger protein SOD1; its mRNA resulted about 1,6 fold higher in Pank2-/- respect to the wt mice, while the peptide was strongly degreased in Pank2-/- mice. In brains, ferritins, catalase and SOD1 evaluation did not show significant differences.
Conclusion: The overall data indicated that the expression of the mitochondrial ferritin, cytosolic H-ferritins and SOD1 resulted affected in Pank2-/- mice testis, suggesting that iron-dependent oxidative damage might occur, at least in specific tissues.

The financial support of Telethon (Grant n°: GGP11088) is gratefully acknowledged.

Poster #214

A CELL MODEL FOR FRIEDREICH ATAXIA USING DORSAL ROOT GANGLIA NEURONS

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(Presented By: Jordi Tamarit)

Introduction: Frataxin is a small mitochondrial protein involved in iron metabolism. Decreased frataxin protein levels cause the neurodegenerative disease Friedreich ataxia (FRDA). The pathologic changes occur first in dorsal root ganglia (DRG), with loss of large sensory neurons. The specific deleterious effects of frataxin depletion on DRGs have been studied using conditional knockout mice and tissue samples from FRDA patients. However, there is not a cellular model to address in detail the molecular and cellular consequences of the lack of frataxin in these type of cells. In this work, cultured DRG neurons from newborn rats have been used as a model for FRDA by repressing frataxin expression using shRNAs and lentivirus vectors.
Materials and Methods: DRGs were extracted from neonatal rats. After enzyme digestion and mechanical dissociation the individual sensory neurons from each ganglion were obtained and cultured. In these neurons, the levels of endogenous frataxin were reduced by two different shRNAs (shRNA34 and shRNA37) using lentivirus vectors. Interference efficiency was measured by western blot. Apoptotic markers and cytoskeleton alterations were evaluated by immunofluorescence and western blot. Mitochondrial membrane potential was assessed by JC1 staining. Plasmid overexpressing human Bcl-xL was delivered using lentivirus vectors.
Results and Discussion: Western blot analysis of DRGs neurons transduced with shRNA34 or shRNA37 demonstrated a 70-80% reduction in frataxin protein levels. Survival analysis of these cells five days post-transduction indicated a drop in cell survival, down to 60% of shRNA34 transduced neurons and to 40% for shRNA37 transduced neurons compared to control neurons transduced with scrambled shRNA (100%). Morphological analysis of frataxin knock-down neurons indicate significant increase of neurite swelling, perykarial accumulation of phosphorylated neurofilament heavy (NF-200), decreased mitochondria membrane potential and apoptotic cell death. We also demonstrated that overexpression of the anti-apoptotic protein Bcl-xL can prevent neurodegeneration and recover mitochondrial function
Conclusion: Frataxin depletion in DRGs neurons induces cytoskeleton interruption, neurodegeneration and apoptotic cell death which can be prevented by Bcl-xL.

Poster #216

AN EXAMINATION OF THE EFFECT OF SEX AND IRON AND COPPER CONSUMPTION IN A MOUSE MODEL OF ALZHEIMER'S DISEASE

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(Presented By: Caitlin Groeber)

Introduction: Age-related diseases are becoming more prevalent because of the increasing ability to live longer, and this comes with increasing dietary enhancements and supplementations to maintain a healthy lifestyle. Previous studies of transgenic Alzheimer's disease (AD) mouse models have found that long-term dietary supplementation of zinc impairs spatial memory, while copper shows remediation of this impairment. Iron shows a similar impairment in spatial and non-spatial, discrimination tasks, but the potential effects of remediation by copper have not been studied. In humans, being female is a risk factor for cognitive impairment in AD, therefore it was important to examine sex differences which are often ignored.
Methods: Male and female transgenic (Tg)CRND8 and wild-type (Wt) mice were raised on four separate types of drinking water: iron enhanced (10ppm FeNO3), iron plus copper enhanced (10ppm FeNO3 + 0.25ppm CuNO3), copper enhanced (0.25ppm CuNO3), and lab tap water to examine the effect of trace metals on cognitive function and brain pathology. Animals were bred at our facility, and tested a 5 months of age. Assessments included the novel object recognition task (NOR; discrimination task) and the Morris water maze (MWM; spatial memory). After a 5-day habituation, NOR was examined at 15 mins, 1 hr, 24 hr, and 48 hr intervals. The MWM, a 7-day paradigm, included a probe trial every other day and a 24-hr probe on day 7. Histological analysis on brain and liver tissue is also being conducted to examine the plaque load and metal content.
Results: Transgenic animals and were impaired in object recognition ($F(1,155)= 6.373$, $p=.013$), as well as the MWM where Wt mice showed significantly decreased latency ($F(1,153)=23.703$, $p=.000$). Females showed better object recognition than males ($F(1,155)=3.9339$, $p=.049$), however, males showed better performance in the MWM with decreased latency ($F(1,153)=4.079$, $P=.045$). There was no overall significant difference across water types, however in transgenic animals metal enhancement appeared to decrease performance in the object recognition task and impair spatial memory in the MWM. Among Tg animals in the NOR, lab water animals performed above chance, but all other metal groups performed at or below chance. In the MWM task, the iron plus copper animals performed the slowest in Wt animals with all Tg animals failing to decrease latency.
Discussion: The data suggest that metal consumption can change the learning and memory processing in both Tg & Wt mice; the effects are complicated by the sex differences, analysis is ongoing. In contrast to Wt mice, Tg females were more impaired in spatial learning and novel object recognition than males. APP mice showed significant spatial learning deficiencies compared to Wt mice by their inability to decrease latency across days. APP mice also showed impairments in novel object recognition.
Conclusions: As expected, this study found that Wt animals showed more novel object recognition and better spatial memory than TgCRND8 animals. But the direction of the sex differences was dependent on the type of task: males showed better spatial memory, but females showed more object recognition. The effect of metal consumption is affected by the sex differences, but brain and liver histology will help determine the impact of these metals histopathologically.

Poster #217

IRON HOMEOSTASIS IN PATHOLOGICAL RETINA: TRANSFERRIN AS NEW THERAPEUTIC AGENT

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Background: Iron is an essential metabolic component for enzymes specifically required for the vision mechanisms. Most of the proteins involved in iron homeostasis have been recently described and localized in the retina. Retinal degeneration has been associated with iron accumulation in age-related macular degeneration (AMD), in several rodent models which had one or several iron regulating proteins impairment or associated with genetic vision defects. To prevent iron accumulation derived damages, we tested the potential neuroprotective effect of human transferrin (hTf) on the photoreceptors (PRs) degeneration in genetic or experimentally induced models.
Materiels and Methods: Eyes from rd10 mice and RCS rats at different stages of photoreceptors degeneration were collected and analyzed by PIXE method for iron contents determination in retina. We intraperitoneally injected human transferrin (hTf) in 5-day-old rd10 mice up to 25 days. To constitutively express hTf in rd10 mice, we have crossbred rd10 mice with transgenic mice for human transferrin (rd10/hTf). We have used intense white LED light on rats to induce retinal degeneration, and intravitreally injected hTf just before exposure. We observed retinal functions *in vivo* by optical coherence tomography and by electroretinography and compared PRs preservation by measurement of the outer nuclear layer thickness on histological sections.

Results: We demonstrated an iron accumulation in PRs segments concomitant with retinal degeneration stages in rd10 mice and RCS rats. At the peak of PRs degeneration, rd10/hTf mice showed less PRs death than rd10 mice. Intraperitoneal injection of hTf in rd10 mice resulted in major PRs preservation. In rats, HTf intravitreal injection decreased oxidative stress and iron accumulation, both resulting from illumination. PRs were protected and theirs physiological functions are preserved.
Conclusion: Tf injected in systemic or directly into the eye, has a potent protective effect on the neurodenegerative process. This effect seems to be due to the iron chelating effect of Tf. This study highlights the therapeutical potential of Tf in retinal diseases associated with iron accumulation such as aging or AMD. Control of iron impairment in tissues could be corrected by adjusting Tf level by different molecular or pharmacological strategies.

Poster #218

THE QUANTITATIVE ASSESMENT OF COMBINATIONS OF THE NOVEL IRON CHELATOR (E)-N´-[1-(2-HYDROXY-5-NITROPHENYL) ETHYLIDEN] ISONICOTINOYLHYDRAZIDE AND THE ANTI-ESTROGEN TAMOXIFEN ON MCF-7 BREAST ADENOCARCINOMA CELL LINE

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(Presented By: Eliska Mackova)

Introduction: Breast cancer is usually treated by simultaneous administration of multiple drugs with different mechanisms of action. Cancer cells have a higher iron requirement and some breast cancer cells are able to produce their own transferrin molecules, whose synthesis is stimulated by estrogen receptors.
Methods and Materials: Therefore, the aim of this study was to examine the antiproliferative effects of combinations of the novel iron chelator (E)-N´-[1-(2-hydroxy-5-nitrophenyl)ethyliden] isonicotinoylhydrazide (NHAPI) or its iron-complex with the antiestrogen agent tamoxifen on MCF-7 breast adenocarcinoma cell line using the Chou – Talalay method for accurate mathematic determination of synergism, additive effect or antagonism. Previously, NHAPI has shown marked atiproliferative action towards cancer cells together with low toxicity towards non-proliferating cardiomyoblasts (Mackova et al. Chem Biol Interact. 2012;197:69-79). Tamoxifen is a clinically used antiestrogen drug for treatment of estrogen-receptor positive breast cancer.
Results: The studied substances were incubated for 72 hours with MCF-7 cells either as single substances or as combinations at multiples of their IC50 concentrations. The cellular proliferations were quantified using the neutral red reuptake test. The IC50 values and the evaluations of the effects of drugs' combinations were calculated by CalcuSyn 2.0 software (Biosoft, Cambridge, U.K.). Furthermore, these combinations were studied in more detail using an electrical impedance measurement and a cell cycle analysis.
Conclusion: The combination of NHAPI and tamoxifen displayed mild to strong synergism (combination indices 0.9 – 0.214) from 30% and higher decrease of proliferation, in comparison with control, and the combination of NHAPI-iron complex and tamoxifen was synergistic (combination indices 0.9 – 0.228) from 45% and higher decrease of proliferation. The electrical impedance measurement and cell cycle analysis confirmed the synergism of the combinations. Both these methods also showed the G1-S cell cycle arrest that was more pronounced by combination than by single substances. An interesting elevation of S phase was observed after NHAPI treatment. Nevertheless, this S-phase elevation was not observed after NHAPI-iron complex treatment. The combination of iron-chelating and antiestrogen therapy could be a new attitude for treatment of estrogen positive breast cancer. Further research is nevertheless needed to assess the potential of this combination in more detail.
This study was supported by the Charles University (grant 299511) and "Support of establishment, development, and mobility of quality research teams at the Charles University" (project number CZ.1.07/2.3.00/30.0022).

Poster #219

CANCER AS A FERROTOXIC DISEASE: WHAT WE HAVE LEARNED FROM ANIMAL STUDIES TOWARD ITS PREVENTION

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(Presented By: Shinya Toyokuni)

Introduction: Iron is abundant universally. During the evolutionary processes, humans have selected iron as a carrier of oxygen inside the body. However, iron works as a double-edged sword, and its excess can be a risk for cancer, presumably via generation of reactive oxygen species. Thus far, pathologic conditions such as hemochromatosis, chronic viral hepatitis B and C, exposure to asbestos fibers as well as endometriosis have been recognized as iron overload-associated risks for human cancer. Indeed, iron is carcinogenic in animal experiments.
Methods: We used a rat renal carcinogenesis model with repeated intraperitoneal injections of ferric nitrilotriacetate (NTA) and rat mesothelial carcinogenesis models by intraperitoneal administration of commercially used asbestos fibers (chrysotile, crocidolite and amosite) or multiwalled carbon nanotubes (MWCNT). The obtained tumors were analyzed with array-based comparative genome hybridization as well as with histological/immunohistochemical analyses. In asbestos-induced mesothelial carcinogenesis, serum ferritin and iron contents of abdominal organs were measured.

Results: Most of the animals in models mentioned above obtained either renal cell carcinoma or malignant mesothelioma in two years. Notably, repeated NTA administration after asbestos injection promoted mesothelial carcinogenesis. During the carcinogenesis, iron accumulation in mesothelial cells and abdominal organs
were evident. Furthermore, these studies unexpectedly revealed that there are common target genes in these iron-induced carcinogenesis (e.g. homozygous deletion of CDKN2A/2B, etc.) with massive genomic amplifications and deletions. MWCNT also induced malignant mesothelioma, with 50 nm diameter MWCNT most carcinogenic.
Discussion: The fact that massive genomic alterations were observed for the first time in the iron overload-associated animal models of wild type animals suggests that iron overload may be a major mechanism in various human carcinogenesis. These genetic changes would be helpful for diagnosing early stage of cancer in pathology specimens.
Conclusion: Recent epidemiological studies reported that iron reduction by phlebotomy decreased cancer risk in the apparently normal population. These results warrant reconsideration of the role of iron in carcinogenesis and suggest that fine control of body iron stores would be a wise strategy for cancer prevention.
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Poster #220

THE INTERACTION OF NOVEL ANTI-CANCER IRON CHELATORS WITH ALBUMIN: MECHANISMS OF CELLULAR UPTAKE

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(Presented By: Danuta Kalinowski)

Introduction: Iron chelators represent a novel treatment avenue to target tumor cells, which have increased requirements for iron due to their rapid proliferation. Importantly, *in vivo* studies and clinical trials have confirmed the potential of thiosemicarbazone iron chelators as potent anti-cancer agents. For example, the chelator, di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone (Dp44mT), has demonstrated potent anti-proliferative activity both *in vitro* and *in vivo* (*PNAS* 2006;103:14901-6). Dp44mT mediates its anti-cancer activity *via* a number of mechanisms, including: ***(1)*** the formation of redox-active iron and copper complexes that result in reactive oxygen species generation (*J Med Chem* 2009;52(2):407-15); ***(2)*** the accumulation of the redox-active Dp44mT complex in lysosomes, resulting in apoptosis *via* the lysosomal pathway (*Cancer Res* 2011;71(17):5871-80); and ***(3)*** the alteration in expression of a number of molecules such as the metastasis suppressor, N-myc downstream regulated gene 1 (*Blood* 2004;104(9)2967-75). However, the mechanism involved in the cellular entry of Dp44mT to induce cell death is unclear. Dp44mT was radiolabeled with ^{14}C in order to assess the mechanisms involved in its cellular uptake in human SK-N-MC neuroepithelioma cells.
Methods and Materials: Uptake studies were carried out in comparison to the more lipophilic chelator, ^{14}C-2-benzoylpyridine 4-ethyl-3-thiosemicarbazone (^{14}C-Bp4eT).
Results: The cellular uptake of ^{14}C-Bp4eT was found to occur *via* passive diffusion (*Mol Pharmacol* 2010;78(4):675-684). In contrast, the temperature-dependent and saturable uptake of ^{14}C-Dp44mT suggested a receptor-mediated process. The uptake of ^{14}C-Dp44mT decreased in the presence of increasing concentrations of its unlabeled counterpart, Dp44mT, and also suggested the involvement of a saturable transport system. On the other hand, the uptake of ^{14}C-Dp44mT was unaffected in the presence of increasing concentrations of unlabeled Bp4eT. Importantly, the uptake of ^{14}C-Dp44mT was found to markedly (p<0.01) increase in the presence of human serum albumin (HSA; 40 mg/mL), a protein that is well known to bind drugs. This effect was inhibited in the presence of excess HSA, indicating that a HSA receptor may be involved. Additional experiments demonstrated that another serum protein, transferrin, did not affect the cellular uptake of ^{14}C-Dp44mT, while the structurally -related protein, bovine serum albumin, resulted in significantly (p<0.01) decreased ^{14}C-Dp44mT uptake. The enhanced uptake of ^{14}C-Dp44mT observed in the presence of HSA was specific to this protein and was found to occur in six different cell types, indicating that this mechanism was not cell type specific. Subsequent drug-protein binding experiments suggested that Dp44mT binds directly to HSA at Sudlow's Site I.
Conclusion: In conclusion, these studies suggest that the ability of Dp44mT to bind to HSA results in enhanced uptake into human cancer cells by a saturable, receptor-mediated process, potentially involving an HSA receptor.

Poster #221

A COMPARATIVE STUDY OF THE ANTIPROLIFRATIVE ACTIVITY OF THIOSEMICARBAZONE IRON CHELATORS IN SKIN CELLS

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(Presented By: Olivier Reelfs)

Introduction: Although the promise of antitumour iron chelation therapy of cancer (ICT) is widely recognised, to date the potential of iron-chelators (ICs) in skin cancer has not been properly explored. A key challenge to be addressed is the observation in both animal and clinical trials that the antitumour properties of currently available ICs may be accompanied by severe toxic side effects upon repeated systemic administration. Further optimization of the dose, period of treatment and mode of administration therefore seems crucial for the success of ICT. The lipophilic nature of thiosemicarbazone ICs of the di-2-pyridyl- (i.e. Dp) and 2-benzoylpyrindine-(i.e. Bp) series makes them potentially attractive for treatment of non-melanoma skin cancer via topical application (1).
Methods and Materials: As a first step towards this goal, in the present study, we investigated the antiproliferative potential of the Dp- and Bp- thiosemicarbazone analogues Dp44mT, Dp4pT, Bp4eT and Bp4pT using the human primary fibroblast cell line FEK4 and the spontaneously immortalised human keratinocyte cell line, HaCaT as models. The HaCaT cell line has proved a useful and reliable in vitro model of human skin cell carcinoma. This cell line is hyperproliferative and shows a significantly higher proliferation rate compared to normal human skin keratinocytes and FEK4 fibroblasts.
Results: The time-course and dose response studies with MTT assay revealed that both Bp- and Dp- analogues have a more pronounced growth inhibitory effect in HaCaT cancer cells than in normal FEK4 fibroblasts. The average IC50 values of the compounds in HaCaT cells were in the nanomolar range (i.e. 0.05-0.5 uM), while in FEK4 cells the average IC50 values were in the micromolar range (i.e. 1-5 uM). In HaCaT cells, Bp4pT appeared to be the most potent antiproliferative chelator with IC50 = 0.05 uM. In contrast in FEK4 cells, both Bp analogues and DP4pT yielded the same IC50 value of 1 uM. Moreover in FEK4 cells, Dp44mT appeared to be the least effective antiproliferative chelator (IC50 = 5uM). Nevertheless the IC50 values of the Bp- and Dp- thiosemicarbazones in both cell lines used in this study were 50-200 times lower than those obtained with desferrioxamine (DFO) and salicylaldehyde isonicotinoyl hydrazone (SIH) chelators with the same cell lines in this laboratory. In an attempt to relate these findings to an in vivo setting, clone forming assays were performed in parallel and the results showed that in agreement with MTT data, both Bp- and Dp-analogues have substantially higher antiproliferative activity than DFO and SIH. We also analysed the impact of the chelators on the cell cycle by flow cytometry, using bromodeoxyuridine incorporation. The results showed that both Dp- and Bp- analogues had an effective impact on the cell cycle in HaCaT cells with concentrations that were 100-200- fold lower than those necessary to obtain the same effect with SIH and DFO.
Conclusion: The strong antiproliferative activities observed for both Dp- and Bp- chelators, together with their lipophilicity, therefore provide a rationale for their use in topical ICT of non-melanoma skin cancer.
1. Yu Y, Kalinowski DS, Kovacevic Z, Siafakas AR, Jansson PJ; Stefani C, Lovejoy DB, Sharpe PC, Bernhardt PV, Richardson DR. J. Med. Chem. 2009, 52:5271-5294.

Poster #222

IMPROVING THE EFFICIENCY OF AMINOLEVULINATE-PHOTODYNAMIC THERAPY OF SKIN CANCER BY COMBINING UVA IRRADIATION AND POTENT IRON CHELATING AGENTS

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University of Bath
(Presented By: Tina Radka)

Introduction: Photodynamic therapy (PDT) is widely used for the treatment of skin cancer. Mechanistically, in delta-aminolevulinic acid (ALA)-mediated PDT, the addition of ALA to cells bypasses the negative feedback control of heme biosynthesis, leading to accumulation of photosensitizing concentrations of protoporphyrin IX (PPIX). Subsequent activation of cellular PPIX with an external light source (usually red light, 550-750nm) leads to generation of reactive oxygen species, resulting in cell death. The major side effect of ALA-PDT treatment is the pain experienced by patients. Management of treatment-related pain still remains a considerable challenge in patients. Further optimization of the treatment protocol including light source, dose and duration therefore seems crucial to try and address this issue.
Methods and Materials: To improve the efficiency of ALA-PDT of skin cells: (i) we changed the conventional light source to UVA (320-400 nm) that is absorbed more efficiently by PPIX and is 40-fold more potent in killing cultured skin cells than red light [1]; (ii) we combined ALA treatment with the potent iron chelators, salicylaldehyde isonicotinoyl hydrazone (SIH), pyridoxal isonicotinoyl hydrazone (PIH) or desferrioxamine (DFO) to further increase the accumulation of PPIX through the depletion of iron available for ferrochelatase-mediated bioconversion of PPIX to heme. Spontaneously immortalised HaCaT keratinocytes were pre-treated (or not) for 18h with SIH, PIH or DFO (20-100 uM), then subjected to ALA (0.5 mM) for 2h and irradiated with low doses of UVA (5-50 kJ/m2). The quantification of intracellular PPIX was carried out by both HPLC and spectrofluorimetry after treatments of cells with ALA alone or combined with chelators. Cell death was examined 24h after UVA exposure of ALA+/- chelators-treated cells by flow cytometry using Annexin V-propidium iodide dual staining assay.

Results: Pretreatment of HaCaT cells with ALA caused a substantial increase in the intracellular levels of PPIX which in turn sensitized the cells to very low non-cytotoxic UVA doses. Pre-treatment with DFO, PIH and SIH followed by ALA treatment further enhanced the PPIX level in HaCaT cells and caused an additional level of photosensitization to low UVA doses. Among the chelators used, SIH combined with ALA provided the most efficient increase in PPIX and cell killing following UVA irradiation, even at a lower SIH concentration of 20 uM. UVA-based ALA-PDT combined with SIH appears therefore to be a promising modality for topical PDT.
Conclusion: The high lipophilicity of SIH which facilitates skin penetration and its potent cytotoxicity at low UVA doses should therefore allow the current modality for topical PDT to be improved, through a reduction of the time of irradiation and therefore the duration of pain experienced through the treatment.
[1] Buchczyk DP, Klotz LO, Lang K, Fritsch C, Sies H. Carcinogenesis 2001; 22:879-83.

Poster #223
THE DOWN-REGULATION OF FERRITIN HEAVY CHAIN AS AN ADJUVANT THERAPY IN HUMAN GLIOMA
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Pennsylvania State University College of Medicine
(Presented By: James Connor)

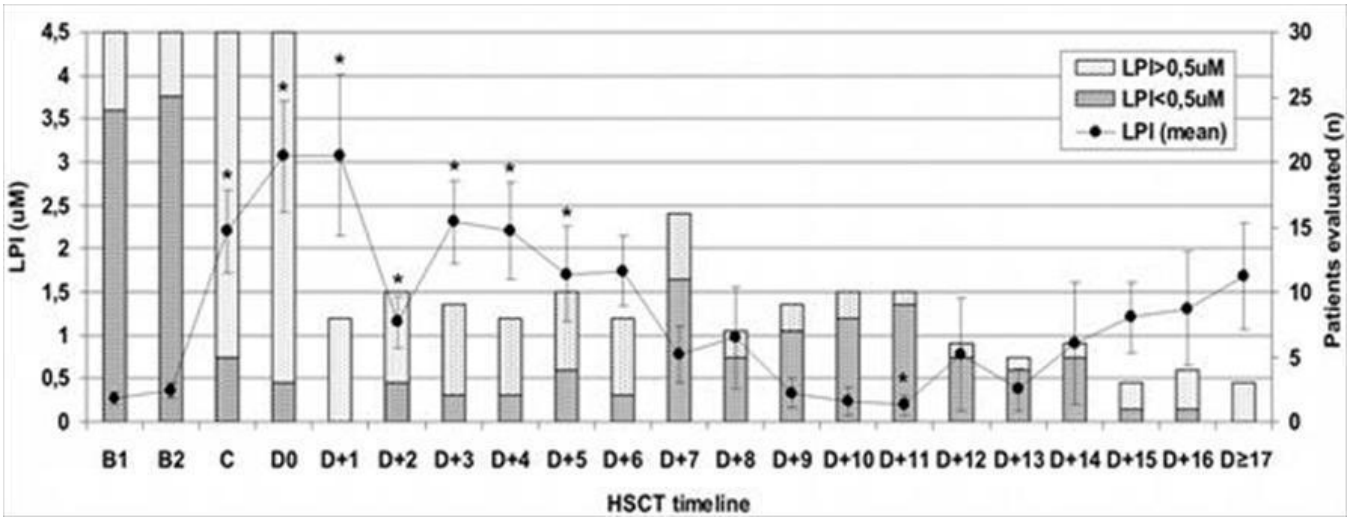
Introduction: Increased iron requirements and heightened iron metabolism is among the key characteristics of highly proliferative cancer cells. As the major iron storage protein, ferritin expression is elevated in cancer cells and we have previously shown that ferritin down-regulation in human glioblastoma cells increases their sensitivity to chemotherapeutic agent both in vitro and in vivo. In this study, we report that the sensitization of H-ferritin down-regulation in glioma cells against radiation and suggest the potential of H-ferritin down-regulation as an adjuvant therapy in human glioma.
Methods and Materials: Down-regulation of H-ferritin was performed in human glioma cell line U251 through a nanotechnology-based transfection platform and effective knockdown efficiency was achieved. A transient decrease in transferrin receptor level was also observed, suggesting a release of intracellular iron with loss of ferritin. This iron release in turn produced intracellular oxidative stress, demonstrated by protein oxidation damage, as well as a decrease in the stability of hypoxia-inducible factors (HIFs), which is an indicator of radioresistance. In U251 cells, exposure to radiation resulted in protein oxidation which could be exacerbated by H-ferritin downregulation.
Results: Previously we have demonstrated a DNA protection role for H-ferritin. Here we report that the down-regulation of H-ferritin is associated with increased DNA vulnerability when cells were exposed to ionizing radiation. Moreover, the activation of DNA repair mechanisms was impaired when H-ferritin was absent. The in vitro data suggest a synergistic effect of radiation and H-ferritin down-regulation.
Conclusion: Additionally, we expand our research into CD133-positive glioma stem cells (GSCs), which are notoriously resistant to anti-cancer treatment. Down-regulation of Hferritin in these cells inhibited cell proliferation in vitro. With an intracranial glioma model established by GSC implantation, we demonstrated that the survival was significantly prolonged by H-ferritin siRNA transfection through intravenous injection, in tumor bearing mice treated with first-line drug Temodar. This study supports the potential of H-ferritin siRNA as an adjuvant therapy in glioma treatment.

Poster #224
ASSESSMENT OF LABILE PLASMA IRON IN PATIENTS SUBMITTED TO HEMATOPOIETIC STEM CELL TRANSPLANTATION
Flávio Naoum, MD, PhD, Breno Espósito, PhD, Lílian Ruiz, MD, Milton Ruiz, MD, PhD, Paula Tanaka, MD, PhD, Juliana Pereira, MD, Rodolfo Cançado, MD, PhD and José Barros, MD, PhD
Academia de Ciência e Tecnologia
(Presented By: Flávio Naoum)

Introduction: Most patients undergoing myeloablative conditioning for hematopoietic stem cell transplantation (HSCT) present with disturbances in iron homeostasis mainly due to suppression of erythropoiesis and release of iron from liver and bone marrow cells. There is a concern that labile plasma iron (LPI), the redox-active and toxic form of iron, can be involved in the occurrence of toxicity and other complications commonly observed in the early post-HSCT period.
Methods and Materials: In order to better understand LPI kinetics and its clinical determinants and implications, we performed sequential LPI determinations before and at least 3 times per week after conditioning until engraftment in 30 HSCT adult patients (25 auto and 5 allo-HSCT) and correlated the time of exposure to increased LPI levels (in days) with transplant characteristics and complications.
Results: Increased LPI levels were present after conditioning in 28 patients for a mean of 8 days (range: 1-18 days), returning to normal range upon engraftment in 25 patients (**Figure**). Time of exposure to increased LPI levels was positively correlated with duration of neutropenia ($p = 0.013$), time to engraftment ($p = 0.005$) and number of red blood cell transfusions after HSCT ($p = 0.02$). Clinical determinants associated with longer exposure to increased LPI were allo-HSCT (vs. auto-HSCT; $p = 0.001$), conditioning with busulfan-based regimens (vs. melphalan; $p < 0.001$) and a higher previous transfusion burden (≥10 vs. <10 units; $p = 0.02$). By logistic regression, baseline LPI levels predicted occurrence of toxicity grade III or IV ($p = 0.038$) and a trend regarding occurrence of fever ($p = 0.063$).

Conclusion: In conclusion, our study shows that LPI kinetics is closely related to clinical parameters of aplasia and engraftment, and the duration of exposure to increased LPI levels is influenced by type of transplant, type of conditioning and transfusional setting. Measuring LPI before starting conditioning offers an opportunity to predict toxicity and, perhaps, the need of chelation therapy.

Figure. LPI levels (mean±SEM) and proportion (increased vs normal) in 30 HSCT patients. LPI levels <0,5µM are considered normal. B1 and B2 are baseline levels (before conditioning) and C represent levels 48h post-conditioning. (*p<0.05 in relation to mean LPI baseline levels).



Poster #225

IRON REDUCTION THERAPY IMPROVES SERUM ALPA-FETOPROTEIN LEVELS IN PATIENTS WITH CHRONIC HEPATITIS C VIRUS INFECTION

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(Presented By: Fumiaki Kimura)

Introduction: Iron reduction therapy (IRT) has been evaluated by its anti-inflammatory and anti-carcinogenic effects in patients with chronic hepatitis C virus infection (CHCVI). Elevation of serum alpha-fetoprotein (AFP) levels in CHCVI has been reported to be one of the most important predictors for hepatocelluler carcinoma. But the change in AFP levels after treatment with IRT in CHCVI is still unknown. In this study we clarify the efficacy of IRT on AFP in CHCVI as anti-carcinogenic effects.
Methodology: In 20 patients who were serologically and virologically diagnosed as CHCVI, IRT was carried out until serum ferritin levels reached 20 ng/mL. Before treatment, the median serum AFP level was 12.5 ng/ml and AFP values ranged from 5.5 to 169 ng/ml. IRT is composed of low iron diet (LID) with phlebotomy or erythrocytapheresis. LID and erythrocytapheresis were carried out along with the methods already shown in Bioiron 2005 and 2007, respectively. Phlebotomy was performed biweekly with 200 ml or 400 ml of blood being removed. Serum AFP, ferritin, ALT, and HCVRNA levels during IRT were investigated. These treatments were approved by the review boards evaluating research in human subjects at our hospital. The patients provided written informed consent prior to this treatment.
Results: During IRT, serum AFP levels were significantly improved from 30.1±41.7 ng/ml to 10.4±7.0 ng/ml (p=0.049) in parallel with significant reduction of ferritin levels from 235.9±129.2 ng/ml to 16.2±3.2 ng/ml (p=3.51E-07) without virological response to treatment. ALT levels were from 86.6±54.0 IU/ml to 55.6±33.9 IU/ml (p=0.16). Any adverse effects were not observed. All procedures around IRT were carried out safe.
Discussion : Conventionally, AFP reduction effects in patients with CHCVI have been observed only in antiviral therapy with interferon, ribavirin, etc. In this IRT study, same effects in patients with CHCVI were observed.
Conclusion: This study suggests that IRT has a possibility to improve serum AFP levels in patients with CHCVI as anti-carcinogenic effects.

Poster Abstracts

Poster #226

DEVELOPMENT AND EVALUATION OF A QUANTITATIVE LATERAL FLOW IMMUNOASSAY FOR THE DETECTION OF FERRITIN AS POINT-OF-CARE TESTING

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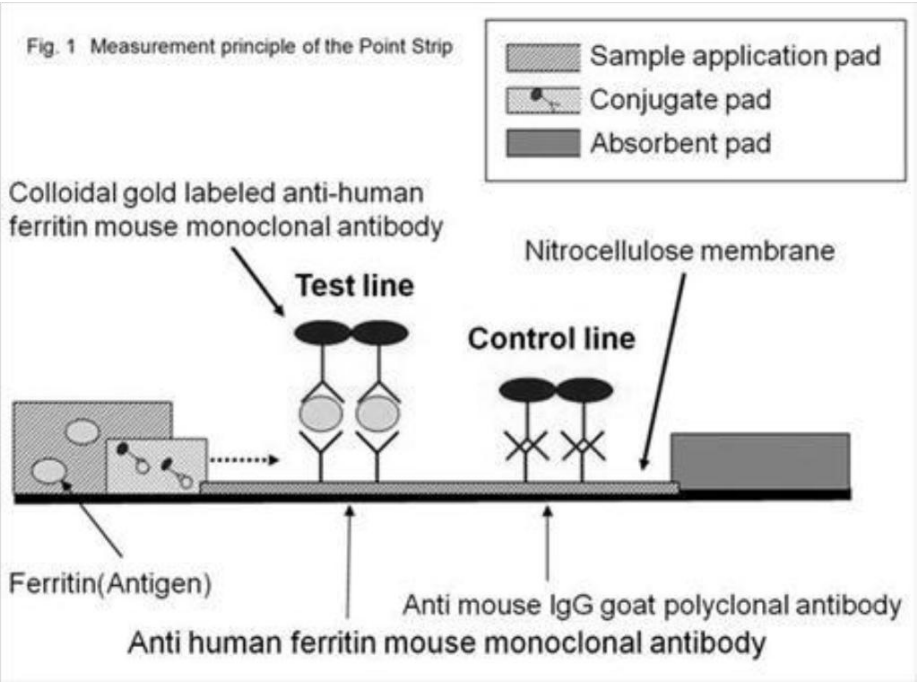
(Presented By: Fumiaki Kimura)

Introduction: Serum ferritin is useful to evaluate the amount of storage iron in the body. Recently, a slight increase of the amount of stored iron has been pointed out to be the risk of inflammation and carcinogenesis in chronic hepatitis C (CHC). In consideration of the importance of the measurement of serum ferritin, we developed a quantitative lateral−flow colloidal gold−based serum ferritin immunoassay system consisted of Point Reader (USHIO INC, Tokyo, Japan) and Point Strip Ferritin (PSF)−100 and 500 (ASKA Special Laboratory, Kawasaki, Japan). In this paper, we evaluated the performance of this system at our hospital.

Methods: PSF-100 and 500 consist of sample pad, conjugated pad, solid face antibody part, and absorption pad (Fig.1). Colloidal gold labeled anti−human ferritin mouse monoclonal antibody is contained within Conjugated pad. Anti human ferritin mouse monoclonal antibody and anti−mouse IgG goat polyclonal antibody is immobilized onto Test line and Control line of the solid face, respectively. When a sample contains ferritin, the ferritin binds to a labeled antibody and forms complex in a conjugate pad. The formed complex and residual labeled antibody move along nitrocellulose membrane, are captured onto Test line and Control line, respectively, and produce red-brown lines. The CCD camera is equipped in Point Reader which is a quantitative analyzer, and quantifies the test line images using a method, namely “the determination of the concentration from the image (JP.PAT)”. Information of Calibration is registered when Point Reader reads QR Code on a Cal. Strip for every lot, and it calculates the concentration of ferritin based on this information. The result is obtained in 5 minutes. In this assay system, the repeatability (intra day), reproducibility (inter day), linearity, and interference were tested and evaluated. Comparability was examined with an automated reference method. This study was approved by the review boards evaluating research in human subjects at our hospital.

Results: The intra−reproducibility were CV6.5% (mean 10.1 ng/ml, n=10), CV6.9% (mean 30.5 ng/ml, n=10), and CV5.7% (mean 69.9 ng/ml, n=10) for PSF−100 and CV7.4% (mean 88.1 ng/m, n=10), CV6.5% (mean 280.5 ng/ml, n=10), and CV5.4% (mean 357 ng/ml, n=10) for PSF-500. The inter−reproducibility were CV3.3% (mean 114.4 ng/ml, n=10), CV3.4% (mean 219.6 ng/ml, n=10), and CV7.7% (mean 408.0 ng/ml, n=10) for PSF−500. No interference with both assays was observed at the tested concentrations in the presence of various interferants such as hemoglobin, bilirubin, chylomicron, rheumatoid factor and ascorbic acid. The ideal linearity extrapolated to the original point in PSF−500 was obtained in a dilution test. Serum samples from patients with various diseases were analyzed by the developed assays. Good correlations were observed in PSF−100 ($y = 0.935x - 0.391$, $r = 0.971$, n=50) and PSF−500 ($y = 1.058x - 2.502$, $r = 0.969$, n=71) when data was compared with that obtained through a laboratory method.

Conclusion: The method is a simple, rapid, and reliable for quantifying serum ferritin levels as point−of−care testing. Therefore, this system will be useful to carry out phlebotomy in patients with CHC.



Poster #227

INCREASED HEPATIC OXIDATIVE DNA DAMAGE IN PATIENTS WITH NONALCOHOLIC STEATOHEPATITIS WHO DEVELOP HEPATOCELLULAR CARCINOMA

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Sapporo Medical University
(Presented By: Koji Miyanishi)

Background: Recently, the rate of onset of hepatocellular carcinoma (HCC) in patients with nonalcoholic steatohepatitis (NASH) has been reported to be comparable to that in chronic hepatitis C cases; however, the precise mechanism contributing to carcinogenesis in such patients remains unclear. Although increased oxidative stress is presumed to play a role in carcinogenesis in NASH, this relationship remains to be directly proven. In this study, we investigated the involvement of oxidative DNA damage in hepatocarcinogenesis in patients with NASH.
Methods: This study included nonalcoholic fatty liver disease patients who visited our university hospital (n=49). The cohort included 30 NASH cases without HCC (NASH without HCC), 6 HCC patients with NASH (NASH-HCC), and 13 patients with simple steatosis (SS). In addition, resected liver tissue samples from the following cases were employed as controls for analyzing the amount of 8-OHdG in the liver tissue: 11 cases with chronic hepatitis B (HBV-HCC), 17 with chronic hepatitis C (HCV-HCC), 11 cases with alcoholic liver disease (ALD-HCC), 9 cases of HCC due to unknown causes, and 8 cases with liver hemangioma and focal nodular hyperplasia who underwent hepatectomy at our hospital. Quantitative immunohistochemistry using with a KS-400 image analyzing system was used for 8-hydroxy-2'-deoxyguanosine (8-OHdG) detection.
Results: In terms of pathological findings, there was a significant difference in intralobular inflammation ($P < 0.001$), ballooning ($P < 0.001$), and NASH activity score (NAS) ($P < 0.001$), as well as in the and grade ($P < 0.001$) and stage ($P < 0.001$) according to Brunt's classification, between patients with SS and those with NASH without HCC. There were no significant differences but not in any category between NASH-HCC and patients with NASH without HCC cases and those with NASHHCC. As the results for the comparison of 8-OHdG content in non-cancerous liver tissue in patients with different background liver diseases, there was no significant difference between HCV-HCC and NASH-HCC cases (relative immunohistochemical staining intensity: 5.820 vs. 8.605, $P = 0.055$), whereas the 8-OHdG content in non-cancerous tissue was significantly greater in NASH-HCC cases versus HBV-HCC cases (8.605 vs. 2.605, $P = 0.002$). Moreover, the level in NASH-HCC cases was significantly greater than in ALD-HCC cases (8.605 vs. 4.075, $P = 0.008$) and undetermined cases (8.605 vs. 2.783, $P = 0.003$).The 8-OHdG content in liver tissue in NASH-HCC cases was significantly different from that in any of the other cases. The median immunostaining intensity was 8.605 in NASH-HCC cases, which was significantly greater than in cases of NASH without HCC (4.845, $P = 0.003$). Multivariate analysis using hepatic 8-OHdG content as a factor in addition to age and FBS showed a significant difference in clinicopathological factors between NASH-HCC and NASH without HCC cases. Old age ($P = 0.015$) and high relative immunostaining intensity for intrahepatic 8-OHdG ($P = 0.037$) were identified as independent factors. The intrahepatic 8-OHdG content showed a significant correlation with serum ferritin (adjusted values) and liver iron staining grade.
Conclusions: 8-OHdG content in liver tissue may serve a marker of oxidative stress as a result of iron overload and it could be particularly useful for predicting hepatocarcinogenesis.

Poster #228

ANAEMIA IN INFLAMMATION-ASSOCIATED COLONIC TUMOURIGENESIS

Anita Chua[1], Desiree Ho[1], Borut Klopcic[1], John Olynyk[2], Ian Lawrance[3] and Debbie Trinder[1]
[1]University of Western Australia; [2]Fremantle Hospital; [3]Centre for Inflammatory Bowel Diseases, Fremantle Hospital
(Presented By: Anita Chua)

Introduction: Inflammatory bowel disease (IBD) is characterised by chronic intestinal inflammation and is associated with an increased risk of colon cancer. Anaemia is a common systemic complication of IBD and is treated with iron supplementation. The aim of this study was to investigate the effects of dietary iron on colonic inflammation and tumourigenesis, anaemia, and the regulation of iron metabolism using a mouse model of inflammation-associated colon cancer.
Methods and Materials: Mice were fed either an iron-supplemented (1% carbonyl iron) or control iron (0.02% iron) diet for 4 weeks prior to administration of a single dose of azoxymethane (AOM; 7.4mg/kg wt i.p.) and 1-3 cycles of dextran sodium sulphate (DSS; 2% w/v in drinking water for 7 days followed by 14 days on water) to induce colonic inflammation and tumourigenesis. Colonic inflammation and tumour development were assessed by colonoscopy and histology. Haematology was measured using standard techniques. Gene expression was determined by real-time PCR, plasma IL-6 by ELISA and phosphorylation of Stat3 by immunoblotting.
Results: In AOM/DSS-treated mice, colonic inflammation was more severe ($p<0.05$) and the number and size of colonic tumours were increased with dietary iron loading ($p<0.05$). Colonic IL-6 gene expression and Stat3 phosphorylation were increased by AOM/DSS treatment and were enhanced by dietary iron ($p<0.05$). Immunofluorescence demonstrated that both ferritin and IL-6 co-localised with $F4/80^+$ macrophages in the lamina propria of the colonic mucosa in iron-loaded mice suggesting that iron had a direct effect on the IL-6 producing-macrophages. Iron deficiency anaemia was observed in both iron-loaded and control mice following AOM/DSS treatment with decreased haemoglobin levels, MCH and MCV and increased reticulocyte count ($p<0.05$). These mice also exhibited hypoferraemia ($p<0.05$) and increased liver iron concentration ($p<0.01$)

indicating the presence of anaemia of inflammation. Dietary iron supplementation, however, did not improve the anaemia. Iron loading increased liver BMP6 gene expression ($p<0.05$) whilst colonic inflammation had no effect. Liver Hamp1 and Smad7 gene expression was upregulated by dietary iron and downregulated by colonic inflammation despite increased plasma IL-6 levels ($p<0.01$) and enhanced liver Stat3 phosphorylation ($p<0.05$). Splenic gene expression of the putative erythroid signalling molecule TWSG1 was elevated with colonic inflammation ($p<0.0001$) whilst GDF15 was increased only when both inflammation and iron were present ($p<0.001$).
Conclusion: In conclusion, these findings suggest dietary iron promotes colonic inflammation and tumour development via an IL-6/Stat3-mediated pathway. Colonic inflammation was accompanied by the presence of both iron deficiency anaemia and anaemia of inflammation which were not alleviated by dietary iron supplementation. Hepcidin expression was downregulated despite the presence of iron loading and inflammation and this is likely to be due to the presence of anaemia. TWSG1 or transferring saturation may contribute to the erythroid-dependent downregulation of hepcidin.

Poster #229
A ROLE FOR THE HEPCIDIN-FERROPORTIN AXIS IN BREAST CARCINOGENESIS?
Oriana Marques, BSc, MSc[1], Alexandra Rêma, BSc[1], Fátima Faria, BSc[2], Berta Martins da Silva, PhD[1], Graça Porto, MD, PhD[2] and Carlos Lopes, MD, PhD[1]
[1]Institute of Biomedical Sciences Abel Salazar; [2]Institute of Molecular and Cellular Biology (IBMC)
(Presented By: Oriana Marques)

Introduction: Iron is an essential functional element, critical in numerous biological processes, such as erythropoiesis, oxidative metabolism and cellular immune response. Although it is a vital biocatalyst, it can also be toxic by producing free radicals through the Fenton reaction. Iron homeostasis is tightly regulated, and in the center of this control lies hepcidin, a 25-amino acid hormone produced primarily in the liver. It regulates negatively the main iron flow by limiting the absorption of dietary iron, the release of recycled iron from macrophages and stored iron by hepatocytes. This is accomplished by hepcidin-mediated phosphorylation and internalization of ferroportin. Cancer cells are thought to have higher requirements for iron, as demonstrated by the higher numbers of transferrin receptors than their 'normal' counterparts. It has been reported that ferroportin is drastically decreased in breast cancer. Our hypothesis is that the hepcidin-ferroportin axis may have a different role in the cellular iron regulation in malignancies, namely in their invasive potential.
Methods and Materials: Hepcidin and ferroportin expression was assessed by immunohistochemistry in 291 tissue microarray spots, consisting of 203 tumor and 88 reduction mammoplasty samples, through a semi-quantitative evaluation. Normal human liver was used as a positive control and liver from Hamp-/- mice as a negative control. Hemosiderin deposits were assessed by Perls' Prussian Blue staining, and evaluated as positive or negative in the epithelial and stromal inflammatory cells. Statistical significance was calculated using Pearson's Chi-Square and One-Way ANOVA test as appropriated. Significance was accepted at $p < 0.05$ Immunoreactivity for hepcidin in breast epithelial cells, lymphocytes and macrophages was found in 25.1%, 37.6% and 46.8% of breast cancer samples and in 5.7%, 3.7% and 3.8% of mammoplasty samples, respectively ($p< 0.001$). None of the control mammoplasty samples presented hemosiderin deposits, either in the epithelial or in the stromal inflammatory compartment, while in tumor samples 39% did ($p= 0.001$). No significant differences for the presence/absence of ferroportin expression were found between sample types.
Results: Hepcidin expression in epithelial cells, lymphocytes and macrophages was significantly different between diagnosed sample groups (Mammoplasty samples, Ductal Carcinomas In Situ [DCIS] and Invasive Ductal Carcinomas [IDC]) ($p< 0.05$). Hepcidin expression is higher in DCIS for the cell types evaluated. Summarily, mammoplasty samples may be distinguished from IDC (Epithelial Cells, $p= 0.024$, Lymphocytes, $p= 0.008$ and Macrophages, $p= 0.004$), and DCIS (Lymphocytes, $p= 0.010$) based on hepcidin expression. Conversely, DCIS presented the lowest ferroportin expression in epithelial cells, although the differences were not significant between groups. The presence of hemosiderin deposits was also positively correlated with hepcidin expression in lymphocytes and macrophages ($R= 0.377$ and $R= 0.273$, $p< 0.01$, respectively).
Conclusion: The recognition of a higher hepcidin expression in breast cancer lesions supports the idea of a cellular iron homeostasis deregulation. Moreover, our results suggest an additional role for resident stromal cells in the local overexpression of hepcidin and iron withholding in the tumor. The absence of correlation between hepcidin and ferroportin expression suggests the existence of alternative targets for the endogenous hepcidin expression.

Poster #230
TUMORAL VECTORIZATION OF NEW IRON CHELATORS FOR ANTIPROLIFERATIVE ACTIVITY: BIOLOGICAL PROPERTIES OF POLYAMINOQUINOLINES
Vincent Corcé[1], Stéphanie Renaud[1], Isabelle Cannie[1], Olivier Loréal[1], David Deniaud[2] and François Gaboriau[1]
[1]Inserm UMR991, CHU Pontchaillou, Rennes, France; [2]CEISAM, CNRS UMR6230, Nantes, France
(Presented By: François Gaboriau)

Introduction : We synthesised a new generation of hybrid chelator-polyamine molecules, designed to decrease iron III in cancerous cells and thus to reduce their proliferation. With these polyaminoquinoline iron chelators that we named Quilamine, the tumor cells are thus targeted in two ways; (i) the uptake of the chelator by the activated polyamine transport system (PTS) in these cells and (ii) the strong dependence of these cells on iron. We demonstrated that the polyamine chain was involved in

the iron(III)-Quilamine complexes and strongly enhanced the iron-chelating capacity of Quilamine compared to that of reference chelators, 8-hydroxyquinolein and O-Trensox.
In the present study we evaluated the biological activity of Quilamines, based on an 8-hydroxyquinoline (8-HQ) scaffold linked by various linkers to various linear polyamine vectors. The influence of the polyamine chain (XYZ) on the selective recognition of different Quilamines by the PTS was evidenced by comparing the antiproliferative activity of nine Quilamines HQ1-XYZ, bearing a single carbon atom linker. The influence of the length and of the chemical structure of the linker on the biological activity was also screened using 6 Quilamines HQN-44, bearing the homospermidine backbone, the most efficient polyamine vector for PTS.
Methods: Selectivity of the Quilamine uptake by PTS was tested by comparing their efficiency on a cell line displaying an efficient PTS (CHO) with that on a PTSdeficient mutant cell line (CHO-MG). In various human tumor cell lines derived from liver and colon, we screened the antiproliferative efficiency of these polyaminoquinolines, including their effect on cell cycle, their cytotoxicity, and their ability to modulate both iron and polyamine metabolisms.
Results: This screening led us to select the Quilamine the most selectively recognized by the PTS, the molecule HQ1-44 which consists of the chelating moiety hydroxyquinoline (HQ) linked to homospermidine (44). We showed that its iron-chelating power is greater than that of O-Trensox, the chelator with the strongest affinity for iron. The antiproliferative effect of HQ1-44 on tumor cell lines originating from human liver carcinoma (HepG2, HUH7, HepaRG) and colon cancer (Caco -2, HCT116) occurs in the micromolar range while the cytotoxicity (LDH release) is only observed at concentrations higher than 100 µM. The antiproliferative activity of this molecule is associated with its selective recognition by the PTS and its combined inhibition of the metabolisms of iron and polyamines, both strongly involved in tumor growth.
Conclusions: Polyaminoquinolines, especially HQ1-44, may be promising compounds for iron depletion as adjuvant treatment in cancer therapies, due to its high affinity and selectivity for iron, and its selective uptake by the PTS. The high antiproliferative activity of HQ1-44, associated with a low cytotoxicity, probably results from its capacity to modulate both iron and polyamine metabolisms.
Acknowledgments : This work was supported by the Conseil Régional Pays de la Loire, the French Ministry of Education, the Ligue National contre le Cancer (Ille et Vilaine/Loire Atlantique committees) and the Association pour la Recherche sur le Cancer (ARC)

Poster #231
NOVEL LIGHT-ACTIVATED CAGED IRON CHELATORS: TARGETED PRODRUGS FOR IRON RELATED-DISORDERS
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(Presented By: Ian Eggleston)

Introduction: Iron chelation therapy (ICT) is a well-established approach for the treatment of a variety of conditions that are associated with harmful levels of iron in the body. The involvement of iron in the pathophysiology of diseases such as cancer, psoriasis, and neurological disorders such as Friedreich's Ataxia has recently attracted significant interest in wider applications of ICT, however such applications require iron sequestration to be targeted to diseased tissues only, thus avoiding depletion of iron in healthy tissue and unacceptable patient side-effects. An ideal solution to this challenge, especially for topically administered therapeutics, is the concept of light -activated caged iron chelators (CICs), which are prodrugs that are inactive themselves as iron chelators, but may be simply activated to release a strong iron chelator in a highly spatially-specific and dose-specific fashion after administration, following targeted exposure to light.
Methods and Materials: To develop our CIC concept [1] further for new therapeutic applications, we have synthesised a range of chelators of the isonicotinoyl hydrazone family, in which a critical iron-binding phenolic function is blocked ("caged") with the photolabile 2-nitrophenylethyl (2-NPE. These are based on the salicylaldehyde (SIH-like), pyridoxal (PIH-like), and 2-hydroxy-1-naphthaldehyde (NIH-like) templates. Upon exposure to a dose of 250 kJ/m2 of UVA, equivalent to an exposure time of 70 minutes midday sunlight, during the summer at northern latitudes, the novel CICs cleanly release the parent chelator and a biologically inert fragment. Extent of uncaging was assessed by analytical HPLC, with comparison with authenticated independently synthesised photoproducts.
Results: The cytotoxicity of the caged and UVA-irradiated derivatives, and the UVA-generated co-product (nitrosophenylketone) was assessed in the spontaneously immortalised human HaCaT keratinocyte cell line. Depending on the precise chelator structure, the toxicity of the parent chelator may be effectively suppressed, but restored upon UVA exposure. To optimise these first generation CICs still further, we have also synthesised prodrugs of salicylaldehyde isonicotinoyl hydrazone (SIH) and pyridoxal isonicotinoyl hydrazone (PIH) in which caging is achieved with groups (6-nitroveratryl, 7-diethylaminocoumarin-4-yl)methyl) that may be more efficiently released at longer wavelengths of light. Clean release of the parent chelator by UVA and modulation of cytotoxicity was again validated by HPLC analysis and MTT assay in HaCaT cells.
Conclusion: This shows the generality of our CIC approach. With suitable caging groups, targeted and wavelength-selective release of chelators can be achieved for a variety of therapeutic applications. In particular, we can target CICs to a specific application (e.g. anticancer, photoprotection) by choosing the optimum caging group-chelator combination for maximum tissue penetration of activating radiation and cytotoxic effect of the released chelator.
[1]. Reelfs O, Eggleston IM, Pourzand C. Cur. Drug Metabol. 2010, 11:242-249.

Poster #232

LIGHT-ACTIVATABLE AND PHOTO-CONTROLLED CAGED-NIH, A PROMISING PRO-IRON CHELATOR FOR THE TOPICAL THERAPY OF SKIN CANCER

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(Presented By: Benjamin L. Young)

Introduction: To date, the behaviour and chemotherapeutic potential of iron chelators in skin tumours has not been extensively studied. For non melanoma skin cancer, a way to meet the need for highly selective, dose-controlled administration of iron chelators is via topical application of light-activatable and photo-controlled 'caged iron chelators' ('CICs') recently developed in our laboratory [1]. The caging groups (e.g. 2-nitrophenyl ethyl, 2NPE) render these compounds temporarily inactive as chelating agents, so 'CICs' do not chelate iron unless activated by external light sources (e.g. UVA), allowing for specific localised release within the targeted tissue and therefore substantially decreasing the need for systemic repeated exposure of the patient to strong iron chelators and their obvious toxic side effects. The marked antitumor activity and the highly lipophilic nature of 2-hydroxy-1-naphtaldehyde isonicotinoyl hydrazone (NIH) [2] make this iron-chelator an attractive candidate ligand for the caged-iron chelation therapy of non-melanoma skin cancer via topical application.
Methods and Materials: In the present study, we first compared the antiproliferative activity of NIH to that of salicylaldehyde isonicotinoyl hydrazone (SIH) and desferrioxamine (DFO) using the spontaneously immortalised human HaCaT keratinocyte cell line, as a model. HaCaT has proved a useful and reliable in vitro model of human skin cell carcinoma. This cell line is hyperproliferative and shows a significantly higher proliferation rate compared to normal human skin keratinocytes and fibroblasts.
Results: The dose response and time course studies with MTT assay revealed that the growth inhibitory effect of NIH is more pronounced than that of SIH but is comparable to that of DFO. In an attempt to relate these findings to an in vivo setting, the colony forming ability assay was performed in parallel and the results showed that in agreement with MTT data, NIH has a much stronger antiproliferative activity than SIH. We also analysed the impact of the chelators on the cell cycle by flow cytometry, using bromodeoxyuridine incorporation. The results showed that compared to SIH, NIH has a higher impact on cell cycle. We then characterised HaCaT cell responses to NIH and its newly synthesized caged derivative i.e. 2NPE-NIH in the presence or absence of extremely low doses of UVA: The MTT assay demonstrated that in the absence of UVA, 2NPE−NIH does not alter the growth of HaCaT cells, but following UVA irradiation provides a significant decrease in growth rate of cells that is comparable to the effect observed with the parental chelator alone. The UVA−irradiated controls did not alter the growth of cells, indicating that the effect observed in CIC−treated irradiated cells is unrelated to radiation but rather related to uncaging of the 2NPE−caging group and release of the active antiproliferative iron chelator NIH.
Conclusion: Taken together, these results suggest that our newly developed caged−NIH which remains inactive inside the cells until its strong iron binding activity and high antiproliferative properties are activated by low doses of UVA, offers a highly selective and dose-controlled alternative for treatment of hyperproliferative skin disorders such as skin cancer.
[1]. Reelfs O, Eggleston IM, Pourzand C. Cur. Drug Metabol. 2010, 11:242-249.
[2]. Yu Y, Kalinowski DS, Kovacevic Z, Siafakas AR, Lovejoy DB, Sharpe PC, Bernhardt PV, Richardson DR. J. Med. Chem. 2009, 52:5271-5294.

Poster #233

MEAT AND HEME IRON AND OESOPHAGEAL ADENOCARCINOMA IN THE EPIC-EURGAST STUDY

Paula Jakszyn
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(Presented By: Paula Jakszyn)

Background: Although recent studies suggest that high intakes of meat intake and heme iron are risk factors for several types of cancer, studies in relation to oesophageal adenocarcinoma (OAC) are scarce. Previous results in the European Prospective Investigation into Cancer and Nutrition (EPIC) based on a relatively small number of cases suggested a positive association between processed meat and OAC. In this study we investigate the association between intake of different types of meats well as heme iron intake, and OAC risk in a larger number of cases from EPIC.
Methods: The study included 481,419 individuals and 137 incident cases of OAC that occurred during an average of 11 years of follow-up. Dietary intake of meat (unprocessed/processed red and white meat) was assessed by validated center-specific questionnaires. Heme iron was calculated as a type-specific percentage of the total iron content in meat.
Results: After adjusting for relevant confounders we observed a statistically significant positive association of OAC risk with heme iron and processed meat intake, with HR: 1.67 (95%CI: 1.05-2.68) and HR: 2.27 (95%CI: 1.33-3.89) respectively for comparison of the highest vs. lowest tertile of intake. After calibration, the positive associations with dietary intake of heme iron (HRmg/2000Kcal: 1.30, 95%CI: 0.98-1.73) and processed meats (HR25g/2000kcal1.33, 95%CI: 0.97-1.82) were not significant.
Discussion and Conclusions: Our results suggest a potential association between higher intakes of processed meat and heme iron and risk of OAC. Further studies are needed in order to confirm this tentative association as well as to elucidate which specific components present in processed meat are responsible.

Poster #234
IRON AND CANCER RISK – A SYSTEMATIC REVIEW OF THE EPIDEMIOLOGICAL EVIDENCE
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(Presented By: Paula Jakszyn)

Background: Iron has been suggested as a risk factor for different types of cancer due to its prooxidant activity which can lead to more oxidative stress and DNA damage.
Objective: To systematically review and analyze the link between iron intake and body iron status and the risk of developing cancer.
Methods: In this article we analyzed 36 publications that responded to our specific search criteria using the PUBMED database between 1995-2012. These criteria included articles with information on dietary iron, total iron, heme iron, iron biomarkers, and cancer risk.
Results: In this review we identified 36 publications and retrieved 53 studies. The reason behind this disparity is because most publications referred to more than one exposure variable and two referred to more than one tumor (although presented results independently). From the 36 publications identified, 25 were prospective and 11 were case-control studies. Many of these publications (21 studies) referred to gastro-intestinal tract cancer (esophageal, gastric and colorectal), 7 related to breast and 4 to lung cancer. The rest of them investigated other cancers (endometrial, pancreatic, prostate, bladder and oral). All studies took into account the dietary intake of iron and only seven of them also conducted biomarkers analysis. From the 24 studies that reported results on heme iron intake and cancer risk, 10 found a positive association (six were statistically significant). From the 19 studies that reported data on dietary iron, the results provided mixed results. Regarding iron biomarkers, seven studies were found, the major part were gastro-intestinal cancer studies (six studies) and mostly showed a negative association with cancer risk, especially with serum ferritin (five were statistically significant).
Conclusion: Despite the heterogeneity of the results obtained in this review, there is some tendency towards the positive association between heme iron intake and cancer risk. Moreover, data on iron biomarkers was insufficient. Nevertheless, serum ferritin clearly showed a negative association with gastro-intestinal cancer risk and TIBC showed a tendency towards a positive association with cancer risk. For this reason, more studies combining research on iron biomarkers, dietary iron intake and also genetic output need to be conducted for better understanding the role of iron on cancer development.

Poster #235
THE POLYAMINOQUINOLINE HQ1-44, A NEW IRON CHELATOR VECTORIZED BY POLYAMINE INHIBITS THE TUMOR GROWTH IN XENOGRAFTED IMUNODEPRESSED MICE
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(Presented By: Stéphanie Renaud)

Introduction: Iron chelation in tumor cells has been reported as potentially useful during antitumoral treatment. Quilamines are hybrid molecules associating one hydroxyquinolein iron chelators moiety with a polyamine backbone. These polyaminoquinoline were designed to target and inhibit the tumor cells growth in which the polyamine transport system (PTS) is overactivated. Among all the synthesised molecules, we demonstrated in vitro, in various tumor cells lines, that the Quilamine HQ1-44 showed the highest selectivity for the PTS and consequently the highest antiproliferative efficiency, especially in the human adecarcinoma cell line (HCT116). The objective of this study is to prove the Quilamine concept by demonstrating in vivo the antitumoral efficiency of HQ1-44 in murine model.
Methods: HCT116, a human colon adenocarcinoma cell line showing the most efficient PTS, were xenografted in Swiss nude mice. Seven days after xenograft, mice were treated with various doses of HQ1-44 by daily intraperitoneal injection for two weeks. In parallel, xenografted mice were also treated with cisplatin (40 mg.kg-1) used as a reference antitumoral agent.
Results: The absence of toxicity and safety of the molecule HQ1-44 was preliminary demonstrated for dose up to 60 mg.kg-1. The treatment with the compound HQ1-44 at 40 mg.kg-1 inhibits the tumor growth by approximately 38%. This inhibition is similar to that observed with cisplatin treatment (4 mg.kg-1). We do not observe any side effects associated to treatment with HQ1-44 while cisplatin causes a sharp decrease in the weight of animals, in a lot of organs including spleen and kidneys as well as a severe anemia. The treatment with Quilamine HQ1-44 modulates both iron and polyamine metabolisms as deduced from their plasmatic levels.
Conclusion: The antitumoral activity of the polyaminoquinoline HQ1-44, associated with its absence of toxicity brings the first line of validation of our Quilamine concept. This compound may be promising as adjuvant treatment in cancer therapies due to its selectivity for tumor cells, capacity to inhibit both iron and polyamine metabolisms.
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Poster #236

DO ALTERED CHOLESTEROL AND LIPID HOMEOSTASIS IN C282Y HFE MUTANT EXPRESSING CANCERS: IMPLICATIONS FOR CANCER THERAPY?

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(Presented By: Sang Lee)

Introduction: Disruptions of both iron and cholesterol homeostasis have been implicated in several human cancers.
Methods and Materials: Therefore, we studied the effects of C282Y HFE mutant on lipid (cholesterol and sphingolipid) metabolism and its possible relevance to cancer phenotypes.
Results: The C282Y HFE mutant expressing human neuroblastoma cells had significantly increased sphingolipid expression/activity and total cholesterol content compared to wild type (WT) HFE expressing cells. Expression of C282Y HFE in the cells also altered expression of a number of genes involved in cholesterol and sphingolipid metabolisms in functional gene arrays. The most robust changes where in the expression of genes and proteins involved in cholesterol uptake and transport which may cause the elevation in cellular cholesterol content observed in C282Y HFE mutant expressing neuroblastoma cells. Since simvastatin, an inhibitor of cholesterol synthesis, has been proposed to be beneficial for the treatment of certain cancers, we determined the effect of decreased cholesterol on the cancer cell survival. Although cells are toxic to simvastatin, a 50% higher concentration of simvastatin was needed to decrease survival of cells expressing C282Y HFE than WT HFE. Because of the significant increase of sphingosine kinase 1 (SphK1) in C282Y cells, we also studied the effect of a SphK1 inhibitor (SKI) on the cell survival. Again, WT HFE cells were more sensitive to the SKI treatment than C282Y HFE cells, reflecting the drug resistant phenotype of C282Y HFE cells. Previously, we reported that iron chelator deferoxamine was cytotoxic to the C282Y HFE cells, but not in WT HFE cells.
Conclusion: In summary our results indicate that C282Y HFE mutant expressing neuroblastoma cells have a phenotype of altered cholesterol and sphingokinase expression that alters their response to chemotherapeutics. They also appear to have an absolute dependence on iron availability. Thus, a combination of decreasing iron availability may be required to increase efficacy of a cholesterol/lipid targeted therapeutic strategy for C282Y HFE expressing cancers.
This work was supported in part by NIH Grant CA167406 and the Tara Leah Witmer Endowment.

Poster #237

HEPCIDIN AND FERRITIN AS PREDICTORS OF BREAST TUMORS

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(Presented By: Italia Bongarzone)

Introduction. Mammography has an acceptable sensitivity, and despite its modest specificity, it locates the tumor for definitive biopsy. Pre-surgical diagnosis of breast cancer is often performed by needle biopsy, an invasive technique that may indeed activate growth factor release and enhance tumor growth. A variety of circulating tumor markers have also been described for breast cancer, such as the CA 15.3 and CA 27.29. However, due to the low sensitivity and specificity, they are used in conjunction with other assessments to monitor treatment response in patients with breast cancer. This study aimed to assess if a proteomic signature obtained from plasma samples could assist in the non-invasive diagnosis of breast cancer.
Methods. This study involved 75 cases (10 patients with benign and 65 patients with malignant breast tumors) before surgical resection and 122 healthy volunteers (controls). Plasma samples were analyzed using surface enhanced laser desorption/ionization-time of flight (SELDI-TOF) mass spectrometry.
Results. Protein profiles were acquired in the m/z range from 2000 to 30,000 and more than 40 signals were found overexpressed in malignant tumors. According to the platform algorithm the most differential peaks were identified as hepcidin-25 and ferritin. The capability of hepcidin-25 in predicting benign and malignant breast tumor, evaluated using the area under the receiver operating characteristic ROC-curve (AUC), was weak (AUC 0.64; 95% CI, 0.42-0.86) and good (AUC 0.81; 95% CI, 0.74-0.89), respectively. In contrast the AUC values estimated for ferritin showed, with respect to hepcidin-25, a higher and lower capability to predict benign (AUC 0.73; 95% CI, 0.51-0.96) and malignant (AUC 0.76; 95% CI, 0.67-0.86) breast tumor, respectively. The comparison of hepcidin-25 levels in a independent set of plasma from 61 benign tumor patients showed comparable intensity level between the different histotypes.
Discussion. We detected statistically significant elevations of hepcidin and ferritin levels in breast cancer patients compared with the healthy controls and benign breast disease patients. This is consistent with recent studies have suggested a crucial role of perturbations in ferritin levels and, tightly associated with this, the deregulation of intracellular iron homeostasis. Very interestingly, they are also in accordance with previous results demonstrating high level of hepcidin and low ferroportin expression in breast cancer tissues. Determination of hepcidin levels in breast disease patients may provide more diagnostic value than ferritin, but further studies are needed. Iron metabolism and inflammation pathways are stimulated in the carcinogenesis. Thus, proteins of both these pathways have to be studied in combination with hepcidin and ferritin in a large number of patients with benign or malignant breast tumor to clarify the underlying mechanisms responsible of their increase.
Conclusion. Measurements of plasma hepcidin and ferritin concentrations may be useful in assisting the differential diagnosis of breast tumors and in detecting those are missed by mammography.

Poster #238
HFE GENOTYPE STUDY IN CANCERS
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(Presented By: Sang Lee)

Introduction: Increased frequency of HFE polymorphisms has been observed in hepatocellular carcinoma, ovarian and breast cancer, and high-grade gliomas. However, HFE genotype and their impact in other cancer types have not been studied systematically. Therefore, we determined HFE genotype in multiple human cancer cell lines using gene sequencing and/or a restriction enzyme digestion method after PCR.
Methods and Materials: Among the tested cell lines, we found the H63D HFE mutation in 1 of 6 melanomas, 1 of 2 pancreatic, and 6 of 8 lung cancer cell lines. In addition, we found the C282Y HFE mutation in cervical (1 of 3), colorectal (1 of 8), ovary (1 of 1), and prostate (2 of 4) cancer cell lines. Because we observed the highest frequency of HFE gene mutations in lung cancer cell lines, we further determined the frequency of HFE polymorphisms in blood from Caucasian lung cancer patients (squamous cell and adenocarcinoma) seen in our Cancer Institute. Among 53 adenocarcinoma patients, the frequency of H63D and C282Y HFE mutations was 32.1% (22.6% heterozygous, 9.4% homozygous) and 13.2% (9.4% heterozygous, 3.8% homozygous), respectively.
Results: These data indicate that there is a significantly higher frequency of H63D HFE homozygosity in adenocarcinoma patients compared to the normal population (9.4% vs 2.4%). In squamous cell lung cancer, the frequency of H63D and C282Y HFE mutations was 14.6% (all heterozygous) and 12.2% (9.8% heterozygous, 2.4% homozygous), respectively. This demonstrates tend to lower frequency of the H63D HFE polymorphism in squamous cell lung cancer patients. Most of the lung cancer patients had low grade (stage 1 or 2) disease, thus survival data is not available at this time to determine any relationship between HFE genotype and patient outcomes. Next, we determined HFE genotype frequency in the genome-wide association study (GWAS) of lung cancer database. H63D HFE mutant status was not available in this database. At present, we found that there was no frequency difference of the C282Y HFE mutation (due to combined dataset for all lung cancer types) except in the EAGLE (Environment and Genetics in Lung Cancer Etiology) study. The frequency of C282Y HFE polymorphisms in lung cancer patients in the EAGLE study was significantly lower ($p=0.03$) than that of controls. Females had a significantly higher ($p=0.04$) C282Y HFE mutation rate than males (5.53% vs 3.23%) in this study. Lastly, we determined HFE genotype in neuroblastoma GWAS. H63D HFE mutant status is not available, but the frequency of C282Y HFE mutant in neuroblastoma was not different compared to normal population. We observed an association between the C282Y HFE mutation and established adverse prognostic factors, particularly MYCN amplification ($p=0.03$), in very young (age <547 days) neuroblastoma patients.
Conclusion: In conclusion, our results suggest that the frequency of H63D HFE variant is altered in lung adenocarcinoma and squamous cell lung cancer. Our data also indicate that the C282Y HFE mutant may suggest a poor prognosis in younger age of neuroblastoma patients.
This work was supported in part by the Tara Leah Witmer Endowment and Pennsylvania Department of Health.

Poster #239
EPIGENETIC REGULATION OF HEPCIDIN EXPRESSION IN NON-VIRAL HEPATOCELLULAR CARCINOMA: RESULTS OF A GENOME-WIDE STUDY
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(Presented By: Michela Corbella)

Introduction: Epigenetics is an emerging field of molecular biology that investigates those heritable and potentially reversible mechanisms able to modulate gene expression without modifying the base sequence of DNA. One of the main epigenetic feature is DNA methylation that consists in the covalent binding of a methyl group to the 5'carbon of cytosine occurring at CpG dinucleotide sequences and affects gene expression. Increasing interest has been given to the role of DNA methylation in cancer development including hepatocellular carcinoma (HCC). A major risk factor for HCC is chronic alcohol consumption, although the mechanisms underlying the alcohol-related liver carcinogenesis are still incompletely understood. Alcohol is linked both to carcinogenesis and to aberrant DNA methylation by interfering with methyl groups transfer within one-carbon metabolism through reactions mainly occurring in the liver. Hepcidin is a liver peptide hormone involved in iron homeostasis and in the innate immune response. Hepcidin (*HAMP*) has been already shown to be repressed in HCC, and a transcriptional down-regulation has been also reported in the liver of alcoholic subjects. Aim of the present study was to evaluate whether *HAMP* is epigenetically regulated by DNA methylation at promoter site and its modulated expression possibly associated to alcohol-related HCC.
Methods: Eight male patients undergoing curative surgery for HCC were selected to be heavy drinkers (≥36 g ethanol/die) and negative for HBV and HCV serologic markers. Other exclusion criteria were: chronic and/or acute inflammatory diseases and haematological disorders (including autoimmune liver diseases and hereditary hemochromatosis) and the presence of decompensate liver cirrhosis (Child-Pugh B, C). Histological confirmed HCC and cancer-free tissues were obtained from each

patient. In HCC tissue compared to homologous cancer-free liver tissue, genome-wide promoter DNA methylation profile was assessed by Methylated DNA Immunoprecipitation (MeDIP, Nimblegen) and microarray analysis, whereas gene expression profile was obtained by an array-based technique. Hepcidin gene expression levels were subsequently validated by RealTime qPCR.
Results: The merging of array-based DNA methylation and gene expression data showed promoter hypermethylation associated with transcriptional repression in HCC tissue in 160 genes. Interestingly, among those genes, for the first time *HAMP* down-regulation was found to be associated with promoter hypermethylation. The finding of a decreased hepcidin gene expression in HCC as compared with cancer-free liver tissue was confirmed by RealTime qPCR in all patients. Validation by bisulfite sequencing is ongoing.
Conclusion: Very interestingly, these results show that *HAMP* is transcriptionally repressed by promoter DNA methylation in non-viral alcohol-related HCC tissue as compared to cancer-free liver tissue. Further investigations are warranted to better elucidate the role of DNA methylation in hepcidin-mediated iron regulation pathway in hepatocellular carcinogenesis.

Poster #240
DIFFERENTIAL EXPRESSION OF MULTI-COPPER FERROXIDASES IN INVASIVE DUCTAL CARCINOMA
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(Presented By: Zouhair Attieh)

Introduction: Increasing evidence of the differential expression of iron regulatory proteins in carcinogenesis has been reported including decreased levels of the tissue iron transporter ferroportin in human breast cancer epithelial cells. Ferroportin-mediated released iron is oxidized by one member of the multi-copper ferroxidase (MCF) family of proteins, which includes ceruloplasmin (Cp), hephaestin (Heph) and zyklopen (Zp), to be transported in plasma by transferrin.
Methods and Materials: In the current study, we investigated MCF RNA and protein differential expression in invasive ductal carcinoma compared to normal tissue samples in addition to MCF10A, MCF7 and MDA -MB231 cell lines. Expression of Zp, Heph and Cp at the RNA level was performed in paraffin−embedded breast reduction mammoplasty samples (normal), invasive ductal carcinoma samples (DC), organoid preparations, MCF10A, MCF7 and MDA-MB231 cell lines using reverse transcriptase polymerase chain reaction (RT-PCR) and quantitative polymerase chain reaction (qPCR) using Zp, Heph or Cp specific primers. MCF protein expression was studied in normal and DC samples employing immunostaining with specific anti-sera to Zp, Heph or Cp.
Results: Obtained results show that, similar to ferroportin, there is a marked decrease in Zp transcript in ductal carcinoma samples compared to organoid preparations. Similarly, less Zp transcript was observed in MCF7 and MDA-MB231 cells compared to MCF10A cells. Immunostaining with Zp-specific antisera showed less staining in ductal carcinoma tissue compared to normal ones. No Heph RNA or protein expression was observed in all tissue samples or cell lines tested. Cp RNA expression was observed in MCF10A, MCF7 and MDA-MB231 cells. Further studies are conducted to verify any differential expression of Cp in normal versus carcinoma samples whether at the RNA or protein level in paraffinated tissue and related cell lines.
Conclusion: Our results conclude that human mammary epithelial cells express two of the three MCF paralogs and that this expression is regulated. Further studies are needed to elucidate regulatory mechanisms that may include miRNA-mediated down-regulation of gene expression. Profound investigation of MCF regulated gene expression, in addition to other iron transport proteins, would greatly impact our understanding of carcinogenesis providing novel markers for the identification of malignant tissues and paves the way for potential therapeutic strategies.

Poster #241
A LINK BETWEEN PREMENOPAUSAL IRON DEFICIENCY AND BREAST CANCER MALIGNANCY
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(Presented By: Xi Huang)

Introduction: Breast cancer (BC) patients diagnosed at a young age (e.g., <45 years old) have lower survival and higher recurrence rates when compared to their older counterparts. Cancers in these young patients are more likely to be of a higher histological grade with mostly estrogen receptor-negative status and more readily metastasize to other organs. However, specific risk factors leading to this poorer outcome have not been identified. One candidate is iron deficiency, as this is common in young women and a clinical feature of young age.
Methods and Materials: In the present study, we used immuno-competent and immuno-deficient mouse xenograft models as well as hemoglobin as a marker of iron status in young BC patients to demonstrate whether host iron deficiency plays a pro-metastatic role. We showed that mice fed an iron-deficient diet had significantly higher tumor volumes and lung metastasis compared to those fed normal iron diets.
Results: Iron deficiency mainly altered Notch but not TGF-beta and Wnt signaling in the primary tumor, leading to the activation of epithelial mesenchymal transition (EMT). This was revealed by increased expression of Snai1 and decreased

expression of E-cadherin. Importantly, correcting iron deficiency by iron therapy reduced primary tumor volume, lung metastasis, and reversed EMT markers in mice. Furthermore, we found that mild iron deficiency was significantly associated with lymph node invasion in young BC patients ($p<0.002$).
Conclusion: Together, our finding indicates that host iron deficiency could be a contributor of poor prognosis in young BC patients. In view of increasing evidence of iron overload in BC, our present study shows that the other end of the iron spectrum, host iron deficiency may play an important role in BC malignancy of young women.

Poster #242
BINDING OF LUMINAL IRON USING ALGINATES AS A THERAPY FOR BOWEL CANCER
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(Presented By: Richard Horniblow)

Introduction: Although iron is essential for homeostasis, epidemiological studies have demonstrated an association with high iron levels and increased cancer prevalence including colorectal cancer. Excess iron in the body can give rise to detrimental effects such as increased reactive oxygen species (ROS) generation through participation in Fenton reaction chemistry. These ROS could potentially result in DNA damage to cells. It is also known that in the presence of a mutated APC gene, a mutation commonly found in colorectal cancer patients, that iron is able to increase tumour cell proliferation. Excess Iron within the bowel therefore not only helps drive nascent tumour cell proliferation but may conceivably also contribute to the formation of tumour initiating mutations in healthy cells.
Methods and Materials: This study sought to determine the effect of luminal iron chelation on colorectal tumourigenesis using the known class of Iron chelators, alginates. Alginates are naturally occurring polymers constructed of alternating manuronic (M) and guluronic (G) acid sub-units and are commonly used in the food and drug industry as stabilisers and encapsulation vehicles respectively.
Results: Here we demonstrated that alginates not only bind iron but also calcium and the level of iron/calcium binding is dependent upon the M:G ratio of the alginate, with a high M:G ratio displaying high Iron binding and reduced calcium binding compared to those with low M:G ratios. Using this knowledge a high M:G ratio alginate was selected for further study in in vivo models of colorectal tumourigenesis. This showed that alginate treatment is able to reduce the tumour burden in APC Min/+ mouse models.
Results: Together this study highlights iron chelation, in particular the use of alginates, as a potential method for the chemoprevention of colorectal cancer.

Poster #243
PRE-TREATMENT WITH THE ORAL CHELATING AGENT ICL670A MAY ENHANCE CHEMOTHERAPY RESPONSE IN OESOPHAGEAL ADEONCARCINOMA
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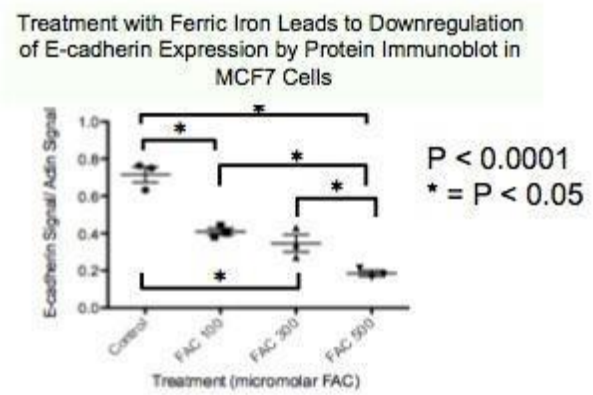
University of Birmingham
(Presented By: Chris Tselepis)

Introduction: The malignant progression of Barrett's metaplasia to OAC is associated with altered expression and function of the pertinent cellular iron transport proteins. These alterations result in increased cellular iron loading which is likely to drive cellular proliferation; a key hallmark of cancer. Experimental iron chelators have demonstrated both anti-neoplastic and chemosensitising properties in a number of cancers; a concept of particular interest to OAC where chemotherapy forms a significant component of the treatment armamentarium but response remains variable. The aim of this study therefore was to investigate the value of the licensed and orally administered iron chelator ICL670A as an adjunct to chemotherapy in OAC. In particular, we wished to ascertain whether or not pre-treatment with the chelator could improve the response of the tumour to subsequent chemotherapy and whether or not it could overcome established therapy resistance.
Methods: The OAC cell lines OE19 and OE33 were treated with ICL670A prior to and/or alongside the standard chemotherapy regimen of Epirubicin, Cisplatin and 5-Fluorouracil (ECF/ECX). In addition, an established OE33 Cisplatin resistant 'clone' was exposed to varying concentrations of Cisplatin with or without ICL670A. Cell viability (MTT) and proliferation (BrdU) assays were performed in order to determine effect in both experiments. A murine xenograft model of OAC was then created by injecting OE19 cells subcutaneously into NOD-SCID mice. Following tumour establishment, mice were treated with intra-peritoneal Cisplatin every 3rd day for 3 weeks with or without ICL670A (given orally on alternate days, either for 1 week before or for 3 weeks alongside Cisplatin treatment). The mice were subsequently culled and the xenograft, liver, spleen, heart and blood harvested for analysis. All animal work was performed under Home Office approved conditions.
Results: Pre-treatment with ICL670A resulted in an additional reduction in cellular viability (38.1 and 25.7%, OE19 and OE33 respectively, $p<0.05$) and proliferation (30.1% and 16.3%, $p<0.05$) compared to ECF alone. ICL670A was also able to overcome established Cisplatin resistance. In the murine model, treatment with ICL670A prior to Cisplatin alone resulted in a statistically significant reduction in tumour burden (81.7%, $p<0.05$) relative to control whereas Cisplatin alone did not. Crucially, no differences were observed in mouse behaviour, weight, serum iron or haemoglobin between groups.

Discussion and Conclusions: Pre-treatment with ICL670A before chemotherapy in OAC appears to result in an improved response compared to chemotherapy alone. Pre-treatment with ICL670A should be considered as an adjunct to chemotherapy in future clinical trials.

Poster #244
TREATMENT WITH FERRIC IRON DOWNREGULATES E-CADHERIN IN A MODEL OF BREAST CANCER IN VITRO AND MAY PROMOTE EPITHELIAL TO MESENCHYMAL TRANSITION
Cody Weston, BS, Ahmed Alkhateeb, BS and James Connor, PhD
Penn State College of Medicine
(Presented By: Cody Weston)

Introduction: The metastasis of carcinomas is believed to require a transition from epithelial to mesenchymal phenotype in order to allow invasion and migration of cells throughout the body. Previous studies have shown that iron chelators are able to inhibit pharmacologically induced epithelial-to-mesenchymal transition (EMT) in colon and prostate cancer cell lines, and that loading of iron in hepatocytes can promote EMT, but the significance of iron overload in the spread of breast cancer is still unclear.
Methods: MCF7 (ATCC, Manassas, VA) cells were seeded at a density of 5x105 cells per well in a six well plate, treated in triplicate with 0, 100, 300, and 500 micromolar concentrations of ferric iron in the form of ferric ammonium citrate (FAC) (Sigma-Aldrich Corp., St. Louis, MO) for 24 hours. Cells were then lysed in RIPA buffer with 1% protease inhibitor cocktail (Sigma-Aldrich) Samples were subjected to protein immunoblot using 4-20% Tris-HCl gels (Criterion, Bio-Rad, Hercules, CA) and PVDF membrane (GE Healthcare, UK). Primary antibodies to E-cadherin and Beta Actin were used (Abcam, Cambridge, MA). Analysis was performed using ImageJ software. To assess cell migration in vitro, the Platypus ORIS Assay (Platypus Technologies, Madison, WI) system was implemented according to the manufacturer's instructions.
Results and Discussion: In preliminary studies, we have found that the addition of Ferric Iron to MCF7 breast cancer cells causes them to downregulate E-cadherin in a dose dependent fashion ($P < 0.0001$, One Way ANOVA). The loss of E-cadherin is a central component of an EMT, so this phenomenon is relevant to the biology of cancer spread. Of note, the application of deferoxamine to chelate iron did not affect E-cadherin. Furthermore, when the cells were exposed to Ferric Iron in a rubber stopper migration assay (Platypus ORIS), application of Ferric Iron was not sufficient to elicit migratory behavior *in vitro*.
Conclusions: Iron loading appears to affect the epithelial-to-mesenchymal transition in this in vitro model of breast cancer. Future work will identify risk factors and concomitant conditions in which the manipulation of iron and its associated proteins are able to augment or resist the transformation to a mesenchymal, aggressive cellular phenotype. In this way, we expect to better understand the therapeutic potential of iron chelators as antitumor agents, and to identify the most promising molecular therapeutic targets within the iron handling signaling network.



Poster #245
THE ROLE OF IRON IN CANCER AND IN THE POLARIZATION OF TUMOR-ASSOCIATED MACROPHAGES
Milene Costa da Silva, Adelheid Cerwenka and Martina U. Muckenthaler
Molecular Medicine Partnership Unit (MMPU), Heidelberg Germany
(Presented By: Milene Costa da Silva)

Introduction: Tumor-associated macrophages (TAMs) play an important role in the tumor microenvironment and their contribution to tumor progression is now well established. In general, TAMs acquire a M2-like phenotype, contributing to tissue remodeling, tumor growth and angiogenesis. Cancer cells have the ability to polarize macrophages although the underlying mechanisms are unclear. Macrophages play a critical role in iron recycling from aging erythrocytes and differential expression of iron related genes is detected in different subtypes of polarized macrophages. Recently, the expression of ferroportin, the only known iron export protein, was correlated with improved metastasis-free survival of breast cancer patients. Here we

investigate whether the macrophage iron content plays a role in TAM polarization and/or influences the tumor microenvironment.
Methods and Materials: We applied the B16-BL6 melanoma model in untreated and iron-supplemented C57BL/6J mice to study the modulation of the tumor microenvironment in response to iron using flow cytometry, tumor size measurements, histology and immunohistochemistry.
Results: We show that mice subjected to iron-dextran injections grow bigger tumors with an increased amount of TAMs and a decrease in the NK cell population, when compared to control mice. The TAMs are hallmarked by a M1-like phenotype and iron-overload in the TAMs did not affect their polarization.
Conclusion: Expression analysis of iron-related genes in the TAMs is consistent with iron retention, similar to human macrophages polarized in vitro to an M1 phenotype. Complementary studies are ongoing to assess the effect of iron depletion on the tumor microenvironment.

Poster #246
ABBERANT REGULATION OF CHECKPOINT RESPONSE IN MOUSE EMBRYONIC STEM CELLS TREATED WITH IRON CHELATOR DEFEROXAMINE MESYLATE
Leona Raskova Kafkova, Lenka Calabkova and Vladimir Divoky
Department of Biology, Palacky University, Olomouc, Czech Republic
(Presented By: Leona Raskova Kafkova)

Introduction: In somatic cells, iron depletion with iron chelators typically results in a G1/S arrest, rarely in G2/M arrest, i.e. in blocks of cell cycle progression that are regulated by checkpoint pathways. Effect of iron chelation on pluripotent stem cell metabolism and fate is apparent, but not well understood.
Objective: We used iron chelation to study possible induction of checkpoint activities in pluripotent mouse embryonic stem cells (mESCs). mESCs are known to lack G1/S checkpoint, but are able to activate checkpoint pathways in S and G2 phase of the cell cycle.
Methods: mESCs and mouse embryonic fibroblasts (MEFs) were cultivated in the presence of 5 μM deferoxamine mesylate (DFO). MEFs were used as a control somatic cell line. Key components of checkpoint pathways − ATR, ATM, Chk1 and Chk2 kinases – were analyzed by western blotting, immunoprecipitation, and kinase assays. Flow cytometry analysis and measurement of ribonucleotide reductase (RR) activity were also used.
Results: MEFs revealed typical response to DFO treatment as described earlier for somatic cells. Our study confirmed activation of cell cycle arrest in G1−phase governed by Chk1 kinase in these cells. Iron depletion in somatic cells has been connected with a decrease in RR activity. RR supplies cells with deoxyribonucleotides (dNTPs) for DNA synthesis. In MEFs, the activity of RR decreased by 80% of its original activity after 48 h of DFO treatment. The lack of dNTPs resulted in apparent increase of DNA damage documented by accumulation of Ser139 phosphorylation on a histone H2AX. The mechanism of activation of cell cycle arrest in MEFs contrasted with events observed in mESCs treated with DFO. The cell cycle distribution of mESC was preserved in iron depleted conditions, without any accumulation of cells in a particular phase. Despite accumulation of phospho−p53 (Ser15), p21 and gammaH2AX (Ser139), we have not observed any cell cycle arrest, and correspondingly, we did not detect activation of the main checkpoint proteins ATR, ATM, Chk1 and Chk2. In fact, the activity of ATR has decreased in these cells. In contrast to MEFs, the activity of RR in mESCs did not significantly decrease in corresponding timepoints, and therefore, our data do not support direct connection between RR activity and accumulation of gammaH2AX (Ser139). mESCs treated with DFO exhibited direct activation of apoptosis without any cell cycle abrogation. This was linked with activation of caspase−3 that, however, was not associated with changes in mitochondrial membrane potential in these cells. Apoptosis was likely mediated by significant increase of phospho−Tyr142 of H2AX.
Conclusion: Treatment of mESCs with 5 μM DFO did not activate the checkpoint proteins and correspondingly, did not activate cell cycle arrest. Instead, apoptosis was activated in DFO−treated mESCs, with accumulation of phospho−p53 (Ser15) and p21 and with phosphorylation of tyrosine 142 of H2AX. Phospho−Tyr142 of H2AX is known to modulate survival/apoptotic decisions and we assume that increased phospho−Tyr142 of H2AX is linked to the activation of apoptosis in DFO−treated mESCs. In contrast, MEFs revealed decrease in phospho−Tyr142 of H2AX under these treatment conditions, in association with the activation of cell cycle arrest.
Grant Support: Projects No. NT12218, P305−11−1745, and institutional project LF_2012_016.

Poster #248
PRION PROTEIN REGULATES IRON TRANSPORT BY FUNCTIONING AS A FERRIREDUCTASE
Neena Singh, Ajay Singh, Swati Haldar, Katharine Horback and Cynthia Tom
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(Presented By: Neena Singh)

Objectives: Prion protein (PrPC) is implicated in the pathogenesis of prion disorders, but its normal function is unclear.
Results: We demonstrate that PrPC is a ferrireductase (FR), and its absence causes systemic iron deficiency in PrP knock-out mice (PrP-/-). When exposed to nontransferrin-bound (NTB) radioactive-iron (59FeCl3) by gastric-gavage, PrP-/- mice incorporate significantly more 59Fe relative to controls, indicating appropriate systemic response to the iron deficiency. Chronic exposure to excess dietary iron corrects this deficiency, but unlike wild-type (PrP+/+) controls that remain iron

overloaded, PrP-/- mice revert back to the iron deficient phenotype after 5 months. Bone-marrow preparations of PrP-/- mice show relatively less stainable iron, and this phenotype is only partially corrected by intra-peritoneal administration of excess iron-dextran. Cultured PrP-/- BM-macrophages incorporate significantly less NTB-59Fe in the absence or presence of excess extracellular iron, indicating reduced uptake and/or storage of available iron in the absence of PrPC. When expressed in neuroblastoma cells, PrPC exhibits NAD(P)H-dependent cell-surface and intra-cellular FR activity that requires the copper-binding octa-peptide-repeat region and linkage to the plasma membrane for optimal function. Incorporation of NTB-59Fe by neuroblastoma cells correlates with FR activity of PrPC, including PrPC in the family of proteins involved in cellular iron metabolism.

Poster #249

THE HEME EXPORTER FLVCR1A LIMITS OXIDATIVE STRESS ASSOCIATED TO HEME BIOSYNTHESIS IN DUODENAL ENTEROCYTES

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University of Torino
(Presented By: Veronica Fiorito)

Introduction: Feline Leukemia Virus, subgroup C, Receptor 1a (FLVCR1a) is a heme exporter and its role has been highlighted in erythroid cells and macrophages. FLVCR1a is expressed in almost all cell types including duodenal enterocytes, suggesting a role for this protein also in these cells. The duodenum plays a crucial role in iron metabolism, accounting for dietary iron supply to the organism in response to body iron requirements.
Methods and Materials: The work investigates the role of FLVCR1a as a heme exporter in duodenal enterocytes. Moreover, it analyses the involvement of FLVCR1a in dietary heme absorption and in the maintenance of the enterocyte redox status.
To this purpose, an intestine specific conditional FLVCR1a-knockout mouse was generated to analyse the role of FLVCR1a in vivo in the duodenum. Moreover, FLVCR1a-depleted enterocyte-like Caco2 cells were used to test FLVCR1a functions in vitro.
Results: Data obtained show that the lack of FLVCR1a results in enhanced cellular heme levels in mice duodenum and increased heme content in enterocyte-like Caco2 cells, indicating for the first time a role of FLVCR1a in heme export in enterocytes, as reported for other cell types.
Conclusion: The lack of FLVCR1a in the duodenum does not affect dietary heme absorption. Nevertheless, data indicates that FLVCR1a is involved in the export of the novo synthesized heme and that cells lacking FLVCR1a suffer from oxidative stress, highlighting the crucial importance of FLVCR1a to reduce enterocytes heme accumulation and, consequently, to limit cell oxidative stress. Accordingly, further data show that FLVCR1a expression is modulated following hypoxia-mediated cell oxidative stress. Thus a role for FLVCR1a in the cell antioxidant response is proposed. The intriguing hypothesis is that FLVCR1a, participating in the maintenance of cellular redox status, should likely collaborate to protect duodenum from gut pathologies related to perturbations of the mucosa oxidative status. Finally, these observations could open the possibility to better understand the impact of heme trafficking in duodenal functions.

Poster #250

NATURE OF CYTOSOLIC IRON POOL IN MAMMALIAN CELLS

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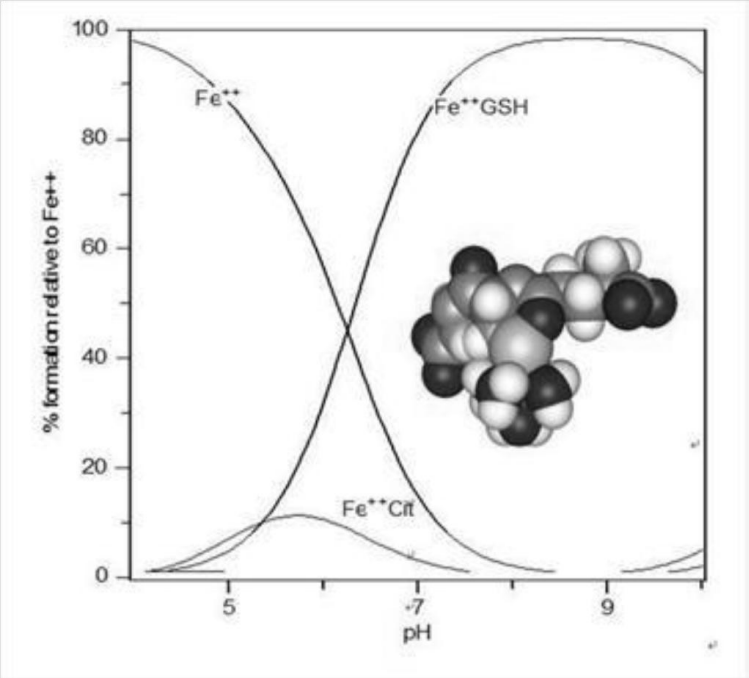
(Presented By: Xiaole Kong)

Introduction: The low molecular weight iron pool has a central role in many metabolic processes1. Evidence from the use of fluorescent probes2, enzyme activity3, and ferritin levels4 indicates that the total iron concentration falls in the range 0.2-2μM. However the ligands involved and their speciation are uncertain. ATP/ADP, citrate and 2,5- dihydroxybenzoic acid have been suggested as candidates and would be suitable if the major form of iron was iron(III). However the cytosolic pool is predominately iron(II)2,3.
Thiol containing ligands are likely to show a higher preference for iron(II) and such cytosolic candidates are cysteine (30μM)5 and glutathione 1-2mM5. As the pKa values for cysteine (8.3) and GSH (8.6) are similar, the 30-60 fold higher concentration of GSH favors this tri-peptide as being the major cytosolic ligand for iron(II).
Iron(II)- glutathione
Few studies have been devoted to the GSH/Fe2+ interaction and no stability constant has been reported. However, by using the Irving-Williams series it is possible to extrapolate a K1 value for FeIIGSH from the corresponding values for ZnII (K1=8.66) CoII and NiII (K1=7.47), resulting with a conservative estimated value of K1 for FeIIGSH as being 5.5±0.5. We have recently determined the logK1 value as 5.18.Using this estimated data, at physiological conditions (pH 7) the majority of iron will be bound to GSH (>80%) as presented in the figure. Due to the relative weakness of hydroxyl anion interaction with iron(II), the glutathione iron(II) species dominates over the pH range 7-10. The autoxidation of iron(II) by trace oxygen is inhibited by GSH.
Conclusion: Glutathione (GSH) plays an important role in intracellular iron(II) speciation, it is probably the major component of the low molecular weight iron pool.



Poster #251

HAEM UPTAKE IN THE HUMAN BLOOD FLUKE SCHISTOSOMA MANSONI

Shu Qin Toh, BSc (Hons)[1], Lucia Gutiérrez, PhD[2], Stephen Karl, PhD[2], Tim St. Pierre, PhD[2], Rob Woodward, PhD[2], David Malagon Martinez, PhD[1], Geoffrey Gobert, PhD[1] and Malcolm Jones, PhD[1]
[1]Queensland Institute of Medical Research; [2]University of Western Australia
(Presented By: Shu Qin Toh)

Introduction: Schistosomiasis is a major public health problem in many developing countries. The disease is endemic in 74 developing countries and affects over 200 million people worldwide. Adult schistosomes are dependent on the blood of their definitive host for nutrition. Schistosomes are blood flukes, parasitic flatworms that feed on red blood cells and other blood components to obtain essential nutrients and amino acids for growth, development and reproduction. In the process of ingesting and breaking down erythrocytes, large quantities of haem are released. Despite its potential toxicity in abundance, haem is a biologically important molecule for schistosomes, as demonstrated by the numerous haemoproteins expressed by the parasites. Work by Rao *et al.*, 2005, suggested that schistosomes are incapable of haem biosynthesis. Thus, it is possible that schistosomes possess a mechanism mediating the uptake of exogenous haem, similar to other haemoparasites (e.g malaria), to support their haem and iron needs. However, knowledge on the uptake and subsequent utilization of host haem in schistosomes is lacking.

Methods and Materials: The aim of this study is to investigate the presence of a haem uptake system and the importance of this host haem in the general biology of *Schistosoma mansoni.* To determine the presence of a haem uptake system, pulse−chase experiment using fluorescent haem analogue, palladium mesoporphyrin is being performed on adult schistosomes. In order to understand how schistosomes utilize host haem and its subsequent iron containing breakdown products, different methods including SQUID (superconducting quantum inference device) magnetometry and Perl's staining were performed on parasites. As numerous transporter proteins have been demonstrated to be involved in the uptake of haem in other organisms, bioinformatics tools are being utilized to identify haem transporter protein from *S. mansoni* genome database.

Results: The results of this study indicate that schistosomes take up exogenous haem which accumulates in the ovary. This suggests that host haem could be involved in supporting embryogenesis in *S. mansoni.* Understanding the uptake and subsequent utilization of haem in schistosomes could reveal potential molecular targets for control of this important group of human parasites.

Reference:

1. Rao AU, Carta LK, Lesuisse E, Hamza I: Lack of heme synthesis in a free-living eukaryote. Proceedings of the National Academy of Sciences of the United States of America 2005, 102(12):4270-4275.

Poster #252

A POSSIBLE ROLE FOR LYSOSOMES IN IRON DISTRIBUTION THROUGH FERRITIN TRAFFICKING AND SECRETION

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Technion – Israel Institute of Technology
(Presented By: Marianna Truman-Rosentsvit)

Introduction: Ferritin in mammalians was mainly considered an intracellular cytosolic iron storage protein, however in recent years possible functions of a secreted form of ferritin have been demonstrated. Recently we have shown that serum ferritin has characteristics of a lysosomally processed ferritin and therefore suggested that ferritin is secreted by a non-classical lysosomal pathway. Moreover, an autophagy mediated pathway for ferritin entering the lysosome has recently been described and in many immunofluorescence images published in the literature ferritin appears to be in a punctate uneven distribution. This punctate appearance could be due to enrichment of ferritin in lysosomes. Once in the lysosome, ferritin can be degraded and iron can be recycled to the cytosol. Alternatively, iron can be stored in the lysosome as hemosiderin for long term storage and detoxification. In addition, lysosomal ferritin may be secreted by cells that have the machinery for lysosomal secretion. Thus, our hypothesis claims that lysosomes play an important role in ferritin trafficking and secretion. We also hypothesize that extracellular signaling agents, such as hormones, may regulate subcellular ferritin distribution by modifying the equilibrium between cytosolic and lysosomal ferritin, thus affecting ferritin secretion.
Purposes: Initially, we wanted to reinforce our previous findings, which showed that ER-Golgi secretion is not a major pathway for ferritin secretion, at the cellular level. Secondly, we wished to determine the ferritin distribution in different subcellular compartments, mainly the cytosol and the lysosome, and to investigate the effect of inhibition of lysosomal function and hormonal signaling on intracellular ferritin trafficking and ferritin secretion.
Methods: Brefeldin-A (BFA) an inhibitor of the classical ER-Golgi secretion pathway was administered to murine macrophage primary cultures and cell-lines. Macrophages and epithelial cell-lines were also treated with chemical inhibitors of lysosomal function and with several relevant hormones. Ferritin from cell lysates and medium was evaluated by immunoprecipitation and Western-blot analysis. In addition, to analyze the subcellular ferritin localization, immunofluorescence was performed using antibodies against ferritin in combination with Golgi- or lysosomal markers.
Results: Immunofluorescence revealed that there is a strong co-localization of ferritin with the lysosomal marker. Inhibitors of lysosomal function or the follicle stimulating hormone (FSH) caused a significant accumulation of intracellular ferritin. This elevation of intracellular ferritin had complex effects on the amount and characteristics of ferritin secreted into the culture medium which is currently under investigation. The addition of BFA did not cause an accumulation of intracellular ferritin and did not prevent its secretion. BFA treatment dissociated the Golgi, but in untreated cells, no co-localization was observed between ferritin and the Golgi marker.
Discussion: The above mentioned results reinforce our previous findings, according to which ferritin secretion does not depend on a functional ER-Golgi system. Our results suggest that much of the cellular ferritin in murine macrophages and epithelial cells is indeed localized in the lysosome. In addition, we suggest that hormonal signaling and interference with lysosomal processing affect the ferritin distribution between cytosol and lysosome and thus manipulate ferritin secretion.
Conclusion: The current research findings support our hypothesis, which claims that lysosomes may play an important role in ferritin trafficking and secretion. This sheds new light on a possible role for ferritin in tissue-iron distribution.

Poster #253

THE FERRITIN-HEAVY–POLYPEPTIDE-LIKE-17 (FTHL17) GENE ENCODES A FUNCTIONAL FERRITIN WITH PREFERENTIAL NUCLEAR LOCALIZATION

Dr. Paola Ruzzenenti[2], Dr. Michela Asperti[1], Dr. Maria Regoni[1], Dr. Ilaria Uberti[1], Maura Poli, PhD[1], Stefania Mitola, PhD[1], Dr. Federica Maccarinelli[1], Dario Finazzi, MD[1] and Paolo Arosio, PhD[1]
[1]DMMT, University of Brescia; [2]Department MMT, University of Brescia
(Presented By: Paola Ruzzenenti)

Introduction: Three functional ferritin genes have been identified so far in mammals, and they encode the cytosolic H and L chain and the mitochondrial ferritin. The expression of a transcript by a forth ferritin-like gene (Ferritin-Heavy–Polypeptide-Like-17, FTHL17) on X chromosome was reported in mouse spermatogonia, but not in somatic tissues. It was found to be expressed in female blastocysts by the paternal allele, as early as the two-cell stage and, because of its specific embryonic expression, FTHL17 was included in the initial screening of genes for induction of pluripotent cells (Takahashi & Yamanaka, 2006). The function of this gene is unknown, and biochemical analyses are needed to verify if the encoded protein has ferritin-like properties which may affect the iron status of embryonic cells.
Methods and Materials: The human FTHL17 was cloned in pET vector and expressed in E. coli. It assembled in a stable 24-mer protein with electrophoretic mobility similar to that of the human H and L ferritins.
Results: As isolated, the FTHL17 did not contain detectable iron, in agreement with the observation that most residues of the ferroxidase centre are substituted. The recombinant FTHL17 showed to be less resistant to chemical denaturation and to heating above 70°C. When transiently expressed in HeLa cells the flag-FTHL17 assembled in ferritin shells that resisted heating, and incorporated iron similarly to flag-ferritin Lchain. Immunocytochemistry with anti-flag antibody showed FTHL17 to have a preferential nuclear localization, while ferritin light-chain accumulated in the cytosol.

Conclusion: We concluded that FTHL17 encodes a functional ferritin that is without ferroxidase activity, but that can co-assemble with H-chain to produce iron storage molecules. The restricted embryonic expression and the nuclear preference suggest that this novel ferritin type may have functions other than iron storage.

Poster #254

CHARACTERISATION OF A NOVEL IRON TRANSPORTER IN MALARIAL PARASITES

Ksenija Slavic, PhD, J. Pittman, S. Krishna, H. M. Staines, M. M. Mota
Instituto de Medicina Molecular
(Presented By: Ksenija Slavic)

Introduction: Malaria, caused by infection with *Plasmodium* parasites, remains a devastating disease killing nearly a million people every year. The discovery of novel antimalarial strategies is crucial, as the development of drug resistance has impaired current treatments. Iron is an essential nutrient for malarial parasites, so intereference with iron homeostasis of parasites may kill them. However, our present understanding of iron acquisition and its homeostasis in malarial parasites is scarce.

Methods and Materials: Here, we functionally investigated a putative plasmodial iron transporter by expressing it in yeast strains deficient for specific divalent metal transporters. We have demonstrated iron-transport by this plasmodial transporter by complementing the mutant phenotype of *Saccharomyces cerevisiae* Δ*CCC1* mutant strain (Ca2+ sensitive cross complementer), susceptible to high iron concentrations in the growth medium.

Results: Expression of this transporter in the Δ*Zrc1* yeast mutant (deficient for a zinc transporter) did not confer phenotype rescue in increased Zn2+ concentrations, suggesting that it does not act as a zinc transporter. In addition, by using transfection strategies in a rodent malaria model, we have successfully targeted its encoding gene in *Plasmodium berghei*.

Conclusion: These transgenic malarial parasites are valuable tools to investigate the role of this transporter for parasite survival and development throughout its complex life cycle. Detailed characterization of the phenotype of the transgenic parasites at various life cycle stages is currently in progress.

Poster #255

FERRIC REDUCTION AND UPTAKE OF NON-TRANSFERRIN BOUND IRON BY K562 CELLS AND PRIMARY RODENT ASTROCYTES

Darius J.R. Lane, PhD and Alfons Lawen, Dr. Rer. Nat
Monash University
(Presented By: Alfons Lawen)

Introduction: Eukaryotic cells possess transplasma membrane electron transfer (tPMET) systems capable of reducing extracellular electron acceptors at the cost of cytosolic electron donors (e.g., ascorbate or NADH). The physiological significance of these systems is not yet fully understood. tPMET has been suggested to be involved in nontransferrin-bound iron (NTBI) reduction and uptake. Transplasma membrane ascorbate/dehydroascorbate (DHA) cycling can promote NTBI reduction and uptake by human erythroleukemia (K562) cells [Lane and Lawen (2008) *J. Biol. Chem*. **283** 12701-12708]. This system involves *i)* cellular import of dehydroascorbate (DHA), *ii)* intracellular reduction of DHA to ascorbate using metabolically-derived reducing equivalents, *iii)* export of ascorbate down its concentration gradient, *iv)* direct reduction of low molecular weight iron chelates by ascorbate, and *v)* uptake of iron (II) into the cell [Lane and Lawen (2009) *Free Radic. Biol. Med*. **47**, 485-495]

Methods and Materials: (Fig. 1). Experiments with both, mouse and rat primary astrocytes show that these cells use the same mechanism for reduction and uptake of NTBI suggesting a universality of our observations made by studying the K562 cell line. In addition to this NTBI uptake system common to both cell types we find in astrocytes an as of yet uncharacterised mechanism for the uptake of ferric NTBI [Lane *et al*. (2010) *Biochem. J.* **432**, 123-132.].

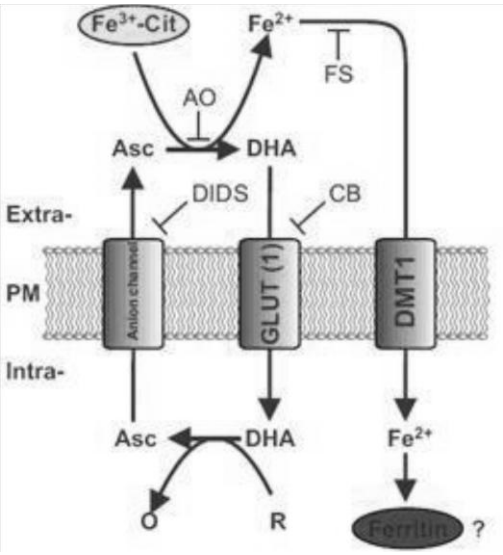
Astrocytes help to protect neurones from oxidative damage, in part by the recycling of ascorbate from its oxidised forms back to neurones for subsequent neuronal uptake. We found astrocytic ascorbate release to be stimulated by glutamate, the major excitatory neurotransmitter in the brain.

Results: When we tested agonists and antagonists of glutamate receptors, we observed no effects on ascorbate release. Glutamate uptake in astrocytes occurs through the excitatory amino acid transporters, EAAT1 (GLAST) and EAAT2 (GLT-1), both of which are predominantly astrocytic in localisation. Glutamate-stimulated ascorbate release can be inhibited by inhibitors of GLAST (EAAT1), but not GLT1 (EAAT2), suggesting that glutamate has to be taken up by astrocytes in order to stimulate ascorbate release and that this uptake involves EAAT1.

Conclusion: Glutamate-stimulated ferrireduction in DHA-loaded astrocytes is furthermore inhibitable by the volume-sensitive organic anion channel (VSOAC) inhibitor dideoxyforskolin (DDF), hypertonic media and chloride channel inhibitors, suggesting involvement of a volume-sensitive anion channel in astrocytic ascorbate release. The effect of glutamate can be mimicked by hypotonic media and again is inhibitable by anion channel inhibitors, suggesting that the direct signal for ascorbate release is hypotonia induced by intracellular glutamate [Lane and Lawen (2012) *Cell Biochem. Biophys*., DOI 10.1007/s12013-012-9404-8].

Fig. 1: Stimulation of iron reduction and uptake by ascorbate in K562 cells.

Present address DJRL: University of Sydney, Pathology, City Rd, Sydney, New South Wales, Australia, 2006



Poster #256

TRANSFERRIN IRON UPTAKE IS STIMULATED BY ASCORBATE VIA AN INTRACELLULAR REDUCTIVE MECHANISM

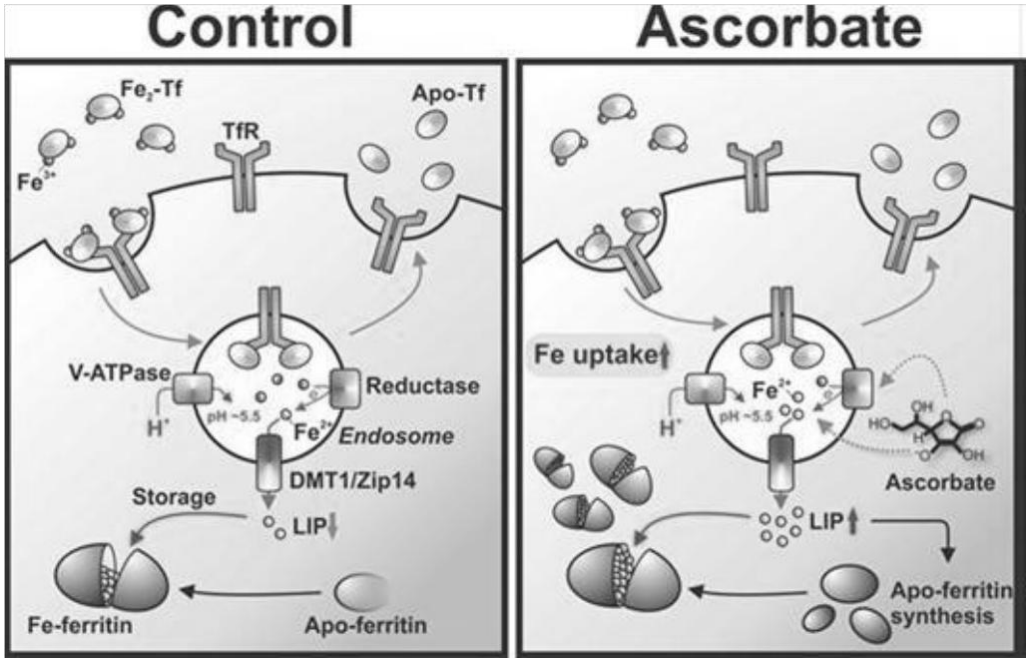
Darius J.R. Lane, PhD, Sherin Chikhani, MSc, Vera Richardson, MSc and Des Richardson, PhD, DSc
The University of Sydney
(Presented By: Darius J.R. Lane)

Introduction: Although ascorbate has long been known to stimulate dietary iron (Fe) absorption and non-transferrin Fe uptake, the role of ascorbate in transferrin Fe uptake is unknown. Transferrin is a serum Fe transport protein supplying almost all cellular Fe under physiological conditions.

Methods and Materials: We sought to examine ascorbate's role in this process, particularly as cultured cells are typically ascorbate-deficient. At typical plasma concentrations, ascorbate significantly increased ^{59}Fe uptake from transferrin by 1.5-2-fold in a range of cells. Moreover, ascorbate enhanced ferritin expression and increased ^{59}Fe accumulation in ferritin. The lack of effect of cycloheximide or the cytosolic aconitase inhibitor, oxalomalate, on ascorbate-mediated ^{59}Fe uptake from transferrin indicate increased ferritin synthesis or cytosolic aconitase activity was not responsible for ascorbate's activity.

Results: Experiments with membrane-permeant and membrane-impermeant ascorbate-oxidizing reagents indicate that while extracellular ascorbate is required for stimulation of ^{59}Fe uptake from ^{59}Fe-citrate, only intracellular ascorbate is needed for transferrin ^{59}Fe uptake. Additionally, experiments with L-ascorbate analogs indicate ascorbate's reducing ene-diol moiety is necessary for its stimulatory activity. Importantly, neither N-acetylcysteine nor buthionine sulfoximine, which increase or decrease intracellular glutathione, respectively, affected transferrin-dependent ^{59}Fe uptake. Thus, ascorbate's stimulatory effect is not due to a general increase in cellular reducing capacity. Ascorbate also did not affect expression of transferrin receptors 1 or 2 (TfRs 1 or 2), 125I-transferrin cellular flux or expression of the endosomal ferrireductase, six transmembrane epithelial antigen of the prostate (Steap3). However, transferrin receptors, endocytosis, vacuolar-type ATPase activity and endosomal acidification were required for ascorbate's stimulatory activity.

Conclusion: Therefore, ascorbate is a novel modulator of the classical transferrin Fe uptake pathway, acting via an intracellular reductive mechanism. We propose a mechanism for ascorbate's stimulatory effect on transferrin Fe uptake whereby it acts to enhance endosomal ferrireductase activity. These findings have ramifications for understanding how transferrin-derived Fe is reduced prior to its transport through endosomal ferrous-selective transporters such as the divalent metal transporter 1 (DMT1).



Poster #257

ROLE OF LYSOSOMES AND DMT1 IN MOBILIZATION OF FERRITIN IRON STORES

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(Presented By: Jessica Morgan)

Introduction: Previous studies indicate that lysosomal degradation is a major rmechanism for mobilization of iron stored in ferritin.

Methods and Materials: Thus, in cell culture models of cells with widely different roles in iron metabolism (hepatocytes, enterocytes and erythroid cells), we determined that ferritin protein and iron concentrations decreased in parallel, with the same kinetics; and inhibitors of lysosomal proteases markedly reduced turnover of both ferritin iron and protein (Am J Physiol Cell Physiol 291: C445–C455, 2006).

Results: Treatment with lysosomal protease inhibitors increased retention of ferritin and iron within the lysosomes. Current research has focused on whether lysosomal "juices" can dissolve the ferrihydrate crystallites exposed by ferritin proteolysis; whether DMT1 is the main or sole transporter of the solubilized iron that results; and whether reticuloendothelial cells also utilize lysosomal degradation for ferritin iron mobilization. Ferrihydrate crystallites within ferritin were purified after heating rat liver ferritin in SDS. Crystallite suspensions were incubated with lysosomal extracts or solutions of glutathione, ascorbate and/or citrate, at pH 5.5 or 7.0 and rates of iron solubilization measured. All treatments resulted in rapid iron solubilization. The relationship of DMT1 to lysosomes was monitored by confocal microscopy before and during ferritin iron mobilization, using LAMP2 as the lysosomal marker. Iron deprivation (by low iron or 10 μM DFO) resulted in increased association of ferritin with lysosomes, an association enhanced by lysosomal protease inhibitors. DMT1 colocalized with LAMP2 in cells of normal iron status, but iron deprivation increased this colocalization within hours, suggesting DMT1 involvement with lysosomal iron metabolism. In further tests, hepatic cells were treated with DMT1 siRNA. Although DMT1 mRNA levels were reduced >90% over 3 days, levels of DMT1 protein were not significantly altered, and there was no consistent increase in iron retention by the lysosomes separated on density gradients. The same was the case after two 3-day specific (versus scrambled) siRNA treatments, although DMT1 mRNA was now almost nil and protein concentrations were reduced about 50%. Effects of additional siRNA treatments are being examined. To extend studies on lysosomal ferritin iron mobilization to reticuloendothelial cells, murine monocytic macrophages (J774a.1) were induced to form ferritin by exposure to 180 μM ferric ammonium citrate, then washed and placed in medium containing 10 μM DFO, in the presence and absence of lysosomal protease inhibitors, leupeptin and chymostatin. Inhibitor presence greatly increased the levels of ferritin iron and protein retained by the cells during DFO exposure.

Conclusion: We conclude that not just in hepatocytes, enterocytes and erythroid cells but also in macrophages, lysosomes are involve in the release of iron from ferritin; that components in the lysosomal matrix are capable of dissolving the iron crystallites in ferritin; and that DMT1 associating with lysosomes in response to iron deprivation is likely to be mediating the transfer of iron released from ferritin back to the cytoplasm, from where it will be utilized by components of other cell compartments or transferred out of the cells by ferroportin to the blood for delivery to other tissues. Supported in part by PHS grant RO1 HD46949 and the Cal State Fullerton MARC U*STAR Program PHS grant 2T34 GM008612-17

Poster #258

THE ABSORPTION OF NANOPARTICULATE IRON HYDROXIDE IS FERROPORTIN-MEDIATED

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(Presented By: Mohamad Aslam)

Introduction: The redox status and chemical form of dietary iron in the human intestine are poorly defined. However, in vitro simulation and animal models suggest that highly amorphous, nanoparticulate iron hydroxides (~2-10nm diameter) form when dietary ferric iron is ingested. Ligand modified ferrihydrite (LM-Fh) is a synthetic mimetic of dietary iron in the intestinal lumen, which may have use as an iron supplement. Unlike ferrous iron and most soluble ferric iron, LM-Fh is endocytosed at the apical surface of the intestinal enterocyte. Moreover, the appearance of LM-Fh-derived iron in the systemic circulation is relatively slow. The handling of the iron derived from LM-Fh at the enterocyte basolateral membrane is not understood.
Objective: To investigate whether ferroportin-1 (FPN-1) is involved in the efflux of LM-Fh-derived iron from small intestinal enterocytes.
Materials and Method: Intestinal iron absorption was measured by the isolated duodenal loop method using intestine-specific FPN-1 knockout (n=3) and wild-type (WT) mice (n=3). Loops were filled with either 500μM LM-Fh or ferric-nitrilotriacetic acid (FeNTA). After a 30 minute incubation, enterocyte and serum samples were collected for iron analysis.
Results: Baseline serum iron levels in WT and FPN-1 knockout mice were 164±29 and 95±17 μg/dL respectively. In WT mice, serum iron levels increased to 251±18 μg/dL and 210±25 μg/dL after 30 minutes incubation with FeNTA and LM-Fh respectively. In contrast, in FPN-1 knockout mice there was no rise in serum iron levels following 30 minutes incubation with either iron preparation, and, in fact, serum iron fell marginally, to give final values of 72±9 and 69±25 μg/dL respectively.
This drop in serum iron may reflect an acute phase response to the surgical procedure. The final serum iron levels were significantly higher in WT mice than in FPN-1 knockout mice for both iron preparations ($p=0.0001$ and $p=0.002$ for FeNTA and LM-Fh respectively), consistent with FPN-1 playing an important role in the absorption of nanoparticulate derived iron.
Conclusion: This small study supports the hypothesis that the intestinal efflux of iron following the enterocyte uptake of nanoparticulate LM-Fh is FPN-1-mediated in a similar manner to that of soluble iron (FeNTA). Thus, nanoparticulate LM-Fh does not bypass the normal homeostatic process that regulates iron traffic across the enterocyte basolateral membrane. This implies that LM-Fh-derived iron joins the common iron 'pool' following its brush border uptake.

Poster #259

NEW DEVELOPMENTS IN MEASURING THE ENZYMATIC ACTIVITY OF AMYLOID-β PRECURSOR PROTEIN AND OTHER FERROXIDASES

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(Presented By: James Duce)

Introduction: Redox cycling is critical for iron biology, as are proteins that regulate this process. Ferroxidases such as ceruloplasmin (CP), hephaestin, heavy-chain ferritin (FtH1) and the recently discovered Alzheimer's-associated Amyloid-beta Precursor Protein (APP) [1] catalyse the oxidation of ferrous iron.
Methods and Materials: Here we demonstrate that the ferroxidase kinetics of APP, CP, and FtH1 are significantly influenced by buffers, salinity, pH, time and temperature when measured by two classic ferroxdiase assays: transferrin loading assay [2]; and Ferene S chromogen assay [3].
Results: Sodium acetate at pH 5.8-6 is traditionally used to buffer assays for CP activity in both ferroxidase assays, however APP and FTH1 performed poorly in these condition. APP and FtH1 had optimal enzyme kinetics when assayed in Good's buffers such as PIPES and HEPES at physiological pH and salinity. Using these more physiologically relevant conditions APP and CP exhibited almost identical enzyme kinetics in the transferrin assay, while in the Erel assay APP had a slower initial rate of reaction but higher maximum velocity compared to CP. Interestingly, the presence of transferrin was also found to influence the enzyme kinetics of the ferroxidase reaction.
Conclusion: We discuss several complexities in performing the transferrin and Erel assays on known ferroxidases and propose more optimized conditions for the future use of each assay.
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Poster #260

THE EFFECT OF MATERNAL BODY MASS INDEX ON EXPRESSION OF GENES OF IRON METABOLISM IN THE HUMAN PLACENTA

Modou Lamin Jobarteh
Rowett Institute of Nutrition and Health
(Presented By: Modou Lamin Jobarteh)

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Introduction: Obesity is associated with increased cytokine and hepcidin levels in blood. Hepcidin is a key regulator of iron metabolism and mediator of anaemia of inflammation [1]. Increased hepcidin levels causes increased iron uptake in macrophages and decreased iron released into circulation. Consequently, obese women may have a higher risk of iron deficiency than their normal or underweight counterparts. Here we tested whether increase maternal BMI has any consequences on iron metabolism during pregnancy.
Methods and Materials: We compared placental iron transporter gene expression levels and birth outcomes between lean (Body Mass Index (BMI) <18.5kg/m2), normal weight (BMI 18.5 -21.52kg/m2) and overweight (BMI >21.52kg/m2) women. These values for BMI were based on the global fat scale calculation developed by researchers from LSHTM. They are lower than Western societies but are appropriate for the Gambian women. These studies were part of the ENID Trial [2]. Ethical permission was obtained and all women gave informed consent to participate. All women were supplemented with iron (60mg) and folate (400 µg) as described by Gambian Government guidelines. They were further supplemented with placebo, MMN, protein energy supplement or both. Maternal anthropometric measurements were performed before the start of supplementation at less than 20 weeks of gestation. The women were monitored throughout pregnancy to the birth of the babies. Neonatal anthropometric measurements were performed within 72hours of birth.
Results: Placental iron transporter gene expression was measured using RT-PCR. Analysis was carried out on lean (N=55), normal weight (N=140) and overweight (N=84) women. There was no significant difference in gestational age at birth between the different BMI categories. However overweight women had babies with a higher birth weight than normal or lean women (3124, 2983 and 2901g, respectively p=0.01). In contrast, there were no changes in placental weight. There was no significant difference in placental iron transporter gene expression between the supplementation groups. However, grouping women according to their BMI revealed higher expression of some of the iron transporters genes in the placenta. There were no statistical significant differences in transferrin receptor (TfR1, P=0.45), ferroportin 1 (Fpn1, p=0.07) and Hepcidin (HAMP, 0.15) gene expression levels between lean and overweight women. However there were significant differences in DMT1 (Lean 0.09, Normal weight 0.16 and Overweight 0.28, p=0.016), Zyklopen (ZP) (0.02, 0.11 and 0.14, p=0.018) and Feline leukemia virus subgroup C receptor 1 (FLVCR1) gene (0.015, 0.139 and 0.146, p=0.022).
Conclusion: Our results indicate that some of the genes involved in iron metabolism in the placenta were more highly expressed among overweight women. This could imply maternal anaemia and suggests a placental adaptive mechanism to maintain fetal iron levels.

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Poster #261

STRUCTURAL MODEL OF HUMAN FERROPORTIN: INVESTIGATION OF IRON BINDING AND TRANSLOCATION

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(Presented By: Giovanna Cece)

Introduction: A combined multiple threading/Monte Carlo replica exchange approach implemented in I-Tasser was used to obtain a high-resolution model of Fpn structure based on the crystal structure of the glycerol-3-phosphate transporter from E. coli (PDB code 1PW4). The latter protein shares with Fpn the 12 transmembrane helices topology and is a member of the major facilitator superfamily of transporters, a class of membrane transporters acting with an alternate 'inward open - outward open' mechanism.
Methods and Materials: Analysis of the Fpn structural model shows the presence of a central channel whose walls are lined by several oxygenated ligands (Asp, Glu and Tyr residues) forming two potential iron binding sites. In particular, a first putative site is located at the center of the intermembrane space and is formed by residues D39, D181, Y318, Y501 while a second one is located towards the extracellular portion of the protein and is formed by residues E52, Y54, E518 and Y331. The model also fits a twostep translocation mechanism typical of other members of the major facilitator superfamily of transporters,

involving exposure of the central binding site to cytoplasm to allow intracellular iron binding to Fpn and a subsequent conformational change which triggers iron translocation to the binding site on the extracellular side of the protein, where Fpn functional partners (e.g., ceruloplasmin) can take up the metal.
Results: Fpn residues targeted for site-specific mutagenesis included aminoacids involved in the putative inward (D39, H43, D181, Y318 and Y501) and outward (E52, Y54, Y331 and E518) iron binding sites, and D325 and R466 as potential 'hinges'. Wild type and mutant Fpn were transiently transfected in HEK293 cells and their iron export ability was assessed by incubation with radioactive 55Fe-NTA and determination of intracellular radioactivity. Subcellular localization of wild type and mutant Fpn-GFP was assessed by fluorescence microscopy. Wild type Fpn and all mutants correctly localized to the plasma membrane, except for D325A which appeared to be partly retained in intracellular compartments.
Expression of wild type Fpn leads to a decrease of intracellular iron, due to export of the metal mediated by the recombinant protein. Substitution of residues D39 and D181 severely impaired iron export from cells compared to Fpn wild type. Mutation of other residues of the predicted internal (H43A, Y318F and Y501C) or external iron binding site (E52A, Y54F, Y331F and E518A) had only modest effects on iron content of cells. D325A and R466M mutations also caused a significative increase in cellular iron content.
Conclusion: These results give robust experimental support to our hypotheses and open new options for the study of Fpn-mediated iron export.

Poster #262
CYTOSOLIC FERROXIDASE (CYTOFOX) ACTIVITY IN RODENT ENTEROCYTES IS INDEPENDENT OF CERULOPLASMIN AND AMYLOID PRECURSOR PROTEIN
Yan Lu, PhD[1], Brie K. Fuqua, PhD[2], Chris D. Vulpe, PhD[2], Gregory J. Anderson, PhD[3] and James F. Collins, PhD[1]
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(Presented By: Yan Lu)

Introduction: Rat and mouse duodenal enterocyte cytosol contains ferroxidase (cytoFOX) activity (Ranganathan, P. N., Lu, Y., et al. *Proc. Natl. Acad. Sci. USA,* 2012). This activity is not membrane-based and is protein-mediated. Furthermore, cytoFOX activity increased in iron-deficient rat enterocyte cytosol, and (in mice) may be ascribed, at least in part, to hephaestin (Heph), which was noted in the cytosolic fraction (Lu, Y., et al., *Biometals*, 2012). Although enterocyte cytoFOX activity was unaffected by copper deprivation of rats, current data cannot exclude a requirement for copper. The present studies were thus designed to test the hypothesis that cytoFOX activity in mouse enterocytes is derived from a known ferroxidase (either multi-copper or otherwise).
Methods: The roles of specific ferroxidases was assessed either biochemically or by utilizing mouse strains in which various FOXs were genetically ablated. Mice were bred at the Queensland Institute for Medical Research (Brisbane, Australia). Western blotting confirmed that the corresponding proteins were absent in the duodenal mucosa of the KO mice studied. Duodenal enterocytes were isolated and spectrophotometric-based, transferrin-coupled ferroxidase assays were run. Blanks were run in each experiment to account for possible auto-oxidation of ferrous iron.
Results: Experiments with *Heph* KO mice demonstrated that cytosolic FOX activity was partially attenuated (~30-40%) (**Fig. 1**). A similar trend was noted in intestine-specific *Heph* KO mice. CytoFOX activity was unaffected by deletion of ceruloplasmin (*Cp*), another multicopper ferroxidase that may play some role in iron absorption, and amyloid precursor protein (*App*). App in the brain has FOX activity and App is expressed in the intestine. Additional experiments were performed with *Atp7b* KO mice. Atp7b is expressed in the small intestine and if cytoFOX is indeed a cuproenzyme, Atp7b could be involved in copper delivery, a role it plays for Cp in hepatocytes. However, enterocyte cytoFOX activity was unaffected by *Atp7b* ablation. Furthermore, to consider the possibility that ferritin (Ft) contributed to cytoFOX activity, cytosol samples were heated at 55-65o C for 15 minutes. FOX activity was abolished, suggesting that Ft is unlikely to contribute, as Ft remains stable and functional up to 75o C (Kim, S.W. et al. *Biophys. Res. Commun*., 2001).
Discussion: Enzymatic iron oxidation in the intestine appears necessary to supply sufficient iron for body iron demands as chemical oxidation is unlikely to be sufficient given the reduction potential of iron at physiologic pH and the oxygen tensions in the intestinal mucosa. CytoFOX activity could complement membrane-bound Heph and the existence of another intestinal FOX seems plausible as *Heph* KO mice still absorb iron, albeit at a reduced rate. This investigation has eliminated several known FOXs as candidates for cytoFOX activity, and confirmed that Heph is partially responsible. Further studies are required to define the nature and origin of the residual cytoFOX activity and to determine its role in intestinal iron homeostasis.

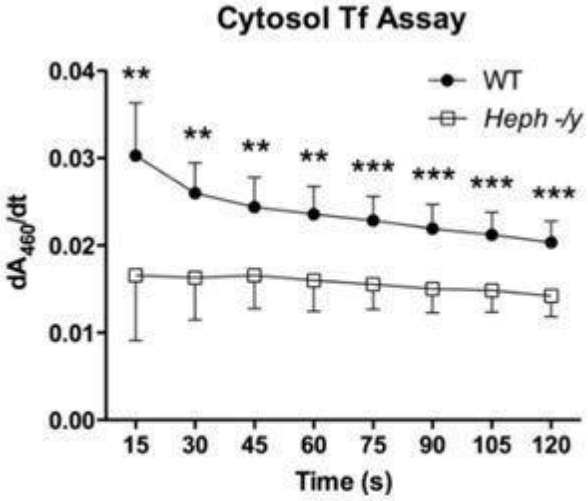

Figure 1: FOX Activity in *Heph* KO (-/y) mice and wild type (WT) littermates. n=7 per genotype; ** p<0.01, ***p<0.001

Poster #263

SPECIFIC INHIBITOR OF DMT1 FAILS TO PREVENT ENTEROCYTE COPPER UPTAKE, AS DETERMINED IN THE CACO2 CELL MODEL

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(Presented By: Maria Linder)

Introduction: Questions about the potential for divalent metal transporter 1 (DMT1) to transfer copper across cell membranes have lingered since discovery of this transporter. Clearly, DMT1 is critical for absorption of dietary iron by enterocytes, and also transports some other metals. Whether it transports copper has been controversial, Cu ions eliciting a transmembrane current in DMT1-transfected cells (Mackenzie et al. Biochem. J. 403: 59, 2007); DMT1 antisense RNA however causing a decrease Cu (I) uptake in the Caco2 enterocyte model (Arredondo et al. Am J. Physiol. 284: C1525, 2003); and enterocytes of Belgrade rats appearing to have a muted response to iron deficiency indicative of less copper uptake (Jiang et al. Am. J. Physiol. 301: G877, 2011). Cu(I) inhibited Fe(II) uptake by Caco2 cells, but with non competitive kinetics (Linder et al., Biol. Res. 39:143-156, 2006). Recent studies on xenopus oocytes transfected with DMT1 showed enhanced uptake of Fe(II) but no significant effects on Cu(I)/Cu(II) uptake (Illing et al. J. Biol. Chem. 287: 30485, 2012). We studied the effects of a specific DMT1 inhibitor to further resolve the matter of DMT1 involvement in absorption of dietary copper by the small intestine.

Methods and Materials: A specific inhibitor (XEN642) developed to block iron absorption for subjects with iron overload was utilized in studies with Caco2 cells grown as impermeable polarized monolayers (with tight junctions) in bicameral chambers on filters precoated with collagen. Radioactive copper or iron chelated with nitrilotriacetate (NTA)
and/or histidine was applied to the apical chamber (representing the intestinal lumen) to follow absorption across the brush border into cells and from there into the basolateral chamber (representing the blood circulation). Monolayers were/were not exposed to 100 or 200 nM XEN642 before and during the uptake phases. The presence of the DMT1 inhibitor decreased rates of Fe(II) uptake about 80% when administered as 5 or 10 μM 59Fe-labeled Fe(II)-NTA. The drug further decreased the release of iron to the basolateral chamber, overall transport (from the apical to basolateral chamber) falling 95-99%. Effects of XEN642 on rates of uptake of the divalent form of copper were then examined. Uptake of 64Cu-labeled Cu(II) was measured in the presence and absence of 100 and 200 nM doses of XEN642 after administering 5, 10 or 25 μM copper as the histidine complex. No effects of the DMT1 inhibitor were observed. The same was the case for uptake studies with Cu(I) in the form of NTA or histidine complexes. Interestingly however, the DMT1 inhibitor reduced transfer of absorbed copper into the basal chamber. Inhibition was about 10% at 5 μM Cu concentrations, but more marked with 10-25 μM copper (about 50% inhibition). In the absence of DMT1 inhibitor, the kinetics of Cu(I) and Cu (II) uptake were similar, but Vmax was higher for Cu(II) than Cu(I).

Conclusion: We conclude that DMT1 plays little or no role in uptake of dietary copper by enterocytes, and speculate that XEN642 may follow DMT1 into enterocyte endosomes in parallel with retreat of this transporter from the apical surface induced by iron exposure.

Poster Abstracts

Poster #264

INTESTINAL DIVALENT METAL-ION TRANSPORTER-1 IS CRITICAL FOR IRON HOMEOSTASIS BUT IS NOT REQUIRED FOR MAINTENANCE OF CU OR ZN

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(Presented By: Ali Shawki)

Introduction: Divalent metal-ion transporter-1 (DMT1)—the product of the *SLC11A2* gene—is a widely expressed iron transporter that serves intestinal iron absorption and erythroid iron utilization. Whereas DMT1 exhibits reactivity with a broad range of transition metal ions, questions have arisen as to the role that DMT1 plays in the absorption of Cu and Zn.
Methods and Materials: We tested the hypothesis that DMT1 is essential for the absorption of Fe, Cu, Mn, and Zn by examining hematological variables, iron status, expression of iron-related genes (by using real-time qPCR), and tissue metal content in a mouse model lacking intestinal DMT1 (i.e. $DMT1^{int/int}$) at age ≈ 120 days. Generation of the $DMT1^{int/int}$ model, by crossing floxed DMT1 and villin-Cre transgenic lines, was described previously [Gunshin et al (2005) *J. Clin. Invest.* **115**, 1258–1266].
Results: The $DMT1^{int/int}$ mouse exhibited a severe microcytic, hypochromic anemia—characterized by profound decreases in hematocrit ($DMT1^{int/int}$, 7% ± SD 2% cf. wildtype, 44% ± 4%; n = 7–9), hemoglobin concentration ($DMT1^{int/int}$, 2.3 ± SD 1.5 g/dL cf. wildtype, 14.6 ± 0.7 g/dL), mean corpuscular volume, and serum iron—accompanied by cardiac hypertrophy, splenomegaly, and severely depleted nonheme iron stores (liver, spleen). mRNA expression of the brush-border ferrireductase Cybrd1 was increased (30 ± 3)-fold in enterocytes of $DMT1^{int/int}$ mice cf. wildtype mice (mean ± propagated SE, n = 3) and mRNA expression of the basolateral iron-exporter ferroportin was increased (2.7 ± 1.0)-fold. Liver Hamp1 (hepcidin) mRNA expression was depressed by 98% ± 42% in the $DMT1^{int/int}$ mouse cf. wildtype. Intraperitoneal iron injection corrected the hematological variables, iron stores, and mRNA expression levels in the $DMT1^{int/int}$ mouse. Analysis of tissue metal content by using ICP–MS revealed strikingly decreased Fe levels in spleen, liver, heart and kidney in DMT1int/int compared with wildtype mice. Mn content was modestly decreased in kidney and spleen but not in other tissues. We observed no changes in tissue Zn or Cu levels, except for a modest increase in spleen Cu content.
Conclusion: Our data reveal that tissue-specific ablation of intestinal DMT1 produces an iron-deficiency anemia, and that DMT1 is critical for iron homeostasis but not required for maintenance of Cu or Zn. PHS Grant DK080047

Poster #265

QUERCETIN DECREASES IRON TRANSPORT ACROSS INTESTINAL CACO-2 CELL MONOLAYERS

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(Presented By: Rukshana Hoque)

Introduction: Polyphenols are known to be major inhibitors of dietary non-haem iron bioavailability. In this study we have investigated the effects of quercetin, the most abundant dietary flavonol, on transepithelial iron flux across Caco-2 cells and on the expression of genes involved in intestinal iron transport.
Methods and Materials: Caco-2 cells grown on Transwell inserts were exposed to quercetin (1 – 100 μM), added to the apical medium for 24 hours. Cells were washed in serum-free uptake buffer to remove excess polyphenol and fresh uptake medium (pH 6.5) containing 55Fe was added to the apical chamber to measure iron uptake into cells and the transepithelial flux of iron into the basolateral medium. In parallel, quercetin-treated cells were harvested and used to isolate RNA. Expression of DMT1, Dcytb, ferroportin and hephaestin were measured by qPCR.
Results: There was no significant effect of quercetin treatment on iron uptake across the apical membrane of the Caco-2 cell monolayer. However, transepithelial flux of iron into the basolateral membrane was significantly decreased in quercetin-treated cells.
Conclusion: Taken together this indicates that the major effect of quercetin is to inhibit release of iron across the basolateral membrane. Consistent with these findings, qPCR analysis of genes involved in intestinal iron transport revealed a significant decrease in ferroportin and hephaestin expression, while DMT1 and Dcytb mRNA levels were unaltered. Current work is investigating the cellular mechanisms underlying the effects of quercetin on intestinal iron transport.

Poster #266

MECHANISM FOR IRON DELIVERY TO HEME: ENDOSOME-MITOCHONDRIA INTERACTIONS AUGMENT FOLLOWING ERYTHROID DIFFERENTIATION

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(Presented By: Tariq Roshan)

Introduction: Iron (Fe) acquisition by cells requires the binding of diferric transferrin (Tf) to membrane transfserrin receptors, followed by the internalization of Tf-receptor complexes by endocytosis. After endosomal acidification and iron reduction, Fe^{2+} is released from endosomes via DMT1. In erythroid cells, more than 90% of iron enters mitochondria where ferrochelatase inserts Fe^{2+} into protoporphyrin IX. The path of Fe^{2+} from endosomes to ferrochelatase is not fully understood.
Methods and Materials: We have shown, using 2D and 3D live confocal imaging that there is a transient interaction of endosomes with mitochondria ("kiss-and-run") and that this interaction is required for efficient iron delivery to heme in reticulocytes . Moreover, we have demonstrated the interaction of these organelles by a novel method exploiting flow cytometry to analyze reticulocyte lysates labeled with Alexa Green Transferrin (AGTf) and MitoTracker Deep Red (MTDR). By using this new technique (flow subcytometry), we identified a double-labeled population representing endosomes interacting with mitochondria. The dynamic nature of this interaction was shown by chase experiments in which a time-dependent decrease of the double-labeled population was observed when reticulocytes were washed and re-incubated with unlabeled Fe_2-Tf. Furthermore, we have shown that the iron status of endosomes governs the efficacy of endosome-mediated iron delivery to mitochondria. In addition, we have used different 'locked' mutants of fluorescence-labeled, recombinant human Tf, which either remain permanently bound to iron (recombinant diferric-transferrin; L-Fe_2-hTf) or cannot bind to iron (recombinant apotransferrin; L-apo-hTf), in flow subcytometry studies.
Results: In these experiments, reticulocytes incubated with MTDR and L-apo-hTf failed to produce a double-labeled population. We also measured ^{59}Fe incorporation from ^{59}Fe-Tf into reticulocytes and their heme and showed that L-Fe_2-hTf, as compared to the wild-type hTf (WT-hTf), significantly decreased ^{59}Fe incorporation into both cells and heme. These results suggest that L-Fe_2-hTf (which cannot release iron) remains associated with mitochondria for a longer time than WT-hTf and thus blocks ^{59}Fe incorporation into heme. This probably slows the transferrin cycle, leading to decreased ^{59}Fe uptake by reticulocytes. Experiments with murine erythroleukemia (MEL) and fetal liver (FL) cells show a significant increase in the magnitude of contacts between endosomes and mitochondria when cells are induced for differentiation.
Conclusion: Hence, we conclude that the mitochondria-endosome interaction is universally involved in iron delivery for heme biosynthesis, but that this process is dramatically augmented in erythroid cells. Taken together our results show that endosomes come in contact with mitochondria to deliver iron for heme biosynthesis not only in erythroid cells but also in their progenitors.

Poster #267

CELL SPECIFIC EXPRESSION OF GENES INVOLVED IN IRON TRANSPORT AND THE EFFECTS OF HEPCIDIN

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(Presented By: Paul Sharp)

Introduction: Iron homeostasis is regulated by a complex series of interconnecting pathways in a number of different cell types. Hepcidin is thought to be the major systemic factor co-ordinating these pathways, acting as the master regulator of iron homeostasis. The aim of this study was to determine the levels of expression of iron metabolism genes in intestinal, hepatic and macrophage cell models and investigate the effect of hepcidin on expression of these genes.
Methods and Materials: Caco-2 cells (intestinal), HepG2 cells (hepatic) and THP-1 cells (macrophages) were exposed to hepcidin (1 μM) for 24 hours. Cells were harvested and RNA isolated. Gene expression levels and the effects of hepcidin were determined using Affymetrix Genechip Human Genome microarray.
Results: DMT1, ferroportin, HFE and hephaestin were expressed most highly in Caco-2 cells. Transferrin, transferrin receptor 2 and ceruloplasmin expression was highest in HepG2 cells. THP-1 cells showed the highest expression of Nramp1 and ferritin-H. Using a 2-fold cut-off, 163 genes in Caco-2 cells, 263 genes in HepG2 cells and 1178 genes in THP-1 macrophages were differentially regulated by hepcidin ($p<0.05$, ANOVA). Interestingly, none of the iron metabolism genes were differentially expressed in hepcidin treated cells.
Conclusion: These data suggest that hepcidin does not regulate iron homeostasis by inducing transcriptional changes in iron metabolism genes. In addition, our data indicate that there are clear transcription effects of hepcidin on other metabolic pathway. Taken together this suggests that hepcidin exerts important physiological actions distinct from its iron regulatory role.
Work funded by BBSRC BB/D015456/1

Poster #268
INACTIVATION OF TRANSFERRIN RECEPTOR (TFR1) IN THE PANCREAS RESULTS IN PANCREATITIS AND DIABETES
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(Presented By: Alan Chen)

Introduction: The pancreas is both an exocrine and endocrine tissue.
Methods and Materials: To explore a possible role for transferrin receptor 1 (Tfr1), we generated and characterized a conditional knockout mouse model in which Tfr1 is deleted throughout the pancreas in a mosaic fashion. All cells of the pancreas are susceptible to inactivation of a floxed Tfr1 allele when co-expressed with Cre recombinase under the control of the Pdx1 promoter. A subset of the Tfr1 pancreas-specific knockout mice began to die as early as 3 weeks after birth.
Results: In these mice, serum insulin levels decreased and, consequently, the mice developed hyperglycemia. Histologic examination of the pancreas showed a disruption of the exocrine tissue and, furthermore, a significant reduction in the number of islets of Langerhans, explaining the elevated glucose and the depressed insulin levels. There was mild bridging fibrosis with collagen deposition. However, we could not distinguish between two possibilities – that Tfr1 was required by insulin-producing cells, or that pancreatitis caused islet destruction. To address this question, we used Cre recombinase expressed under the control of the RIP promoter to conditionally delete Tfr1 in insulin producing β-cells. These animals developed normally, with no increased lethality. Histology revealed the presence of normal islets of Langerhans. Serum glucose and insulin levels were comparable to wildtype, indicating that the phenotype of the global pancreatic knockout mice was most likely attributable to the disruption of exocrine tissue.
Conclusion: We conclude that Tfr1 is required for pancreatic homeostasis because of an essential role in exocrine cells. The loss of islets of Langerhans and hyperglycemic phenotype appear to be secondary effects. Future studies will be directed towards understanding why pancreatic exocrine cells require Tfr1.

Poster #269
ACTIVATION OF THE ESSENTIAL DIIRON ENZYME DEOXYHYPUSINE HYDROXYLASE BY THE IRON CHAPERONES PCBP1 AND PCBP2
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(Presented By: Caroline Philpott)

Introduction: Cells contain hundreds of metalloproteins, yet the mechanisms through which these proteins receive their metal cofactors remain largely unknown. Ferritin, an iron storage protein, depends on the iron chaperone PCBP1 for metallation in Huh7 cells. PHDs are iron-dependent, prolyl hydroxylases that regulate hypoxia-inducible factor1. PHDs are dependent on PCBP1 and its paralog, PCBP2, for metallation of their mononuclear iron sites.
Methods and Materials: Here we show that an essential enzyme of the oxodiiron class, deoxyhypusine hydroxylase (DOHH), is also dependent on PCBP1 and PCBP2 for metallation in cells.
Results: Depletion of PCBP1 and 2 by siRNA led to loss of DOHH activity and the loss of the metal-bound holoenzyme in cells. DOHH was found associated with PCBP1 by co-immunoprecipitation, but only in cells treated with iron. PCBP1 and 2 depletion did not affect the activity of Fe-S cluster enzymes, including c-aconitase, m-aconitase, and xanthine oxidase. Heme synthesis was similarly unaffected.
Conclusion: These findings indicate that PCBPs serve as iron chaperones for multiple classes of cytosolic non-heme enzymes, but are not required for delivery of iron to mitochondria.

Poster #270
A LOOK AT LIVER IRON TRANSPORT BY FOLLOWING RADIOLABELED MONODISPERSE IRON OXIDE NANOPARTICLES IN VIVO
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(Presented By: Denise Bargheer)

Introduction: The major proteins and mechanisms in iron transport have been elucidated in the last years and we have today a well-accepted view on the iron homeostasis in mammals. However, astonishing few in vivo studies have been performed so far showing the fate of larger iron compounds such as intravenous (i.v.) iron preparations. Iron transport in the liver is thought to involve mainly hepatocytes for the handling of transferrin bound iron and Kupffer cells for red cell degradation. The roles of sinus lining endothelial cells as well as intercellular iron transport ways are not known in detail.
Methods and Materials: Iron based nanoparticles (MIONs) are regarded as promising tools in future nanomedicine as they can be functionalized and used as specific MRT diagnostics or in drug delivery. We synthesized ^{59}Fe-labelled superparamagnetic nanoparticles (1) and followed their distribution and degradation in mice after i.v. injection as well as

in cell culture models including primary liver cells. All liver cell types can in general take up these nanoparticles. After i.v. injection in mice, the liver was the main organ for nanoparticles uptake and degradation.
Results: Using transelectron microscopy (TEM), the monodisperse particles are found in endocytic vesicles in Kupffer cells and also in sinus lining endothelial cells but not in hepatocytes. Pre-treating mice with i.v. “Clodronate liposomes” largely depleted the actual population of phagocytosing cells in the liver and the spleen. This had almost no effect on the overall uptake of nanoparticles into the liver, on the ^{59}Fe-whole-body-retention, and the faecal excretion of ^{59}Fe. However, as indicated by the ^{59}Fe-erythrocyte incorporation, the degradation of the iron core of the test MION was substantially delayed and diminished by pre-treatment with Clodronate indicating the important role of Kupffer cells in the degradation of iron based nanoparticles. In the absence of Kupffer cells, most of these particles are taken up by sinus-lining endothelial cells which must also be partly capable to degrade the 10 nm iron oxide cores.
Conclusion: It can also be speculated that the acute and chronic toxicity of high-molecular weight iron compounds in vivo is greatly influenced by the uptake, storage/and or processing of nanoparticles in specific cells. Detailed studies in primary liver cells from mice injected with radiolabeled MIONs are under way to clarify the role of different cell types in the storage or degradation of iron-based nanoparticles in the liver.
(1) Freund B et al. ACS Nano. 2012 Jul 20. DOI: 10.1021/nn3024267

Poster #271
DUODENAL CYTOCHROME B PLAYS A ROLE IN COPPER UPTAKE
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(Presented By: Solomis Solomou)

Introduction: Iron and copper are absorbed in their reduced forms (ferrous and cuprous, respectively). Duodenal cytochrome b (Dcytb) functions as an intestinal ferric reductase; however, the nature of the putative intestinal cupric reductase remains unclear. We have shown previously that cells over-expressing Dcytb show not only enhanced ferric reductase activity but also display significant cupric reductase activity, suggesting that Dcytb may function as a dual metallo-reductase in intestinal enterocytes (Wyman et al FEBS Lett 2008, 582:1901-6). The aims of this study were to investigate whether intestinal Dcytb expression was regulated by copper, and whether Dcytb might play a role in cellular copper uptake.
Methods and Materials: Human intestinal epithelial cells (Caco-2 and HuTu-80) were exposed to either the copper chelator TETA (0.5 mM) or copper (50 μM) for 24 h. Cells were harvested for RNA and expression of Dcytb and the copper transporter Ctr1 determined by qPCR. To determine whether Dcytb plays a role in copper uptake we employed a MDCK cell line which over-expresses Dcytb under the control of a tetracycline-repressible promoter (TET-Off MDCK, Clontech; Wyman et al 2008). Copper uptake was measured by ICP-OES.
Results: In Caco-2 cells, Ctr1 expression was significantly increased by TETA treatment ($p< 0.01$) and significantly decreased by exposure to high copper medium ($p<0.05$). Basal Dcytb expression in Caco-2 cells was below the detection threshold in our studies so we used HuTu-80 cells as an alternative intestinal model. Dcytb expression was significantly increased in TETA-treated cells ($p<0.01$). Copper treatment did not significantly alter Dcytb mRNA compared with untreated controls. Copper uptake in Dcytb-overexpressing MDCK cells was significantly increased compared with untransfected control cells (+6.7-fold; $p<0.01$). Treatment with doxycycline (20ng/ml) significantly reduced copper uptake to levels seen in untransfected cells ($p<0.01$).
Conclusion: These data demonstrate that Dcytb is a copper-regulated gene and that it plays a role in copper uptake. Taken together with our previous work we hypothesize that Dcytb functions as an intestinal cupric reductase providing Cu+ to Ctr1 for cellular uptake. This work forges a further link between mammalian iron and copper metabolism.

Poster #272
DIETARY BERRY POLYPHENOLS MODULATE EXPRESSION AND FUNCTION OF INTESTINAL IRON TRANSPORTERS
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(Presented By: Fawaz Alzaid)

Introduction: Berries are a rich dietary source of polyphenolic compounds, such as anthocyanin flavonoids (1). Previous research has demonstrated that the transport of iron across Caco-2 cell epithelial monolayers is impaired in the presence of flavonoids (2). However, the long-term effects of flavonoids on expression and function of intestinal iron transport have not been previously addressed.
Methods and Materials: In light of this, the present study investigated the effects of flavonoids, in the form of an anthocyanin-rich berry extract, on the expression and function of genes involved in iron transport in human intestinal Caco-2 cells. Caco-2 cells were cultured for 19 d, then treated either acutely (15 min) to determine the effects on iron transport when both iron and flavonoids are present; or chronically (16 h) to determine the effects of flavonoids on gene expression and the resulting functional implications. The source of flavonoids was a commercially available anthocyanin-rich berry extract (OptiBerry; InterHealth Nutraceuticals, Benicia, CA, USA) at a final concentration of 0.125% (w/v). Gene expression was analysed by qRT-PCR; functional effects on iron transport were determined by applying radiolabelled Iron-55 to Caco-2 cells grown on Transwell® insert membranes. All gene expression data were normalised to 18S and GAPDH as housekeeping genes and

presented as mean normalised expression ratio and SEM. Statistical significance was determined by Student's unpaired t-test with significance indicated at $p \leq 0.05$.
Results: Addition of iron in the presence of flavonoids (acute treatment) caused an overall decrease in iron transport across Caco-2 cell monolayers ($p < 0.05$); this occurred despite apical iron uptake being significantly increased ($p < 0.05$). Following chronic treatment, flavonoids also resulted in decreased transepithelial flux of iron ($p < 0.05$); however, this was associated with a significant decrease in apical iron uptake ($p < 0.05$). Chronic exposure to berry extract also resulted in decreased expression of iron influx genes [Divalent metal ion transporter (DMT1), $p < 0.001$; and Duodenal Cytochrome B reductase (DCYTB), $p < 0.05$], while expression of ferroportin and hephaestin was not significantly altered.
Conclusion: These results indicate that dietary flavonoids have the potential to influence the expression and function of the intestinal iron transport pathway. Further studies will investigate cellular responses and aid in developing a model to elucidate the biological relevance of these effects in relation to the bioavailability of dietary iron.
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2. Kim EY, Ham SK, Shigenaga MK et al. (2008) Bioactive dietary polyphenolic compounds reduce nonheme iron transport across human intestinal cell monolayers. J Nutr. 138, 1647–1651.

Poster #273
A PRIMARY CULTURE OF CARDIOMYOCYTES WITH FRATAXIN DEFICIENCY EXHIBITS A METABOLIC SWITCH BEFORE IRON DISARRANGEMENTS
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(Presented By: Elia Obis)

Introduction: Friedreich ataxia (FRDA) is a rare hereditary disorder which usually begins earlier in live and has a progression of symptoms that lead patients wheelchair bound in adulthood. Some of the patients develop diabetes and most of them die from heart problems. It is caused by mutations in the frataxin gene that lead to a decreased expression of this mitochondrial protein, which has been involved in iron sulphur cluster (ISC) homeostasis. Heart specific problems are still not well known, so we attempted to establish a cardiomyocyte model to study frataxin deficiency in these cells, determine if they were intrinsically affected and analyze the particular effects in this singular cell type.
Methods: we used primary cultures of newborn rat ventricular myocytes and frataxin knockdown was conducted with lentiviral vectors containing shRNA. Cardiomyocyte purity was assessed by immunofluorescence of a specific sarcomeric protein. Frataxin expression was analyzed by Real-Time PCR and Western blot, and following experiments were carried out 7 days after viral infection. We analyzed the network and function of mitochondria *in vivo* with Mitotracker® and JC-1® probes. Energy status was checked by nucleotide phosphates content measured with high-performance liquid chromatography (HPLC). Electron transport chain complexes activity and levels were also assayed. Iron content was measured photometrically in digested cells and expression of iron regulated mRNAs by Real-Time PCR. Aconitase activity was also evaluated as an ISC protein. Metabolomic analysis was performed by liquid chromatography-quadrupole time-of-flight (LC-Q-TOF) -based approach. Lipid droplets were stained with BODIPY®.
Results and Discussion: we succeed to knockdown frataxin in cardiomyocytes to levels similar to those found in patients and to analyze the primary effects of this deficiency. Under such conditions, we found an impaired mitochondrial network, with giant mitochondria with low membrane potential. However, AMP/ATP and ATP/ADP ratios were not affected and neither were the electron transport chain activities. Metabolic fingerprinting indicated that frataxin knockdown cardiomyocytes exhibited a metabolic shift. A dysfunction in lipid metabolism was also observed. Despite all this alterations, intracellular iron, aconitase activity and expression of iron regulated mRNAs were not altered.
Conclusions: Cardiac myocytes with depleted frataxin protein levels exhibit a metabolic switch, dysfunction in lipid metabolism and disarrangement of the mitochondrial network, which could compromise the heart contractile function and the survival of FRDA patients.

Poster #274
REGULATION OF IRON TRANSPORTERS AND REGULATORY PROTEINS IN DEVELOPING WILD TYPE (HFE+/+) AND KNOCK OUT (HFE-/-) NEONATE MICE
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(Presented By: Sara Balesaria)

Introduction: Iron status of the neonate is important in ensuring adequate development and growth. Previous studies have demonstrated that the Hfe status of the foetus is important in determining how much iron is transported across the placenta. Hfe knockout pups have been shown to have higher body iron stores than their WT counterparts in utero, possibly due to dysregulation of hepcidin in the Hfe knockout pup liver affecting iron transport across the placenta. In this study we investigated whether knockout of Hfe would affect iron parameters in the neonate and how iron transporter gene expression would differ in both the liver and duodenum compared with WT mice.

Methods and Materials: Hfe knockout and WT pups were sacrificed 0, 2, 6, 10 and 20 days after birth. Dams were fed an iron replete diet (150ppm iron) throughout pregnancy and were maintained on the same diet during suckling. Duodenal and hepatic transporters were measured by real-time PCR and non-heme liver iron levels were also determined.
Results: Our results showed that in the duodenum, expression of DcytB, Dmt1 and ferroportin was not dependant on Hfe genotype. Expression of these genes differed only at various stages of development with DcytB and Dmt1 increasing markedly at day 20, coinciding with pups weaning. Hif2alpha levels in the duodenum were dependant on Hfe genotype with levels being lower in Hfe knockout than in WT at every time point measured.
Liver iron levels were higher in Hfe knockout than in WT at day 0 and continued to be elevated until day 20 where non-heme liver iron levels were the same in both Hfe knockout and WT pups. These levels however, are markedly lower than their adult counterparts. Interestingly hepatic hepcidin levels were significantly higher in Hfe knockout pups at day 0 but levels continued to decline after this time point to the same level as WT. Tfr1 levels increased at day 2 but continued to decline in pups
of both genotypes although levels were higher in WT at almost every time point. Tfr1 and hepcidin levels were positively correlated in WT pups ($p<0.001$, $R2=0.61$) but there was no correlation in Hfe knockouts. Expression of Dmt1, Tfr2 and ferroportin followed a similar pattern in pups of both genotypes until day 10 where levels of all three genes were markedly upregulated in Hfe knockout pups. Tfr2 levels were positively correlated with both Dmt1 and ferroportin in Hfe knockouts ($p<0.001$, $R2=0.78$; $p<0.001$, $R2=0.86$, respectively) and to a lesser degree in WT.
Conclusion: Taken together our results demonstrate that during development, especially during suckling, the duodenum plays a diminished role in iron regulation. It would appear that the liver plays a major role in iron distribution during suckling and that this regulation can differ depending on genotype.

Poster #275
TRACING IRON BALANCE FROM MOUSE TO HUMAN
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(Presented By: Fudi Wang)

Introduction: Iron is an essential element for most living organisms on earth, including human beings. An inability to maintain iron homeostasis may lead to death or a disease. In fact, it is estimated that nearly one quarter of population worldwide are suffered from iron deficiency anemia. In contrast, iron overload induces hemochromatosis, and many neurodegenerative diseases. Therefore, maintenance of iron homeostasis is crucial for human health. Recently, emerging evidence supports iron transporters play important roles in regulate iron homeostasis. However, the underlying molecular mechanisms are not well defined.
Methods and Materials: To explore the mechanistic network of iron regulators in maintenance of iron homeostasis, we initiated our investigations with knockout mouse models and epidemiology.
Results: Recently significant findings are listed as followings: 1) By using macrophage Fpn1 deletion mouse models, they provided solid data to support Fpn1 plays important roles in macrophage iron release and in modulating innate immune responses (*Blood*, 2011); 2) Functionally characterized Fpn1 as a major iron exporter in hepatocytes, and further defined hepatocyte Fpn1 as one of the important players in iron mobilization, iron storage, and intestinal iron absorption to maintain systemic iron homeostasis (*Hepatology*, 2012); 3) We observed metalloreductase Steap3 coordinates with Fpn1 to regulate systemic iron homeostasis and inflammatory responses (*Haematologica*, 2012); 4) Defined significant association between TMPRSS6 polymorphisms and decreased iron status, and refined genetic risk factors for iron deficiency and iron-deficiency anemia (*Hum Mol Genet*, 2012); and 5) Discovered *TMPRSS6* variants, which mediated by plasma ferritin, were significantly associated with lower risk of type 2 diabetes in Chinese Hans (*Am J Clin Nutr*. 2012). Furthermore, recently we measured the level of hepcidin expression in cultured cells treated with sixteen different medicinal plant extracts, all of which are used to treat anemia-related disorders in traditional Chinese medicine. Among the extracts tested that of Caulis Spatholobi, also called Jixueteng, showed the most potent inhibitory effect on hepcidin and was therefore selected for further mechanistic study.
Conclusion: Our data indicated that the extract of Caulis Spatholobi as a novel, potent hepcidin inhibitor, which may be further modified and optimized to become an effective herb supplement or treatment option for diseases in which hepcidin is overexpressed, such as ACD or IRIDA. We believe our studies pave ways to translate the findings to therapeutic target stratification in iron metabolism related diseases.
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Poster #276
ROLE OF QUERCETIN ON IRON HOMEOSTASIS
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(Presented By: Marija Lesjak)

Introduction: Iron is crucial in processes such as oxygen binding in haemoglobin, electron transport in mitochondria, cell proliferation and differentiation. As there is no regulated physiological mechanism for excretion of iron, iron homeostasis is maintained primarily by regulating iron absorption. Therefore dietary iron content and other dietary constituents can have a major impact on iron homeostasis. Quercetin, one of the most abundant flavonols in the diet, acts as an antioxidant by

modulating expression of antioxidant enzymes and is also able to chelate iron. Quercetin therefore can have an effect on iron absorption either by chelating iron and/or by virtue of its antioxidant properties. The aim of this study was to determine the acute and chronic effects of oral quercetin administration on duodenal iron uptake, serum iron levels and duodenal iron tranporters and effect of introducing quercetin with radioactive iron.
Methods and Materials: Sprague Dawley male rats fed on an iron deficient diet (25ppm) for two weeks, prior to quercetin administration (50mg/kg) and then during the period of study, 10 days for chronic and 5/18 h for acute effect. . Real-time PCR was used to determine the expression of duodenal iron transporters (DMT1, FPN and Dcytb) expressions. Intestinal iron absorption was measured in another group of animals using the in situ loop method containing quercetin (50mg/Kg) and radioactive iron. Levels of iron and transferrin saturation in serum, and nonheme iron in duodenum, liver and spleen, were also determined.
Results: Chronic oral treatment of rats with quercetin (for 10 days), resulted in significant decrease in both liver and spleen iron levels. These results confirmed the negative effects of chronic polyphenols consumption on iron adsorption when consumed in the diet for a longer time. Acute oral administration of quercetin affects iron homeostasis by changing expression of proteins involved in iron absorption, primarily by reducing DMT1 and FPN. Moreover, in uptake experiments where quercetin was introduced into the duodenum together with radioactive iron, resulted in a significant difference in levels of radioactive iron accumulated in duodenal mucosa (increase 2.7 fold) or transferred to serum (decrease 7.3 fold), compared to controls. The most probable explanation is that quercetin chelates iron, and that the complex is transported into enterocytes, but is not transported further into the circulation. In order to test this conjecture we repeated the above experiments substituting quercetin with pentamethyl-quercetin (unable to chelate iron), Iron uptake into mucosa and transfer into circulation was not affected by non-iron chelating Pentamethyl-quercetin when compared with control.
Conclusion: In summary we have demonstrated that dietary quercetin has an effect on duodenal iron absorption by virtue of its ability to chelate iron. Acute effect of quercetin is to reduce the expression of iron importer and exporter levels. In the long term dietary quercetin results in lower body iron stores, most likely due to its ability to chelate iron, however systemic effect of quercetin on iron absorption can not be ruled out and requires further studies.

Poster #277
CHARACTERISATION OF FERROXIDASES IN HUMAN LEUKAEMIC K562 CELLS
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(Presented By: Kenneth White)

Introduction: The human erythroid cell line K562 has been widely used to study erythroid differentiation and globin gene expression. Several reports have demonstrated that this cell line expresses the membrane ferroxidase hephaestin, which, together with its homologue serum ferroxidase ceruloplasmin, plays a role in facilitating export of iron from cells that have a key role in systemic iron homeostasis such as duodenal enterocytes and macrophages. It is not clear why K562 cells should express hephaestin since they are an erythroid precursor line that can be induced to differentiate by addition of excess iron, especially hemin, and it seems paradoxical that they should have iron export capability. Nevertheless K562 cells present a convenient model to study hephaestin since they are easy to grow and transfect. One key aspect of hephaestin cell biology is its localisation in the cell. Early models assumed hephaestin would be expressed on the plasma membrane where it would have access to the iron transporter transferrin. However, studies on enterocytes or polarised epithelial cells have shown a marked and extensive intracellular localisation of hephaestin, despite its role of mediating cellular iron export. More recent work has demonstrated clear localisation of hephaestin on the plasma membrane of enterocytes, but limited studies on K562 cells suggest a distinct intracellular localisation of hephaestin.
Methods and Materials: As part of an ongoing programme of studies on the interactions between ferroxidases and iron transport proteins we carried out a biochemical analysis of ferroxidase expression in K562 cells.
Results: Ferroxidase activity was assayed using a ferrozine based method that measures consumption of Fe2+ substrate. A marked masking of ferroxidase activity was found in whole K562 cells since addition of TX-100 in the assay buffer stimulated measurable activity about 20 fold, confirming that hephaestin was mostly inside K562 cells. Fractionation of K562 cells into membrane and cytosol fractions revealed roughly equal amounts of ferroxidase activity in each fraction.
Membrane activities were investigated further by non-denaturing electrophoresis followed by ferroxidase staining and western blotting to detect hephaestin. A strong band of ferroxidase activity was found to run more slowly than a ceruloplasmin control, and was confirmed by western blotting of the non-denaturing gel to be hephaestin.
A second, faster running band was consistently found, accounting for a small proportion, about 7%, of the total membrane activity. The identity of this activity remains to be characterised, but appears not to be a fragment of hephaestin.
We attempted to analyse the cytosolic ferroxidase by non-denaturing electrophoresis but could not identify a clear activity, probably because the sample contains a smaller number of cell equivalents (about 105) compared with membrane sample (about 106).
Conclusion: In conclusion we have confirmed that the main membrane ferroxidase activity of K562 cells is hephaestin but note the existence of a potentially novel second membrane ferroxidase. The cytosol also contains a substantial amount of ferroxidase activity of as yet unknown origin. Candidates include xanthine oxidase, ferritin, amyloid precursor protein. The last could also account for the presence of a second membrane ferroxidase.

Poster #278
IRON EXCHANGE PROPERTIES OF DIFERRIC TRANSFERRIN
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(Presented By: Robert Woodworth)

Introduction: The iron-transport protein transferrin found in the blood plasma of vertebrates binds two ferric ions with an estimated binding constant of 1023 at physiological pH. A binding constant implies two competing reaction rates, one for association and one for dissociation.
Methods and Materials: This study was designed to discover whether diferric-tranferrin, Fe2Tf, dissociates at a measurable rate under "physiological" conditions.
The protocol for these studies was based on the fact that isotope exchange reactions,

$$AX+BX^*=AX^*+BX$$

no matter what the detailed reaction mechanism, generally follow a first order rate equation

$$\ln[xinfin/(xinfin-x)]=(R/ab)(a+b)t$$

where: $a=[AX]+[AX^*]$; $b=[BX]+[BX^*]$; $x=[AX^*]$; $a-x=[AX]$
In our case $Fe2Tf+Fe^*=Fe^*2Tf+Fe$
where: Fe is Fe56 and Fe* is Fe59
Because the binding constant for the binding of iron to transferrin is ~10^{23}, the rate controlling step in the dynamic equilibrium is likely to be the dissociation of Fe from Fe_2Tf. The approach was to saturate the transferrin in normal human serum with Fe^{56}, then to add Fe^{56} and follow the incorporation of the Fe^{59} isotope into the Fe_2Tf complex at 37^oC by counting the incorporated activity. This was accomplished by drawing aliquots of the reaction mixture at increasing times and precipitating the iron-transferrin complex with an anti-human-transferrin antibody produced in rabbits. The precipitate was washed twice in normal saline then counted for radioactivity.
Results: Controls showed that the Fe^{59} was effectively locked into the precipitate. Fresh ascorbate was added to the human serum in the study to give a concentration of 1 mg/ml. During the exchange reaction pH was maintained by keeping the reaction mixture under an atmosphere of 5%CO2/95%N2. Concentrations of Fe_2Tf, Fe(II), buffer, H^+ and EDTA were varied in these experiments in order to analyze the involvement and molecularity of each in the exchange reaction. Plots of l+log[1-(x/xinfin)] vs t revealed two phases, a rapid exchange reaction with a zero intercept of 1.0 and a slower exchange reaction with a zero intercept of 0.5. Plots of the faster reaction rate vs $[Fe_2Tf]$ gave a straight line with a slope of 1.0. Plots of the slower reaction rate vs $[Fe_2Tf]$ gave a slope of ~0.2. Plots of the faster reaction rate vs. $[H^+]$ gave a slope of 1.0 and vs $[CO_2+HCO_3^-]$ gave a slope of 1.0. Plots of the faster reaction rate vs. concentration for other buffers, e.g., Tris, glycylclycine, gave plots with a slope of zero. Chelation of the excess, labeled iron with EDTA had no effect on the reaction rate. Increasing the reaction temperature increased the reaction rate.
Conclusion: The rate dependences of the isotope exchange rates for Fe^{59} into Fe_2Tf show that for the N-lobe the reaction rate is first order in $[Fe_2Tf]$ and zero order in $[Fe^{59}]$. Therefore, the overall exchange reaction is controlled by simple dissociation of Fe from Fe_2Tf. The radioisotope then rapidly binds to the empty binding site. The first order dependence of the dissociation rate on $[H^+]$ implies the direct involvement of a proton in the iron-release mechanism. The first-order dependence of the dissociation rate on $[CO2+HCO_3^-]$ implies a direct involvement of one of these species in the release mechanism. Recall that the binding of Fe(III) to Tf requires a synergistic anion, normally HCO_3^-. Other buffers lack this effect. The meaning of the roughly zero-order dependence of the slow-exchange rate on $[Fe_2Tf]$ is not clear, but it likely arises from isotope exchange in the C-lobe of transferrin.

Poster #279
PROXIMITY LIGATION ASSAYS INDICATE THAT HEMOCHROMATOSIS PROTEINS HFE AND TRANSFERRIN RECEPTOR 2 (TFR2) DO NOT INTERACT
Gautam Rishi, Msc (Medical Genetics), MSc (Human Genomics), Daniel Wallace, PhD and V. Nathan Subramaniam, PhD
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(Presented By: Gautam Rishi)

Backgrounds and Aims: Mutations in HFE and transferrin receptor 2 (TFR2) are associated with the iron overload disorder hereditary haemochromatosis, characterized by an inappropriate expression of hepcidin relative to body iron levels. Earlier studies using transient over-expression systems have suggested that an interaction between HFE and TFR2 is required for the correct regulation of hepcidin. Studies from our group using the Hfe/Tfr2 double knockout mouse and others suggest that an interaction between Hfe and Tfr2 is not required for the regulation of hepcidin (1-3). We used a novel co-expression system to study the interactions between Hfe, transferrin receptor 1(Tfr1) and Tfr2 at a molecular level using a recently developed proximity ligation assay.
Methods: A stable co-expression system in the mouse hepatoma cell line,Hepa 1-6, was established with a single plasmid expressing both Flag-tagged Hfe and myctagged Tfr2. Confocal microscopy was used to determine the localisation of Hfe, Tfr1 and Tfr2. Co-immunoprecipitation (Co-IP) studies using anti-Flag beads and Tfr2 antibody was performed on lysates of cells cultured in the presence or absence of holo or apo transferrin (Tf). The proximity ligation assay (PLA) (DuolinkTM) was used to determine the interactions at a molecular level.

Results: Using confocal microscopy we observed co-localisation of Hfe and Tfr1 but minimal co-localisation between Hfe and Tfr2. Treatment of cells with holo or apo-Tf did not affect the degree of co-localisation of Hfe and Tfr2. Co-IP with anti-Flag beads or Tfr2 antibody revealed an absence of binding with Tfr2 or Hfe respectively. The Duolink assay is used to detect weak and transient interactions between two proteins. Duolink analysis indicated the absence of any interaction between Hfe and Tfr2, plus or minus holo or apo transferrin.
Conclusions: These studies indicate an absence of interaction between Hfe and Tfr2 in mouse hepatoma cells stably co-expressing these proteins. These results are similar to a recently published study using transgenic mice (2). In our study we systematically investigated the co-expression and localization of Hfe, Tfr1 and Tfr2.
Earlier published studies which have shown an interaction between TFR2 and HFE have used transient overexpression systems; the co-expression of Hfe and Tfr2 from a single construct in our study keeps the relative expression of the two proteins similar and consistent which may explain the different results. Recent studies support this and have gone a step further in suggesting that the interaction between Hfe and Tfr2 is not required for hepcidin regulation (1-3). Our results indicate that Hfe and Tfr2 do not form a complex with each other.
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Poster #280
A NEW CLASSIFICATION OF GENETIC IRON OVERLOAD SYNDROMES
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(Presented By: Motoyoshi Yano)

Introduction: In addition to hereditary hemochromatosis (HH), aceruloplasminemia (aCP) and ferroportin disease B (FP−B) may be complicated by iron−induced multiple organ damage; therefore, clinicopathological features should be evaluated in a wider range of genetic iron disorders.
Methods and Materials: This study included 15 Japanese patients with genetic iron overload syndromes. The responsible genes were CP in 4, HAMP in 1, HJV in 3, TFR2 in 5, and SLC40A1 in 1 patient. A patient with FP−B was free from mutation in SLC40A1. Their clinical features, biochemical parameters including hepcidine25 and liver histologies were compared among the genotypes.
Results: Diabetes was a major disorder common to all genotypes. Transferrin (TF) saturation was low in aceruloplasminemia (aCP) patients. All patients, except those with FP−B, displayed unusually low serum hepcidin25 levels. Altered FP proteins in the enterocytes may resist hepcidin25. Liver pathology showed phenotype−specific changes; isolated hepatocyte (H) iron loading in aCP, periportal fibrosis associated with heavy iron overload in both H and Kupffer cells (K) of FP−B, and H−dominant iron−loading fibrosis or cirrhosis in HH of TFR2, HJV and HAMP. The impaired hepcidin−FP system may divide genetic iron overload syndromes into 3 types of pre−hepatic aCP, hepatic HH, and post−hepatic FP−B, as summarized in the Table. HFE−hemochromatosis may be included in hepatic HH.
Conclusion: In conclusion, based on clinicopathological features, we proposed a new classification of genetic iron overload syndromes.

Table. Three types of iron overload syndromes and their characteristics

	Disease Entity	Serum Hepcidin25	Disrupted System	Hepatic Iron Overload	Hepatic Histology
Pre-Hepatic	aCP	low	TF saturation	H-selective	fibrosis free
Hepatic	HH	very low	hepatic signals	H-dominant	cirrhotic
Post-Hepatic	FP-B	normal	resistant FP	mixed H and K	fibrosis

Poster #281

HETEROZYGOUS DELETION OF THE HFE GENE CONTRIBUTES TO HEPATIC IRON LOADING BUT NOT STEATOSIS IN MICE FED A HIGH FAT DIET

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(Presented By: Laurence Britton)

Background: Altered iron metabolism and heterozygosity for *HFE* (haemochromatosis) gene mutations are common in non-alcoholic fatty liver disease (NAFLD) and are associated with more severe disease. We have previously shown that *Hfe*$^{-/-}$ mice develop steatohepatitis and fibrosis when fed a high fat diet (HFD). Paradoxically, a HFD was associated with reduced hepatic iron stores and other investigators have confirmed these findings and shown that a HFD reduced intestinal iron absorption. Since heterozygosity for *HFE* is common in adults with NAFLD, we explored the effect of feeding a HFD to *Hfe* heterozygous (*Hfe*$^{+/-}$) animals. Methods: Eight week old *Hfe*$^{+/-}$ and wild type (WT) C57BL/6J animals were fed either a HFD or chow for eight weeks and then sacrificed. Liver tissue was analysed for histology, markers of iron metabolism, hepatic fibrosis and lipid metabolism. Results: *Hfe*$^{+/-}$ animals fed a HFD had a higher hepatic iron concentration (HIC) than WT mice (6.35 vs. 3.80 µmol Fe/g dry weight) representing partial phenotypic expression. Consistent with previous studies, HFD was associated with a trend towards reduction in HIC in both *Hfe*$^{+/-}$ and WT animals. HFD was associated with steatosis in both groups, although this was more pronounced in WT mice. Hepatic triglyceride content and serum alanine aminotransferase (ALT) however were not significantly different between groups. mRNA expression of regulators of de novo lipogenesis: Srebp-1 (sterol regulatory element binding protein-1), Scd1 (stearoyl coA desaturase 1) and fatty acid oxidation: Ppar-α (peroxisome proliferator-activated receptor-α), Cpt1 (carnitine palmitoyl transferase 1a) was also similar between genotypes.
Conclusions: *Hfe*$^{+/-}$ mice fed a HFD show evidence of partial phenotypic expression of the genetic defect. Despite the increase in HIC, *Hfe*+/- animals do not show increased susceptibility to HFD. Unlike homozygous deletion, heterozygosity for *Hfe* in this mouse model does not influence the severity of NAFLD.

Poster #282

HFE MUTATIONS IN OCEANIAN PEOPLE (MELANESIAN AND POLYNESIAN)

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(Presented By: Gerard Dine)

Introduction**:** Homozygous mutations of the HFE gene lead to hemochromatosis. In Europe, it is mainly associated with the C282Y mutation. Other mutations are involved in the disturbance of the iron metabolism, particularly H63D. Previous international studies reveal significant frequency, notably for the H63D mutation, among European populations.
Methods: We carried out a preliminary study in 2011 aiming at measuring the HFE mutations frequency among an Oceanian population from South Pacific in New-Caledonia. Each volunteer provided a full written consent, approved by the Necker Hospital Ethic Committee. The blood tests were done at the elbow fold. The following biological analyses were completed on the blood samples: complete blood count, serum iron, transferrin saturation, ferritin, glycated hemoglobin. Among the collected blood samples, 2 EDTA tubes per subject were repatriated for DNA extraction and gene assays in the IBT laboratory.
The study included 119 subjects (66 Melanesian and 53 Polynesian), range 13-35 years (mean age 23±4.47). The participants were considered of Melanesian or Polynesian origin on the basis of two-generation autochthonous ancestry. Subjects born from miscegenation were excluded, in particularly European. The repartition gave a male/female ratio of 0.78 for Melanesian subjects (29 men and 37 women) and of 1.4 for Polynesian subjects (31 men and 22 women). The genetic assays done with the Vienna Lab method covered the following mutations: HFE mutations (V53M, V59M, H63D, H63H, S65C, Q127H, P160delC, E168Q, E168X, W169X, C282Y, Q283P), TFR2 gene (E60X, M172K, Y250X, AVAQ594-597del) and FPN1 gene (N144H, V162del). To assess the different biological parameters between mutated and non mutated subjects, we conducted t-tests.
Results: No C282Y mutation has been found. However, at the heterozygotic state, three H63D mutations have been identified in the Melanesian group (2 women and 1 man) and four in the Polynesian group (1 woman and 3 men). There is no significant difference between both groups. The frequency in the general Oceanian population is 7/119 and more precisely 3/66 in the Melanasian group and 4/53 in the Polynesian group. Concerning the biological datas, hemoglobin levels of mutated male subjects appear slightly higher than the levels of non mutated male participants (p=0,09). Non mutated subjects have lower Stfr level than the mutated subjects (p=0.03). The mutated subjects don't have any difference with the non mutated participants for the glycated hemoglobin.
Discussion: This preliminary work has been done among an Oceanian population in differentiating Melanesian from Polynesian origin. The C282Y, the HFE allele most commonly associated with the hereditary hemochromatosis among persons of European descent, has not been identified in the Oceanian population. But we highlight the H63D at the heterozygotic state without any deleterious consequence on the iron metabolism or the glycemic regulation.
Conclusion: This work is the first to inform about the situation in the native population of Pacific. This study suggests the presence of the H63D mutation in subjects with a lower frequency than in the already studied European populations.

Poster #283

ATYPICAL JUVENILE HEMOCHROMATOSIS ASSOCIATED WITH HETEROZYGOSITY FOR NOVEL HEMOJUVELIN MUTATIONS

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(Presented By: Serena Pelusi)

Introduction: Juvenile hemochromatosis (JH) is a rare autosomal recessive disorder characterized by severe iron overload presenting in the 2-3 decade, due to hepcidin deficiency related to mutations in the hemojuvelin (HJV) and in the hepcidin genes.
Case 1: a 12 year-old male from Central Italy was discovered high levels of aminotransferases and hepatosplenomegaly. Blood tests showed a beta-thalassemic trait, ferritin 9035 ng/ml and transferrin saturation 84% with negative HFE mutations and no other liver disease. Histopathological liver evaluation demonstrated massive parenchymal siderosis and bridging fibrosis. Cardiac ecocolordoppler was normal.
Case 2: A 12 year-old female from Northern Italy was incidentally discovered with increased iron parameters (ferritin 467 ng/ml, transferrin saturation 87 -95% at two different evaluations, normal blood count and aminotransferases). Evaluation of hepatic iron by T2* MRI revealed iron overload.
Aims & Methods: genetic tests with direct sequencing of hemochromatosis causing genes (HFE, TfR2, HJV, Hepcidin and Ferroportin-1) was performed in the children and their parents.
Results: in case 1, we detected heterozygosity for a newly described HJV mutation (g.3659_3660insG), which was inherited together with the thalassemia trait from the father, who (like the mother) had normal iron parameters. In case 2, heterozygosity for another novel HJV mutation (g.2297delC) was detected. The mutation was inherited from the mother, which had mild iron deficiency. The father had normal iron stores. Both mutations caused a frameshift in the coding sequence determining premature stop codons.
Conclusions: This report illustrates examples of atypical JH. The beta-thalassemic trait was likely a cofactor influencing iron overload in case 1, but iron overload could hardly be explained by simple heterozygosity for HJV mutations in both cases, and other genetic factors should be investigated.

Poster #284

HEREDITARY HEMOCHROMATOSIS UNRELATED TO KNOWN MUTATIONS IN IRON GENES

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(Presented By: Raffaela Rametta)

Introduction: We report the case of a 49-year old man who was referred at our center for suspected hereditary hemochromatosis (HH) because of increased ferritin levels (4071 ng/ml) and transferrin saturation (TS >80%) with increased liver enzymes (ALT 121 and AST 181 UI/mL) in 2001. His parents were not consanguineous and he had no family history of liver disease. He first came to medical attention at 34 years because of insulin-deficient diabetes, elevated liver function tests and serum iron. Embolic stroke associated with dilated cardiomyopathy was diagnosed at 37 years, associated with chronic liver disease.
Methods and Results: Ferritin levels were first tested and resulted increased at 44 years, when hypothyoridism was also detected. At presentation the patient was also found to be affected by hypogonadotropic hypogonadism, osteoporosis and specific arthropathy associated with HH. He was negative for other liver diseases or alcohol abuse. Liver histology demonstrated the presence of cirrhosis, with massive hepatic siderosis with pan-nodular and pan-zonal hepatocellular iron deposition. Liver iron concentration (LIC) was 1413 mg/100mg of dry tissue and hepatic iron index 5.2. He was negative for mutations in the HFE coding sequence. He was started on phlebotomy and removed >17g of iron before reaching depletion, with normalization of liver enzymes and cardiac contractility, and improvement in diabetes control and bone mineral density. He was subsequently tested for the presence of mutations in the TFR2, FPN1, HJV, and HAMP coding sequences, and the HAMP promoter sequence, which all resulted negative.
In 2012, a 62.5 mg oral iron tolerance test was performed under iron depletion (basal TS<30%, ferritin <100 ng/ml), showing increased TS afer oral iron administration (>70%) similarly to HFE C282Y+/+ HH patients, which was persistent at 24 hours, whereas healthy controls with similar iron stores had only a modest increase in TS (<50%) at 4-8 hours.
In order to identify other genetic causes of iron overload, we searched for missense/nonsense mutations in candidate genes, such as BMP6, ALK2, ALK3, BMP2, NEO1, PCSK7, but no potential disease causing mutation was found.
Conclusion: In conclusion, this is a typical patient presenting with phenotypic HH but without mutations in genes known to be responsible for HH, and in some other candidate genes. Oral iron tolerance test may be useful to diagnose HH in the absence of known mutations in iron genes. New genetic variants responsible for HH should be searched for by innovative approaches such as whole exome / whole genome sequencing in well characterized patients.

Poster Abstracts

Poster #285

HIGHFERRITIN: A MEDICAL WEB TOOL FOR DIAGNOSIS AND RECOMMENDATIONS IN PATIENTS WITH HYPERFERRITINEMIA

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(Presented By: Mayka Sanchez)

Introduction: A high level of serum ferritin is a common finding in medical biochemical analysis and might be associated with several disorders, such as Hereditary Hemochromatosis, inflammatory diseases, metabolic syndrome, neoplasia and hepatic diseases. Diagnosis and management of this clinical finding depend on the underlying cause and requires medical expertise to proper address it.

Methods: To help in the diagnostic of hyperferritinemia we have created a new medical web tool named HIGHFERRITIN. HIGHFERRITIN web is implemented in HTML 5, CSS 3 for the static content. The client-side program uses JavaScript 1.4 and jQuery 1.7.2 libraries. The dynamic content of the web page is implemented in PHP 5.4. The database is implemented in SQL, using a SQLite 3 database server.This tool guides medical doctors with detailed steps towards a proper characterization of the causes of hyperferritinemia, provides treatment recommendations and general information. The web includes a restricted area for collection of a database in hyperferritinemia to evaluate the tool and for future studies.

Conclusion: The algorithms and recommendations provided within this tool have been agreed among a group of medical experts and are open to improvements from external medical evaluations.

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Poster #286

ASSESSING THE MITOCHONDRIAL IRON METABOLISM IN HEREDITARY HEMOCHROMATOSIS

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(Presented By: Sonia Distante)

Introduction: The mitochondrion has a crucial role in energy transduction and apoptosis signaling, but its role in iron metabolism has been underappreciated; it is now believed that mitochondrial iron metabolism modulates cytosolic iron metabolism. Iron is the most abundant metal in mammalian cells and can easily reduce/ oxidize between its two oxidation states, which is critical for generation of reactive oxygen species (ROS). Dysregulation of mitochondrial iron metabolism can lead to severe clinical conditions such as Friedreich's ataxia (frataxin deficiency) and X-linked sideroblastic anemia (defect hematopoiesis). The effects of systemic iron overload on mitochondrial iron metabolism in hemochromatosis patients have not previously been studied.
Aim: to study systemic iron overload effects on mitochondrial iron metabolism in hemochromatosis patients before and after treatment by venesectio compared to controls and to evaluate if lymphocytes are appropriate cell type for these studies.
Methods: Patients (n=12) from the hemochromatosis outpatient clinic were sequentially recruited to this study. They were either homozygous for the C282Y mutation or compound heterozygous for C282Y/H63D mutations and had raised iron parameters. Blood samples were collected before and after treatment. Age and sex matched controls (n=12) were also recruited. Mitochondrial and total cellular lymphocyte extracts from patients' blood before and after treatment and matched controls were analyzed. Subcellular levels of the following mitochondrial iron binding proteins are/will be determined by western analyses: Frataxin, Ferritin light and heavy chain, mitochondrial Ferritin (mt fer), Mitoferrin, Iron sulfur cluster assembly proteins IscU1/2 and Iba57, and the bottleneck enzyme in the mitochondrial heme synthesis; Aminolevulinate Synthase (ALAS1).
Results: We find considerable differences in the levels of distinct mitochondrial iron binding proteins (mt fer and ALAS1) in hemochromatosis patients compared to controls. We are currently evaluating the effects of venesectio therapy. Further examination is under way and may unravel if such alterations reflect mitochondrial dysfunction in hemochromatosis.
Conclusions: Lymphocytes from hemochromatosis patients seems to provide a suitable model for studying the effect of systemic to mitochondrial iron metabolism before and after venesectio.

Poster #287

BLOODLETTING THERAPY IN HEMOCHROMATOSIS: DOES IT AFFECT TRACE ELEMENTS HOMEOSTASIS?

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(Presented By: Sonia Distante)

Introduction: Trace elements are prevalent in all living organisms. Essential elements are vital for biological functions, while others are toxic even in small concentrations. Hemochromatosis is the most common hereditary disorder in the Nordic population, if left untreated can result in severe and sometime lethal parenchymal iron accumulation. Bloodletting is mainstay treatment; 450-500 mL blood is withdrawn weekly in the acute phase, patients can undergo up to 20-40 bloodlettings to achieve normalisation of iron parameters. Maintenance treatment to prevent further iron accumulation is lifelong and consists of bloodlettings; two to four times a year. Iron and trace elements partially share their cellular uptake and transport mechanisms; the effect of iron removal on trace elements homeostasis is unclear.
Aim: To study trace elements homeostasis in hemochromatosis patients, before and after iron removal via bloodletting.
Methods: We recruited patients (n=20) referred to our clinics for diagnosis and treatment of hemochromatosis. Two women and 18 men, from 23 to 63 years of age (mean 44 years) were included. Nine were C282Y homozygote, one was C282Y heterozygote, three were H63D homozygote, five were compound heterozygote and two had none of the mutations above. Iron and liver function tests were performed, serum level of the elements B, Ba, Co, Cs, Cu, Mn, Mo, Ni, Se, Sr, Y and Zn were measured using inductively coupled plasma mass spectrometry. Results before the start of treatment and after normalization of iron parameters were compared.
Results: Serum ferritin before the start of treatment ranged from 249 – 2555 µg/L (mean 764 µg/L) and fell to 18-140 µg/L (mean 63 µg/L) after treatment. On completion of the bloodlettings the serum concentration of cobalt had increased in all patients (from average 5,5 to 12,4 nmol/L), serum copper increased in 19 out of 20 patients (from average 15,8 to 17,5 µmol/L) and serum nickel increased in 16 of the patients (from average 51 to 54 nmol/L). Serum manganese declined in 16 of the patients (from average 32 to 29 nmol/L). All changes were statistically significant (by paired t-test). Serum levels of zinc, selenium, yttrium, boron, strontium, molybdenum, cesium and barium were not significantly changed.
Conclusions: Bloodletting for hemochromatosis does affect the homeostasis of some trace elements. The pathophysiological implication of this finding is yet to be elucidated.

Poster #288

THE FRENCH REFERENCE CENTRE FOR RARE IRON OVERLOAD DISEASES OF GENETIC ORIGIN: A MULTI-COLLABORATIVE APPROACH

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(Presented By: Lenaick Detivaud)

Introduction: The acquired expertise of Rennes in the field of iron metabolism and iron-related disorders led in 2007 to its official recognition as the French Reference Centre for Rare Iron Overload Diseases of Genetic Origin. In 2008, regional qualified Centres, officially named “Centres of competence”, were created in order to collaborate with the Reference Centre with the special mission to take in charge the patients in their home vicinity.

Objectives: As defined by the first and second national plans for rare diseases, our purpose is to help for diagnosis and treatment, to inform patients and health professionals, to improve knowledge on these rare diseases, with specific actions on data collection, to coordinate research in this domain and to improve the relationship with patients’ associations.

Methods: Multidisciplinary meetings take place once a week to discuss unexplained or non classical iron overloaded patient cases, originating from Rennes hospital, from Competence Centres, from individual French MDs or from foreign physicians. Futhermore, four videoconferences are organized each year with the Competence Centres. Clinical, biochemical, genetic, imaging, and pathological aspects are analyzed in the light of recent research knowledge. Depending on the results, complementary genetic studies are performed and, according to the detected mutations, functional studies can be proposed. Each case is recorded in a dedicated databank named logifer.

Results: To inform patients and physicians, the Reference Center created a web site: www.centre-reference-fer-rennes.org To improve diagnosis and treatment, the Reference Centre and the Competence Centres have defined specific guidelines. Furthermore, genetic and functional studies are available to determine the impact of the detected mutations in transcription, in splicing, in cell localization and protein function for different genes involved in iron metabolism such as HAMP, SLC11A2, SLC40A1 and TFR2. The Reference Centre coordinates different research studies, especially regarding unexplained forms of hepcidin deficiency.

Conclusions: The active collaboration between the Reference Centre and Competence Centres allows to increase the knowledge in the field and improve patient care management. We wish now to reinforce international collaborations in close link with patient associations (FFAMH, EFAPH, IAHA).

Keywords: iron overload diseases, hemochromatosis, guidelines

Poster #289

TFR2-RELATED HEREDITARY HEMOCHROMATOSIS AS A FREQUENT CAUSE OF PRIMARY IRON OVERLOAD IN CENTRAL-SOUTHERN ITALY

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(Presented By: Francesca Clementina Radio)

Introduction: Hereditary hemochromatosis (HH) is a common genetic disorder that shows both clinical and molecular heterogeneity. It is mainly caused by the HFE p.C282Y mutation in the homozygous condition. Less often, the disease is associated to the HFE p.H63D polymorphism (present both in the homozygous condition or in the compound heterozygous state with p.C282Y).

The global geographic distribution of the p.C282Y mutation, due to a founder effect, is not homogeneous and overlaps with the route of Celtic invasion (North-South). Accordingly, its prevalence varies with latitudes, as it is high in Northern-Europe and less frequent between Southern Europeans, Native Americans, Africans, and Asians. Therefore, an important percentage of patients with HH from Central-Southern Italy result to be HFE negative. Other 4 forms of HH are described so far worldwide. These rarer types are caused by different genes (HAMP, HJV, TFR2 and SLC40A1) and show some clinical peculiarities. Among non HFE related HH cases, about 30 TFR2 pathogenic mutations, mainly private, have been reported to date, in less than 50 families.
Methods and Materials: This study was performed on 47 individuals (45 Italian and 2 from other Mediterranean countries) affected by HH but without HFE mutations, evaluated in a dedicated outpatient clinic.
Results: TFR2 sequence analysis (Genbank # NC_000007.13) discovered biallelic pathogenic mutations in 7/47 (14,9%) of the investigated patients permitting to diagnose TFR2-related HH. Moreover, several subjects presented monoallelic deleterious defects in TFR2 (8/47 - 17%) or polymorphic variants with unclear meaning (16/47 – 34%). Besides, our study revealed 10 novel TFR2 variants with different pathogenic significance and 9 already described changes.
Conclusion: In conclusion, present findings support the hypothesis of a possible central role of TFR2 gene in HH pathogenesis especially in those countries, as Italy, where a high percentage of HH patients are HFE negative. Further studies, including worldwide systemic investigations, of the whole TFR2 are needed to verify this assumption.

Poster #290
A NOVEL HFE FRAMESHIFT MUTATION IN A PATIENT WITH HEREDITARY HEMOCHROMATOSIS TYPE 1
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(Presented By: Silvia Majore)

Introduction: Hereditary hemochromatosis (HHC) type 1 is the most frequent cause of primary iron overload in Northern Europe. The majority of patients with HHC type 1 are homozygous for the HFE p.C282Y mutation. More rarely, p.C282Y/p.H63D and p.H63D/p.H63D genotypes may result in mild HHC phenotypes. In addition, rare HFE variants, mostly private and inherited in trans with the p.C282Y mutation, have been shown to be sometimes related to the disorder.
Methods and Materials: We report a 42-year-old man originating from Central Italy with minor features of HHC in whom we clinically diagnosed a mild form of HHC. The patient was an habitual blood donor and was ascertained by the occasional observation of slightly increased ferritin plasma levels (460 ng/ml). Further laboratory evaluations revealed raised iron serum levels (220 µg/dl) and transferrin saturation (100%). At ultrasound scan liver was enlarged and showed diffusely inhomogeneous structure.
Results: Targeted HFE mutation analysis (p.C282Y and p.H63D) revealed the heterozygous p.C282Y mutation. Clinical evidence prompted further molecular studies by HFE direct sequencing, which revealed, on the second allele, the not previously described c.757_760delinsTTGCC frameshift mutation. This novel mutation was predicted to generate a truncated protein possibly subjected to nonsense mediated decay.
Conclusion: Presented molecular findings further support the concept that in patients in whom biochemical parameters are strongly indicative of primary/idiopathic iron overload the identification of a heterozygous p.C282Y mutation in a single allele is predictive for the possible presence of a second HFE mutation. In these patients targeted HFE testing should be followed by HFE direct sequencing.

Poster #291
ORTHOTOPIC LIVER TRANSPLANTATION FOR HEREDITARY HAEMOCHROMATOSIS IN IRELAND
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(Presented By: John Denis Ryan)

Introduction: Ireland has the highest reported prevalence of Hereditary Haemochromatosis (HH) worldwide. In HH, hepatic iron accumulation can lead to fibrosis, cirrhosis and hepatocellular carcinoma (HCC) formation. Approximately 5% of patients with HH progress to cirrhosis, which is associated with a significantly increased risk of HCC development.
Methods and Materials: In this study, we sought to characterise the role of liver transplantation for HH in Ireland. Data was obtained from the transplant database at the National Liver Transplant Unit, St Vincent’s Hospital, Dublin.
Results: A total of 634 patients underwent liver transplantation between January 1995 and April 2012. 27 of these had a diagnosis of HH. 93% of HH patients were male. HCC was the primary indication in 15 patients (56%), of whom 4 out of 15 (27% were non-cirrhotic). Significant risk factors for liver disease were present in 11 out of 15 (41%; alcoholic liver disease or hepatitis C infection in 9 and 2 patients, respectively). Only 5 patients (19%) had undergone successful therapeutic venesection prior to transplantation. The one year and 5 year survival for individuals with HH was 79% and 62%, respectively, compared to 88% and 81% for the entire transplant cohort.
Conclusion: Despite the prevalence of HH in Ireland, it remains an uncommon indication for liver transplantation, particularly in females. The vast majority of HH patients have concomitant HCC or other risk factors for liver disease, and post-transplant survival appears reduced compared to other indications. Early detection and treatment to prevent complications such as HCC remains the cornerstone of HH management.

Poster #292

HEPATOCYTE DIVALENT METAL-ION TRANSPORTER-1 IS DISPENSABLE FOR HEPATIC IRON ACCUMULATION AND NON-TRANSFERRIN-BOUND IRON UPTAKE IN MICE

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(Presented By: Chia-Yu Wang)

Introduction: Divalent metal-ion transporter-1 (DMT1) is a transmembrane protein that is known to be essential for iron uptake by enterocytes and erythroid precursors. DMT1 is also present in the liver, where it is believed to play a role in hepatic iron uptake, either through transferrin-bound iron (TBI) or non-transferrin-bound iron (NTBI), which appears in the plasma during iron overload.

Methods and Materials: To test the hypothesis that DMT1 plays a role in hepatic iron uptake, we examined mice with the *Dmt1* gene selectively inactivated in hepatocytes ($Dmt1^{liv/liv}$) and found that levels of hepatic iron (total and nonheme) or plasma iron status parameters did not differ from controls ($Dmt1^{flox/flox}$). To examine the role of DMT1 in iron overload conditions, we crossed $Dmt1^{liv/liv}$ mice with models of genetic iron overload, i.e., $Hfe^{-/-}$ and hypotransferrinemic ($Trf^{hpx/hpx}$) mice.

Results: We found that the double-mutant $Dmt1^{liv/liv}$;$Hfe^{-/-}$ and $Dmt1^{liv/liv}$;$Trf^{hpx/hpx}$ mice accumulated similar amounts of hepatic iron as did their respective $Hfe^{-/-}$ and $Trf^{hpx/hpx}$ controls. Perls' Prussian blue staining of the double-mutant livers demonstrated that DMT1 is not required for iron accumulation in hepatocytes. To directly access the role of DMT1 in NTBI and TBI uptake, we injected ^{59}Fe-labeled ferric citrate (for NTBI) or ^{59}Fe-transferrin intravenously into $Dmt1^{liv/liv}$ and $Dmt1^{flox/flox}$ mice and measured hepatic ^{59}Fe uptake. We found that hepatocyte-specific inactivation of *Dmt1* had no effect on liver NTBI uptake, but did result in a 40% reduction in TBI uptake. Hepatic transferrin receptors 1 and 2 and ZIP14 (ZRT/IRT-like protein 14), which may also participate in iron uptake by the liver, were unaffected in $Dmt1^{liv/liv}$ mice.

Conclusion: We conclude that DMT1 is dispensable for hepatic iron accumulation and NTBI uptake. Although DMT1 is partially required for hepatic TBI uptake, hepatic iron levels were unaffected, suggesting that under normal conditions, this pathway is a minor contributor to the iron economy of the liver.

Poster #293

DECIPHERING THE HFE-INTERACTION NETWORK FOR THE REGULATION OF IRON HOMEOSTASIS

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(Presented By: Flavia D'Alessio)

Introduction: The Hereditary Hemochromatosis-associated membrane proteins HFE, TfR2 and HJV are required for adequate hepatic expression of the iron hormone hepcidin to maintain tissue iron homeostasis. HJV acts as a bone morphogenetic protein (BMP) co-receptor driving hepcidin transcription via the BMP-SMAD pathway. However, the molecular circuitry emanating from HFE and TFR2 has remained more enigmatic.

Methods and Results: Recently, we showed that HFE, TfR2 and HJV physically interact with each other on the surface of hepatocytes (D'Alessio et al, 2012). These findings help explain previous observations that the BMP/SMAD signaling cascade is impaired in HFE and TfR2 knockout mice and HFE-HH patients (Corradini et al., 2009; Kautz et al., 2009; Wallace et al., 2009, Ryan et al., 2010) and that HFEinduced hepcidin transcription requires functional HJV (Schmidt et al., 2010). Our data also indicate that high molecular weight protein complexes may form with one or more of the HH-associated proteins. Such proteins may act as scaffolds or as regulators of iron surveillance triggering signal transduction for hepcidin production.

To define the composition of the iron-sensing complex and its connections to signalling networks, and to discover novel interacting proteins that may not have previously been linked to the regulation of iron homeostasis, we combined the biochemical purification of protein complexes under native conditions with protein mass-spectrometry. HUH7 cells were transiently transfected with HFE-YFP, Flag-HJV and TfR2-HA expression vectors, and the protein complexes were purified by immunoprecipitating HFE-YFP from the cell lysate using the single-chain antibody GFP-Trap. Proteins that co-purified with HFE-YFP were subsequently analyzed by liquid chromatography in combination with tandem mass spectrometry (LC-MS/MS). Selected proteins are undergoing validation by 1) conventional immunoprecipitation assays and 2) RNAi to analyze their function in the regulation of hepcidin transcription.

Conclusion: With this study we expect to better understand the mechanisms by which circulating iron levels are being sensed to prevent iron accumulation by activation of hepcidin transcription and the role of the HH-associated proteins in this process.

Poster #294

DIFFERENTIAL CARBOHYDRATE PROCESSING OF THE WILD TYPE AND C282Y-MUTANT HFE PROTEINS

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(Presented By: J.V. Fleming)

Introduction: Type I hereditary Haemachromatosis is associated with a mutation to the HFE gene that leads to misfolding of the translated protein and an inability for it to bind to its ß2 microglobulin chaperone. The mutant protein (HFE-C282Y) is retained in the endoplasmic reticulum where it induces the Unfolded Protein Response (UPR) and undergoes proteasomal degradation.

Methods and Results: Our recent studies now show that HEK293T cells transfected to express HFE-WT and HFE-C282Y proteins exhibit slightly different fractionation patterns when lysates are subjected to SDS-PAGE. Specifically, for cells expressing the wild type protein it is possible to observe a higher molecular weight protein that is not normally detected in cells expressing the mutant. Treatment of cells with castanospermine to inhibit glucosidase enzymes leads to detection of the higher molecular weight band also in the HFE-C282Y expressing cells, suggesting that the mutant protein is normally undergoing carbohydrate trimming.
The mutant protein also exhibits lower molecular weight isoforms, which we believe to represent fully or partially deglycosylated forms of the protein. There is an increase in the expression of these lower molecular weight isoforms when cells are treated with pharmacological inducers of the UPR. This suggests that UPR signalling actively promotes the deglycosylation of HFE-C282Y, possibly in advance of proteasomal degradation. To test this hypothesis cells expressing the mutant protein were incubated in the presence and absence of the proteasome inhibitor lactacystin. While this led to modest increases in the expression of the glycosylated HFE-C282Y protein, the largest increase in steady state expression was observed for the deglycosylated form of the protein.
Conclusion: We conclude that wild type and C282Y mutant isoforms of the HFE protein undergo differential carbohydrate processing to facilitate proteasomal degradation and clearance of the misfolded variant.

Poster #295
MUTATION ANALYSIS OF SEVEN GENES INVOLVED IN IRON METABOLISM IN A MENNONITE POPULATION
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(Presented By: Monique Zaahl)

Background: We have previously reported 15 variants, one of which is novel, in "non(C282Y)-HFE" hemochromatosis affecting 16 individuals (within the Mennonite community in the lower Fraser Valley of British Columbia, Canada) from 3 unrelated families. These variants include: *HFE*: 5'UTR-1206C/G, 5'UTR-467G/C; *SLC40A1*: 5'UTR-750G/A, 5'UTR-98G/C, 5'UTR-8C/G, V221; *HAMP*: 5'UTR-511A/G, *CP*: 5'UTR-365delT, 5'UTR-354T/C, IVS2+20C/T, H977, T551I; *CYBRD1*: 5'UTR-624G/A and *HMOX1*: IVS4+51delTGGCTGTCTGACT, IVS5+71C/G.
Methods: We looked for these mutations in 49 control subjects (33 women and 16 men) from the Mennonite community and correlated the findings with serum iron, total iron binding capacity and transferrin saturation percentage (TS%). The genetic analysis was performed by PCR amplification of promoter and coding regions of the genes followed by mutation analysis using heteroduplex single-stranded conformation polymorphism (HEX-SSCP) analysis and subsequent bi-directional DNA sequencing analysis. Restriction fragment length polymorphism (RFLP) analysis was performed where appropriate.
Results: None of the control subjects were found to have transferrin saturation levels above the reference range. All of the variants except the novel *HMOX1* IVS5+71C/G were identified in the control population. The C282Y and H63D HFE mutations were also present in the control cohort. Further analysis of the initial 3 families showed that common haplotypes in *HFE* and *SLC40A1* (ferroportin) appeared to inherit with the iron overload phenotype. Correlations, which were statistically significant, could also be observed between the presence of the variant(s) and general trend(s) of iron loading.
Conclusion: The genetic variants identified in the *HFE*, *SLC40A1*, *HAMP*, *CP* and *CYBRD1* appear to be polymorphisms since they have been found in the control population. However, analysis of the haplotypes identified warrants further investigation as well as the novel variant identified in the *HMOX1* gene.

Poster #296
LISTENING TO THE PATIENTS' VOICE: THE FIRST EUROPEAN SURVEY ABOUT GENETIC INFORMATION IN HEMOCHROMATOSIS
Emerência Teixeira, Pierre Brissot, Barbara Butzeck, Françoise Courtois, Robert Evans, Janet Fernaud, Margaret Mullett, Brigitte Pineau, Graça Porto, Mayka Sánchez, Júlio Borlido-Santos, Robert Sorrill, Dorine Swinkles, Ketil Toska, Judit Várkonyi and EFAPH (European Federation of Associations of Patients with Hemochromatosis) IBMC – Instituto de Biologia Molecular e Celular, Universidade do Porto
(Presented By: Emerência Teixeira)

Introduction: With the advent of genetic testing for Hereditary Hemochromatosis (HH) in the late nineties, screening of patients at pre-symptomatic stages became a common practice, contributing to reduced disease morbidity and mortality (*Aleman et al 2011. Scand J Gastroenterol*. 46:1118-26). Nevertheless, it has been recently recognized, by the European Commission, that the disease is still largely underdiagnosed (*E-012656/2011*). Early diagnosis depends on increased awareness and proper information not only among health professionals but also among the patients themselves, who are key players in the motivation and spread of information among family members. The patient-centered design of information strategies is an approach that has been shown to improve effectiveness on health-promotion campaigns. Nevertheless, no studies have previously explored the HH patients' perspectives on genetic information neither the type of sources they use to obtain that information.

Methods: In order to fulfill this gap, European Federation of Associations of Patients with Hemochromatosis (EFAPH) designed and applied a European online survey on genetic information about HH. National Hemochromatosis Associations from nine European countries, coordinated by EFAPH, were involved in the study.
Results: Validated responses to the survey were obtained from a total of 1019 Associations' members, 895 of them having performed a genetic test. 58% of respondents were males and 42% were females and the average age was 56 years. The survey content included questions regarding the types of information sources they used, preferred or trusted more. Notably, 70% of respondents declared to feel sufficiently informed about the genetic test and its implications. When these were asked about their information sources, the majority (66%) reported to have got the information from a specialist doctor but would like (66%) to obtain it from the family doctor, although the specialist was still the one they trusted more (69%). Regarding the remaining 30% of members who did not feel sufficiently informed, the majority (78%) would like to have information from the family doctor although they also trusted much more on the specialist (75%) than on the family doctor (35%). In conclusion, this exploratory study elucidates the patients´ needs for information and identifies the General Practitioner (GP) as the preferred source to obtain information about HH. These results may have important implications in future strategies for health promotion, with a special emphasis on GP awareness to enhance early case detection, as previously discussed by others (*Young et al, 2004. Australian Family Physician*, 33: 1040-1043). The results also highlight the importance of including the subject of genetic information from the patients' perspective in future clinical guidelines in order to promote patient self-management of the disease and related needs.
Conclusion: Further qualitative evaluations studies are being undertaken in order to deeper understand the processes on information handling/appropriation by the patients, as well as the sources networks and material specification that should be produced in a patient-centered and GP-centered method.

Poster #297
LIVER TRANSPLANTATION NORMALIZES SERUM HEPCIDIN LEVEL AND CURES IRON METABOLISM ALTERATION IN HFE P.CYS282TYR HEMOCHROMATOSIS
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(Presented By: Edouard Bardou-Jacquet)

Background and Aim: Iron overload in *HFE* related hemochromatosis (HH) is induced by a deficient hepatic secretion of hepcidin. Liver transplantation (LT) is a key treatment of potential complications of HH such as Hepatocellular Carcinoma (HCC). Thus, the outcome of hepcidin secretion and iron burden after LT is a unique model to study *HFE* related hemochromatosis physiopathology. Our aim was to describe the long term outcome of hepcidin secretion and iron metabolism parameters in a cohort of transplanted patients.
Methods: All p.Cys282Tyr *HFE* homozygotes patients who had LT for complications of hemochromatosis between 1999 and 2008 were retrospectively included in the study. Files were reviewed for mortality and causes of death. Determination of biological serum iron parameters, serum hepcidin level and hepatic iron concentration (MRI) were done at the end of follow-up.
Results: 18 patients were included (mean age 56.2±7 years). The median follow up time was 57 (IQR: 12-93) months. Indication for LT were HCC (16 patients), liver failure (1 patient), and biliary hamartomas (1 patient). 16 patients were Child-Pugh A5. Survival at one year was 83.3%, and 66.6% at 5 years. Causes of death were sepsis in the 3 months post-LT (3 patients), HCC recurrence (1 patient, 18 months after LT), lung cancer (1, 2 years post LT) and stroke (1). Before LT: 7 patients had serum ferritin < 50 µg/L, serum hepcidin levels were evaluated in 11 patients and found very low in 9 patients and at the lower limit of normal in 2 patients: median: 0.54±2.5 (normal range : 4-30 nmol/L) , ii) After LT: 11 patients had iron parameters evaluation at end of follow-up, none of them had iron depletion therapy since LT. Mean serum ferritin was 185 (±99)µg/L, and all patients had normal transferrin saturation. MRI showed no iron overload in 9 patients, one patient had mild iron overload (70 µmol/g), likely related to by metabolic syndrome and excessive alcohol consumption, one patient had high iron overload (180 µmol/g), but also had hereditary spherocytosis. At the end of follow-up serum hepcidin was normal in 10 patients and low in one patient who had iron deficiency anemia: median 11.12±7.6 nmol/L ($p<0.05$ versus before LT value).
Conclusion: This study demonstrates, in patients with *HFE* related hemochromatosis, that liver transplantation normalizes hepcidin secretion in the long term, thus preventing recurrence of hepatic iron overload in those patients. Therefore LT cures phenotypic expression of *HFE* hemochromatosis.

Poster #298
A NOVEL TMPRSS6 MUTATION IN 2 BULGARIAN SIBLINGS
Patricia Bignell, BSc Hons[1], Mimi Sheikh, MD[1], Anna Schuh, PhD[1] and Kathryn Robson, PhD[2]
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(Presented By: Patricia Bignell)

Introduction: Iron refactory iron deficiency anaemia (IRIDA) is a rare autosomal recessive disorder, characterised by; congenital, hypochromic, monocytic anaemia, very low mean corpuscular volume (MCV), low serum iron and low transferrin saturation, normal ferritin or ferritin levels in the lower limit of normal, no response to oral iron treatment and inappropriately

high levels of hepcidin, which has been linked to mutations in the TMPRSS6 gene. TMPRSS6 encodes for matriptase-2, a type 2 transmembrane serine protease identified as a modifier of iron homeostasis because it regulates the expression of the systemic iron regulatory hormone hepcidin, inhibiting hepcidin activation by cleaving membrane hemojuvelin.

Methods and Results: We identified a Bulgarian family with asymptomatic non-consanguineous parents with 2 children presenting with IRIDA (Table 1), not responding to oral but to intravenous iron supplementation. After excluding all known causes responsible for iron deficiency anaemia we screened for mutations in the TMPRSS6 gene.

	Reference range (children)	Son 10yrs	Daughter 5yrs
Hb g/l	115-155	80	78
MCV fl	78-102	56.3	57.5
Serum ferritin µg/l	15-200	16.9	11.8

Conclusion: A novel mutation was found in the TMPRSS6 gene as the cause of the children's IRIDA. A homozygous nonsense mutation in exon 17, c.2161 C>T, p.Q721*. The mutation is in the carboxy-terminal serine protease domain. Several mutations in the TMPRSS6 gene have been characterised in IRIDA families of different ethnic origins and this shows the allelic heterogeneity of this disorder.

Poster #299

JUVENILE HAEMOCHROMATOSIS MUTATIONS ARE NOT THAT SIMPLE

Patricia Bignell, BSc Hons[1], Anna Schuh, PhD[1] and Kathryn Robson, PhD[2]
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(Presented By: Patricia Bignell)

Introduction: Juvenile Haemochromatosis (JH) is a rare autosomal recessive disorder of iron overload that leads to organ damage before the age of 30. JH is characterised by severe iron overload usually associated with liver damage, cardiomyopathy and/or hypogonadotrophic hypogonadism. The course of the disease is more rapid and severe than classic HFE haemochromatosis. Men and women are equally affected. JH is subdivided in types 2A and 2B, which are caused by mutations in the HFE2 and HAMP genes.

Over the last few years we have analysed patients with suspected JH and severe iron overload.

Methods and Results: We now have a cohort of 22 patients with HFE2 mutations and 10 patients with HAMP mutations. 4 novel HFE2 and 2 novel HAMP mutations were found. The novel HFE2 mutations are p.L294H and a 5.2Mb deletion, removing the HFE2 gene in a Caucasian woman, p.N372I in a South African man and p.A143Pfs*112 in an Indian woman. Both novel HAMP mutations were found in males, p.R59Q in a Caucasian and a Pakistani who is homozygous for p.C69Sfs*112. In our Asian families homozygosity for the p.G99R mutation appears to be a common cause of JH whereas in Caucasians homozygosity for the p.G320V mutation appears to be the most common as reported by others. We found a wide range of mutations in our patients, finding 14 different HFE2 and 6 different HAMP mutations. This demonstrates a wide allelic heterogeneity in the UK population that includes a significant Asian community.

One observation we have made is the variation in phenotype in p.G320V homozygotes, we have seen a wide range severity, a 22 year old with JH died suddenly with cardiomyopathy to a 74 year old with later adult onset haemochromatosis. Another 2 patients with the p.G320V homozygous genotype also have late adult onset haemochromatosis. They also seem not to have any of the characteristic JH-associated organ damage, cardiomyopathy and/or hypogonadotrophic hypogonadism. An intermediate iron overload phenotype has been observed in some of our patients who have digenic inheritance, heterozygous for HFE2 and HFE mutations or heterozygous for HAMP and HFE mutations. The ages of these patients are generally older and mainly of Caucasian origin. However in the HAMP/HFE group we have 1 Chinese patient with p.R56X (HAMP) and p.[H63D];[C282Y] (HFE) and a Caucasian/Chinese patient with p.G71D (HAMP) and p.C282Y (HFE). His symptoms seem much worse than the majority of our patients with p.G71D and who have the p.C282Y mutation. This could be due to modifying SNPs on the Chinese haplotype. Another patient, a female is homozygous p.[C282Y];[C282Y] and the age of onset of symptoms is much lower 40 yrs when compared to the heterozygous group. It appears that p.G71D is contributing to her haemochromatosis.

Conclusion: From our intensive genetic analysis of the HFE2 and HAMP genes, there is considerable heterogeneity. Two groups for the majority of JH cases, Pakistanis who have p.G99R mutation and Caucasians with the p.G320V mutation. Of importance is the finding that patients with a later age of onset have HFE2 mutations normally associated with a much earlier age of onset. There is in addition an intermediate group with digenic inheritance who have an HFE mutation with an HFE2/HAMP mutation.

Poster #300

COMPREHENSIVE GENETIC TESTING IS REQUIRED FOR PATIENTS WITH UNEXPLAINED IRON OVERLOAD

Patricia Bignell, BSc Hons[1], Alison Merryweather-Clarke, PhD[2], Chun Yu Lok, DPhil[2], Anna Schuh, PhD[1] and Kathryn Robson, PhD[2]
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(Presented By: Patricia Bignell)

Background: About 80% of European patients with idiopathic iron overload who have increased serum ferritin concentrations are homozygous for the mutant p.(C282Y) allele of HFE. Mutations in four further genes have been reported, but these genotypes are considered rare and are not investigated routinely.
Methods: We performed detailed genetic analysis of 458 patients with iron overload who were not simple p.(C282Y) homozygotes. We sequenced 5 known haemochromatosisassociated genes and analysed copy number variation by multiplex ligation-dependent probe amplification.
Findings: Mutations in one or more of the 5 genes analysed were present in 96 (21%) of the 458 patients investigated. There were 22 novel mutations in the five known haemochromatosis-associated genes, 35 examples of digenic inheritance and the first two reports in the literature of deletions of exons in HFE2 and TFR2.
Interpretation: Our results show the value of comprehensive genetic studies in iron-loaded patients who lack two copies of the p.(C282Y) HFE mutation. The findings emphasize the phenotypic and genetic heterogeneity of haemochromatosis patients in the UK. They indicate the need for patients with unexplained iron overload who are not p. (C282Y) homozygotes to undergo comprehensive sequence and copy number analysis of all five currently known haemochromatosis-associated genes. New guidelines are required for investigating patients in whom iron overload cannot be attributed to homozygosity for the p.(C282Y) allele of the HFE gene.

Poster #301

NOVEL GENE DELETIONS RESULT IN HAEMOCHROMATOSIS

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(Presented By: Patricia Bignell)

Introduction: We report here the first large deletions of HFE2 and TFR2 in patients with haemochromatosis. The vast majority of case of inherited iron overload are due to missense mutations or small deletions in one of five genes: HFE, HFE2, HAMP, TFR2 and SLC40A1. To date the only report of a large deletion resulting in inherited iron overload was in a female patient who was homozygous for the complete deletion of HFE. Gene deletions involving the loss of exons are not routinely screened for in patients with haemochromatosis. Here we describe two patients whose disease is attributable to gene deletions.
Methods and Results: Patient P, a woman (serum ferritin at diagnosis >12,000 µg/ml), had been treated for haemochromatosis for a number of years. Detailed genetic analysis showed that she was a compound heterozygote for two novel HFE2 mutations. At first she appeared to be homozygous novel missense mutation p.L294H which was located close to a second polymorphism. As neither had previously been described before we were concerned that this apparent homozygosity masked a deletion of the second allele. Multiplex ligation-dependent probe amplification revealed the complete deletion of the second allele thus explaining the apparent homozygosity. SNP analysis revealed that a further 73 genes from this region of chromosome 1 were also deleted. Both her daughters have inherited the deleted chromosome and currently show no phenotype. Patient S is a 38 year male who presented with an out-of-hospital cardiac arrest from which he was successfully resuscitated. Biochemical tests on hospital admission revealed iron overload (serum ferritin 7672 µg/ml, TSat 75% and serum iron 71µmol/L). His heart was shown to contain iron. He was found to be a compound heterozyote for the HFE mutations p.H63D and p.C282Y. Further genetic analysis revealed a single nucleotide deletion in exon one, p.(G6Vfs*51) together with deletion of exons 1-3 in TFR2. RNA analysis has demonstrated that he is unable to make any mRNA that corresponds to the α transcript of TFR2. The β transcript can be synthesized from the allele containing the single nucleotide deletion.
Conclusion: Both patients have been successfully treated.

Poster #302

ESTIMATING DISEASE PENETRANCE IN P.C282Y HOMOZYGOTES IN THE UK

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(Presented By: Patricia Bignell)

Introduction: Since the discovery of the HFE gene we have received over 4000 requests for HFE mutation studies. Of these 75% were for suspected genetic haemochromatosis and 25% were for family studies. The referral area corresponds to a population of ~2.4 million. The ethnic mix in the study area varies, with high concentrations of those of South Asian ancestry living in Slough and Wolverhampton. From our data we demonstrate that disease penetrance in people aged over 40 years old

is at least 12.5% in the UK. We know this to be an underestimate in that we have excluded all family members who were p.C282Y homozygotes.
Methods and Materials: The population of catchment area studied includes Wolverhampton, Swindon, Northampton, Kettering, Warwick, Milton Keynes, Aylesbury, Reading, Slough, Newbury, Oxford, Banbury and High Wycombe. The number of people in this population of 2.4 million who have the HH/YY genotype is predicted to be 7920 if there 1 in 300 are p.C282Y homozygotes.
Results: We have observed that only those over the age of 40 years are likely to show signs of iron overload. 50% of the UK population is over the age of 40yr, therefore there are 3960 P.C282Y homozygotes at risk of iron overload. We have had 3152 requests for HFE gene testing of who 495 were found to have the genotype HH/YY.
Conclusion: These figures suggest that ~12.5% (495/3960) of those over 40 years of age and are p.C282Y homozygotes have received a diagnosis of haemochromatosis.

Poster #303
SEVEN NEW CASES OF TYPE 3 HEMOCHROMATOSIS WITH EIGHT PREVIOUSLY UNDESCRIBED TFR2 MUTATIONS EMPHASIZE THE HIGH VARIABILITY OF CLINICAL PRESENTATION
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(Presented By: Edouard Bardou-Jacquet)

Introduction: Since the discovery of the HFE gene and of the p.Cys282Tyr mutation, new genes involved in iron metabolism have been described and related to peculiar forms of hereditary hemochromatosis. Mutations in the TFR2 gene lead to type 3 hemochromatosis whose clinical picture mimics type 1 hemochromatosis. Several reported cases affecting young patients have been described. It has been clearly demonstrated that in type 1 hemochromatosis cofactors play an important role by modulating the phenotypic expression of the disease since the clinical penetrance of p.Cys282Tyr homozygosity is very low. In type 3 hemochromatosis, clinical expression seems higher but the number of reported cases is too small to definitely assess penetrance. We report here seven cases of type 3 hemochromatosis with eight unpublished mutations in the TFR2 gene.
Methods: Over the 2002-2012 period, TFR2 mutations were looked for in 287 patients with iron overload as part of the diagnostic activity of the French Reference Centre for Rare Iron Overload Diseases of Genetic Origin in collaboration with Competence Centers. All patients were tested for the HFE mutations p.Cys282Tyr and p.His63Asp. The entire coding region and intronic flanking sequences of TFR2 (GenBank no. NM_003227.3) were sequenced. To exclude other mutation(s), analysis of the hemojuvelin (HJV), hepcidin (HAMP), and ferroportin (SLC40A1) coding sequences was performed.
Results and patients are reported in Table 1.
Discussion: Our data which concern a significant number of new type 3 hemochromatosis cases contribute to further document the clinical description of the disease and confirm that TFR2 related hemochromatosis should be assessed in children with unexplained iron overload. Indeed six out of seven patients described here were diagnosed before the age of 30, two of them being less than 18 years old. Our results are in favor of a more variable phenotypic expression than previously thought. It is therefore possible that the phenotypic expression of type 3 hemochromatosis is a spectrum with highly variable severity, reflected by the widespread age at diagnosis even in the absence of cofactor, possibly depending on the “severity” of the mutation impact. We are still lacking an efficient and quantitative in vitro model to assess TFR2 function on hepcidin synthesis in order to validate this hypothesis.
Conslusion: We confirmed here that looking for TFR2 mutations should be part of the screening process in case of adult as well as juvenile hemochromatosis.

	1	2	3	4	5	6	7
Sex	F / 10	M / 17	M / 39	M / 25	M / 27	F / 28	F / 29
Clinical signs	Asthenia		Hypogonadism	Asthenia Bronzed Skin	Asthenia Arthropathy	Arthropathy	Amenorrhea dark skin
Cofactors	None	Gilbert Disease α1 Antitrypsin deficiency	Dyslipidemia	None	Dyslipidemia	Ferroportin Disease (p.Gly204Ser)	None
Liver biopsy		Metavir F3	Cirrhosis		Metavir F3		Cirrhosis
Liver Iron Content (µmol/g)	200 µmol/g	>350 µmol/g		450 µmol/g			350 µmol/g
Transferrin sat %	98	Initial value NA	99	92	Initial value NA	Initial value NA	92.5
Serum ferritin µg/l	586	Initial value NA	4784	3116	Initial value NA	Initial value NA	3308
Iron removed (g)	NA		> 15g	NA	> 26g	19,5	Iron chelation
HFE	H63D +/-	H63D +/-	None	None	None	none	H63D +/-
TFR2	p.Arg678Pro +/+	p.Asn412Ile +/+	p.Gly430Arg +/+	p.Leu85_Ala96delinsPro +/- p.Gly735Ser +/-	p.Met705Hisfs*87+/- p.Gly792Arg+/-	p.Ala444Thr +/- p.Gly792Arg +/-	p.Arg730Cys +/- p.Trp781* +/-

Poster #304

CONSERVATION OF MAJOR HISTOCOMPATIBILITY COMPLEX (MHC) HAPLOTYPES IN HFE C282Y HOMOZYGOUS HEMOCHROMATOSIS PATIENTS AND ITS EFFECT ON THE PHENOTYPE OF LOW CD8+ T-LYMPHOCYTES: A STUDY IN 3 POPULATIONS FROM PORTO, ALABAMA AND TRONDHEIM

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(Presented By: Mónica Costa)

Introduction: Hereditary Hemochromatosis (HH) is a major histocompatibility complex (MHC)-associated autosomal recessive disorder that increases risk to develop iron overload. It is characteristically associated with homozygosity for the C282Y mutation in *HFE* gene localized to chromosome 6p21.3, 4 Mb telomeric to the human leukocyte antigen (HLA)-A. *HFE* encodes a non-classical MHC-class I molecule. C282Y is in strong linkage disequilibrium with particular HLA alleles and haplotypes. A phenotype of low $CD8^+$ T-lymphocyte numbers is common in HH patients and it is transmitted in association with other MHC markers, namely *HLA-A*. This supports the postulate that MHC-class I genes may influence $CD8^+$ T-lymphocyte numbers.

Methods and Materials: We analyzed 608 MHC haplotypes (using *HLA-A*, *-B* and three SNPs in genes *PGBD1, ZNF193* and *ZNF165*, localized between *HLA-A* and *HFE*) and $CD8^+$ T-lymphocyte numbers in three populations of C282Y homozygous HH patients from geographically distant regions: Porto (Portugal), Alabama (USA), and Trondheim (Norway). The respective frequencies of common MHC haplotypes, degrees of haplotype conservation and genotype/phenotype associations were variable, supporting the existence of different founder effects and different recombination histories among the three populations.

Results: Thus, we evaluated whether those differences could affect other traits encoded in the same chromosomal region, namely the determinants of $CD8^+$ T-lymphocyte numbers. The low $CD8^+$ T-lymphocyte phenotype was significantly associated with the most conserved ancestral haplotype carrying B*07-A*03-A-A-T in the cohorts from Porto and Alabama, supporting our postulate that a major genetic determinant of $CD8^+$ T-lymphocyte numbers is transmitted in linkage disequilibrium with this ancestral haplotype. Our observations favor the localization of a putative locus that influences numbers of $CD8^+$ T lymphocytes between *HLA-A* and the SNP in *PGBD1*: the association is lost not only in chromosomes without the A-A-T SNP microhaplotype but is also lost in A-A-T conserved haplotypes that do not carry the ancestral HLA-A*03 allele. This is also supported by the results of a high-density mapping using 62 SNP markers in Porto patients, in whom the association with the low $CD8^+$ T-lymphocyte phenotype is lost or maintained according to recombination events close to *HLA-A.* Data from the highdensity mapping allowed us to approach further HH haplotype genealogies using phylogenetic analyses implemented in MEGA. Haplotypes carrying HLA-A*03-B*07 or HLA-A*01-B*08 appear in distinct phylogenetic branches supporting different founder effects and an apparently more recent origin for the chromosomes carrying HLA-A*03-B*07.

Conclusion: In conclusion, our results contribute to positioning further a candidate locus associated with the transmission of low $CD8^+$ T-lymphocyte phenotypes. The strategy of selecting "non-conserved" chromosomes may help to identify individual loci contributing to the low $CD8^+$ T-lymphocyte phenotypes in *HFE* C282Y homozygotes.

Poster #305

OXIDATIVE STRESS DECREASES HFE PROTEIN LEVELS

Louise Foley and J.V. Fleming, BSc, PhD
University College Cork
(Presented By: Louise Foley)

Introduction: The HFE protein is one of the key regulators of cellular iron uptake. It functions at the cell surface to decrease the binding of holotransferrin to the transferrin receptor. Genetic mutations that leave the HFE protein functionally inactive are associated with cellular and tissue iron overload. Cellular iron overload is also a feature of many oxidative stress associated diseases. Nevertheless, the effects of oxidative stress specifically on HFE expression and function have not been well studied. It remains possible therefore that reactive oxygen species could alter HFE expression is such a way as to increase iron uptake.

Methods and Materials: In order to examine this we transfected HEK293T cells to express HFE (and its associated ß2 microglobulin chaperone). Transfected cells were treated for sixteen hours in the presence or absence of hydrogen peroxide (H2O2) before harvesting and fractionation of lysates by SDS-PAGE.

Results: Immunoblot analysis indicated that steady state expression of HFE decreased in a concentration dependent manner. This effect was not limited to H2O2 and could be detected also when reactive oxygen species were produced over a similar

time period by menadione. Shorter exposure times (5hr) to higher concentrations of the oxidizing agents showed a similar effect on HFE levels, but had no effect on the steady state expression of the transferrin receptor, confirming the specificity of our observations.
Conclusion: We conclude that decreases in HFE levels could contribute towards the iron overload observed in some diseases where reactive oxygen species are produced.

Poster #306
EXTRA-HEPATIC IRON MEASUREMENTS IN HEREDITARY HEMOCHROMATOSIS
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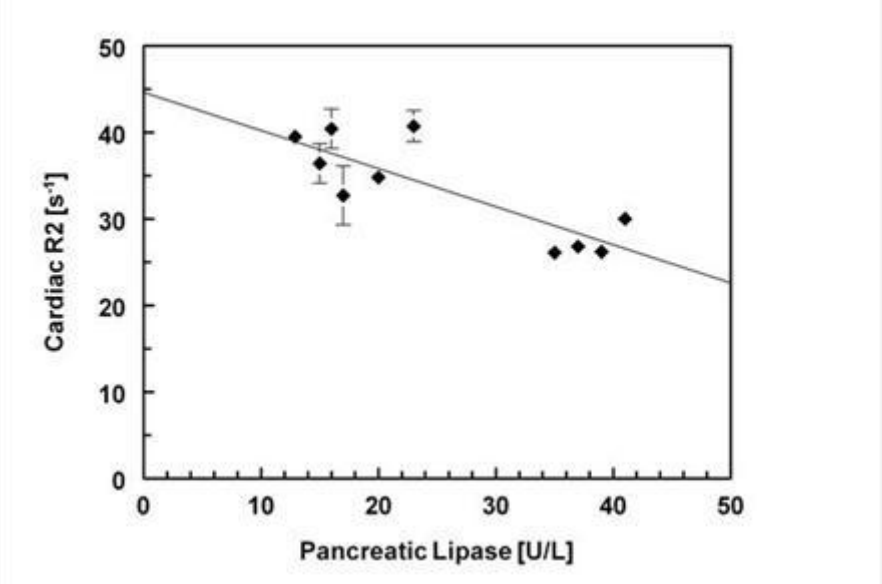
(Presented By: Roland Fischer)

Background: In untreated hereditary hemochromatosis (HHC), iron accumulation in other organs than the liver is claimed to lead to cardiomyopathy and other iron induced organ damage without real evidence. In addition to routine liver iron measurements by biomagnetic liver susceptometry (BLS), patients with genetically proved HHC also underwent quantitative magnetic resonance imaging (MRI-R2*) in liver, spleen, heart, pancreas, bone marrow and cardiac function assessment by 3D cine MRI.
Methods: So far, 12 patients with HHC (C282Y++: n=10, H63D++: n=2, age: 39-78 y, untreated: 10/12, females: 6/12) were measured. A multi-purpose multi-echo MRI-R2* breathhold sequence was used. Signal intensities ± SD averaged over global ROIs were fitted by a mono-exponential relaxation and/or by a water-fat relaxometry model with constant signal offset.
Results: Liver iron concentration by BLS (LIC: 191-5708 µg/g-liver, dry weight conversion factor = 6) reveals a curvilinear relationship with transverse liver relaxation rate R2* (R2*: 42-1314 s^{-1}, r^2=0.89). Fatty liver (fat content: 15-34%) was especially detected in the two HHC patients homozygous for H63D by water-fat shift relaxometry. Interventricular septal R2* ranged from 26.1 to 40.7 s^{-1} (controls < 40 s^{-1}), with cardiac R2* > 40 s^{-1} in 2/4 HHC with LIC > 4000 µg/g-liver, but without any correlation with LIC. Likewise no correlation of cardiac R2* was seen with pancreatic (tail) R2* (20-121 s^{-1}), but the exocrine pancreatic function parameter lipase did correlate (Spearman rank rS = -0.66, p=0.038), see Figure 1. Fatty infiltration of the pancreas (fat content > 10 %) was present in 50% of the patients. Left ventricular function with consideration of papillary muscles was unaffected in all patients (LVEF: 63-77%, normal: > 60%). R2* of the vertebral bone marrow was found in the range of controls for patients with the C282Y++ mutation (R2* = 57-120 s^{-1}, controls: 62-137 s^{-1}), but elevated for the two H63D++ patients (R2* = 165, 189 s^{-1}).
Discussion and Conclusion: Of notice are fatty infiltrations in liver and pancreas, especially for homozygous H63D patients although liver iron overload was mild. Cardiac iron accumulation in HHC patients seems to play a minor role on this pilot study level. As was already seen in thalassemia (Yamamura et al (2011)), serum lipase may become a sensitive screening parameter even for low levels of cardiac iron.
Figure 1. Pancreatic lipase predicts low cardiac iron (R2*± SE) in hereditary hemochromatosis (Spearman rank correlation rS = -0.66, p < 0.04).



Poster #307
FINE-TUNING OF SYSTEMIC IRON LEVELS BY MACROPHAGE-HFE
Naveen Kumar Tangudu, Dilay Lai, Richard Sparla, Matthias Hentze, Martina Muckenthaler and Maja Vujic Spasic, PhD
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(Presented By: Maja Vujic Spasic)

Introduction: Hfe deficiency in mice associates with severe iron overload as a consequence of diminished expression of the principal iron hormone hepcidin. Mice with the lack of hepatocytic-Hfe phenocopy major iron-related alterations seen in constitutive Hfe-deficient mice. By contrast hepatocytic Hfe is dispensible for the hepcidin response to LPS-triggered inflammation, which requires Hfe expression in macrophages.
Methods and Results: To further investigate putative Hfe functions in macrophages we systematically analyzed tissue iron levels in macrophage-specific Hfe mutant mice aged up to one year. These mice show significantly lower hepatic, splenic and duodenal iron levels compared to control mice, while plasma iron levels remain unchanged.
Conclusion: These data suggest that macrophage-Hfe serves to control systemic iron homeostasis by fine-tuning iron levels at the sites of iron storage, recycling and absorption. Experiments are ongoing (i) to identify molecular mechanisms that explain altered tissue iron levels (ii) to address whether local iron dysregulation affects the susceptibility of macrophage-Hfe mutant mice to inflammatory stimuli, and (iii) whether systemic iron injections overcome malfunctions in mice as a consequence of macrophage-Hfe deficiency.

Poster #308
EFFICACY OF DEFERASIROX IN THE TREATMENT OF SEVERE IRON OVERLOAD IN A PATIENT WITH HFE-HAEMOCHROMATOSIS AND ß-THALASSEMIA MINOR
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University Medical Center Hamburg-Eppendorf
(Presented By: Peter Nielsen)

Introduction: Phlebotomy is the standard treatment procedure in haemochromatosis. In single patients in which the repeated removal of larger volumes of blood is impossible due to technical reasons or the bad medical conditions of a severely affected patient, iron chelators can be used as second line treatment. We have published the efficacy of the parenteral iron chelator deferoxamin in two patients with severe hemochromatosis (1). Recently, a phase I/II study of using the new oral chelator deferasirox (ExjadeR) was published in a group of patients with haemochromatosis with relative moderate iron overload. The progress of iron removal was followed mainly by serum ferritin measurements.
Methods and Results: In this case report, iron overload was diagnosed in a so far untreated female patient (58 y old German) with HFE-haemochromatosis and ß-thalassaemia minor. At diagnosis, a liver iron of 5.7 mg Fe/g liver (normal 0.1-0.4 mg/g, Ferritin 6700 µg/l) was measured noninvasively by SQUID-biosusceptometry indicating a very severe liver siderosis. T2*-measurement by MRT excluded increased excess heart iron. As the hemoglobin values due to thalassaemia minor were only around 10 g/dl, repeated removals of larger blood volumes were not possible. We decided to use a split dose of 16 mg/kg/day deferasirox (500 mg in the morning, 750 mg in the evening). A higher dose was discontinued because of increasing creatinine values. Liver iron measurements after 5 months (3.3 mg/g, Ferritin 1530 µg/l) and 15 months (1.9 mg/g, Ferritin 642 µg/l) showed a relative fast reduction of the excessive iron storage to the actual degree of moderate iron loading. A clear clinical progress was seen under this treatment, the patient felt much better, liver enzymes normalized and the grey skin coloration disappeared. Due to some side-effects which the patient accounted to the medication, it was decided to stop the chelator treatment and to continue with a therapy using moderate blood removal (100 ml every 1-2 weeks) in order to achieve a negative iron balance and to slowly normalizes the remained iron excess.
Conclusion: This case documents the effective iron removal also in severe cases with iron overload in haemochromatosis by using a still moderate dose of deferasirox.
(1) Nielsen P et al. Brit J Haematol. 2003;123: 952–953
(2) Phatak P et al. Hematology 2010;52:1671-1679

Poster #309
NEXT GENERATION TARGETED DEEP SEQUENCING OF HEMOCHROMATOSIS GENES IN IRON OVERLOADED PATIENTS: A PILOT STUDY
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(Presented By: Daniele Manna)

Introduction: Hereditary Hemocromatosis (HH) is a common and genetically heterogeneous disorder with relevant geographical differences. While in people of North European descent nearly all cases (up to 95%) are associated with homozygosity for the C282Y mutation in the HFE gene, in the Mediterranean area up to one third of patients do not carry this genotype. Other genes associated with HH include HFE2, HAMP, TFR2 and SLC40A1, but mutations are generally “private”

and require sequencing. The advent of high-performance next generation sequencing (NGS) techniques, whose costs are rapidly declining, represents a possible approach for future molecular diagnosis in patients with iron overload. This pilot study was aimed to explore this approach in patients attending a tertiary care center for iron overload disorders.
Materials and Methods: We re-sequenced targeted regions (340Kb) of 5 HH-related genes (HFE, HFE2, HAMP, TFR2, SLC40A1) in 27 patients attending to our Regional Referral Centre for Iron Overload Disorders. Of them, some had known mutations in the HH genes (3 C282Y homozygotes, 1 C282Y/H63D compound heterozygote, 2 with mutations in TFR2, 3 with mutations in SLC40A1), previously determined by standard techniques or traditional sequencing. The remaining had relevant biochemical signs of iron overload that could be considered of "unknown origin" (including some with simple C282Y or H63D heterozygosity). A similar number (n=27) of subjects with normal serum iron parameters were included as controls. Exon capture was performed by a new technology, i.e. the HaloPlex Target Enrichment System (Agilent), which produces circularized fragments suitable for Illumina paired-end sequencing (using the Illumina HiSeq 1000 platform). Raw reads were filtered by quality and then aligned against human reference HG19 version 37 using BWA with standard parameters. On average we had a 354x sequence coverage for captured regions. These alignments were used to detect variants in each sample using Genome Analysis ToolKit (GATK): on average, GATK found about 1500 variants in each sample. The putative effects of the called variants were evaluated using the Variant Effect Predictor by the EnsEMBL consortium.
Results: In the iron overloaded patients, a total of 49 potentially deleterious variants were found in the 5 selected genes (2 missense and 1 splicing variants for HFE, 10 missense variants for HFE2, 0 variants for HAMP, 28 missense and 5 splicing variants for TFR2, 2 missense and 1 splicing variants for SLC40A1). Comparison with control samples as well as the functional relevance of these variants and their possible pathogenic role in each individual patient is currently under further investigation.
Conclusions: high-coverage selective exon capture is a powerful methodology for rapid DNA analysis in patients with iron overload of uncertain origin. Due to the high number of variants found, proper bioinformatic analyses are needed to reveal variants that are interesting for further clinical investigation. Anyway, as costs for NGS analyses keep to decline, they are expected to become useful tools in the near future for clinicians.

Poster #310
IMPACT OF D181V AND A69T ON THE FUNCTION OF FERROPORTIN AS AN IRON EXPORT PUMP AND HEPCIDIN RECEPTOR
Roman Praschberger, Melanie Schranz, Martin Hermann, William J. H. Griffiths, David J. Lomas, Antonello Pietrangelo, Timothy M. Cox, Wolfgang Vogel and Heinz Zoller
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(Presented By: Roman Praschberger)

Introduction: Mutations in the only known mammalian iron exporter ferroportin cause a rare iron overload disorder in humans, which is termed ferroportin disease. Two distinct clinical phenotypes are thought to be caused by different disease mechanisms: Mutations in ferroportin either cause loss of iron export function or gain of function due to resistance to hepcidin, the peptide hormone that normally downregulates ferroportin. The aim of the present study was to examine the disease mechanism of the so far unclassified A69T and the D181V ferroportin mutations.
Methods and Materials: We overexpressed wild-type and mutant ferroportin fused to green fluorescent protein in human embryonic kidney cells 293T and used a 59Fe-assay, confocal microscopy and flow cytometry to study iron export function, subcellular localization and the responsiveness to hepcidin.
Results: While the A69T ferroportin mutation seems not to affect the iron export function it causes partial hepcidin resistance in our assay. We further found that D181V mutated ferroportin is iron export defective and hepcidin resistant, similar to the loss of function control mutation A77D. This indicates that intact iron export might be necessary for the hepcidin response. We further investigated this hypothesis by studying the hepcidin response under modulation of extra- and intracellular iron availability. Incubation of wild-type ferroportin overexpressing cells with holo-transferrin increases the hepcidin effect whereas chelating extracellular ferrous iron causes hepcidin resistance. We also confirm the previously reported hepcidin resistance disease mechanism of the C326Y ferroportin mutation and the partial hepcidin resistance of the N144H mutation.
Conclusion: In this study we present data that suggest to classify the D181V ferroportin mutation as loss of function and the A69T mutation as partial hepcidin resistant and outline a possible causal link between iron export function and the hepcidin effect.

Poster #311
HOMOZYGOSITY FOR COMMON HFE MUTATIONS IS AN INDEPENDENT PREDICTOR OF IMPROVED SURVIVAL IN LIVER CLINIC PATIENTS
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Medical University of Innsbruck
(Presented By: Armin Finkenstedt)

Introduction: Hemochromatosis is the commonest genetic iron overload syndrome in caucasians, which is associated with homozygosity for the C282Y polymorphism in > 80 % of patients. Compound heterozygosity for C282Y/H63D and homozygosity H63Dare known to confer a risk for elevated serum iron parameters but are insufficient to cause frank hemochromatosis. Patients with HFE hemochromatosis have normal life expectancy, when adequately treated before tissue damage has occurred,.

Methods: Between 1997 and 2012, 8500 patients underwent HFE genotyping at our liver clinic, which is a tertiary referral centre in Western Austria. Inclusion criteria for this retrospective analysis were availability of follow-up information and biochemical laboratory parameters including ferritin concentration and transferrin saturation at the time of HFE genotyping.
Results: The cohort finally included 3920 patients (70% men) with a mean age of 51 years (± 15) at the time of HFE genotyping. HFE genotyping revealed C282Y homozygosity in 104 (2.7%), C282Y/H63D compound heterozygosity in 177 (4.5%), H63D homozygosity in 264 (6.7%) and other HFE genotypes (wildtypes and heterozygotes for C282Y or H63D) in 3375 (86.1%) patients. H63D homozygosity was significantly more common than predicted from the Hardy-Weinberg equation. Median ferritin concentration was 429 µg/l in C282Y homozygotes, 394 µg/l in compound heterozygotes (C282Y/H63D); 561 µg/l in H63D homozygotes and 316 µg/l in patients with other genotypes. Median transferrin saturation was 45%, 49%, 75% and 34%, respectively. After a mean follow-up of 7 years, patients with non hemochromatosis associated HFE genotypes had a significantly worse survival than patients with hemochromatosis associated genotypes of H63D homozygotes. Mean survival was 12.4 years for 'non hemochromatosis associated genotypes' versus 13.2 years, 14.3 years and 14.0 years for C282Y homozygotes, C282Y/H63D compound heterozygotes and H63D homozygotes, respectively. A ferritin concentration of > 300 µg/l was associated with worse survival (12 vs. 13.3 years; $p < 0.001$). In a multivariate Cox proportional hazards model, the risk of death was 2.8 (95% CI: 2.1 – 3.8) for patients with wild type or single heterozygous HFE genotypes as compared to those with homozygosity for C282Y, H63D or compound heterozygotes. This was independent of age, ferritin concentration and transferring saturation.
Discussion: The majority of patients who underwent HFE genotyping in our liver clinic population did not have HFE mutations associated with increased serum iron parameters. Our results show that HFE mutations can be considered a benign cause of iron overload. Patients without HFE mutations are at an increased risk of death, where other diseases like liver cirrhosis or malignancies may be accountable for imbalances in iron homeostasis. Furthermore, C282Y/H63D compound heterozygosity and H63D homozygosity are commonly associated with increased serum iron parameters in this selected population.
Conclusion: Patients who present with increased ferritin concentration/transferrin saturation and do not have HFE mutations should be carefully evaluated for other causes of disturbed iron homeostasis as they are at an increased risk of death.

Poster #312
THE PHENOTYPE OF TFR2-TMPRSS6 DOUBLE KNOCK OUT MICE ONLY PARTIALLY OVERLAPS THAT OF TMPRSS6 NULL MICE
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(Presented By: Antonella Roetto)

Introduction: TMPRSS6 encodes matriptase-2, a liver serine protease that, cleaving the BMP co-receptor hemojuvelin, downregulates the expression of the iron regulator hepcidin (HAMP). Loss of TMPRSS6 activity in humans and mice results in anemia due to elevated HAMP levels and impaired dietary iron absorption. The in vivo role of TMPRSS6 in hereditary hemochromatosis has been previously studied by deleting the gene in both Hfe-/- and Tfr2-/- mice (Finberg et al, Blood 2010; Lee et al, BCMD 2012). The phenotype of Tmprss6-/-Hfe-/- mice is indistinguishable from that of Tmprss6-/- animals, demonstrating that HFE acts genetically upstream of TMPRSS6 in the modulation of the BMP-SMAD pathway. Similar results were obtained in the Tmprss6-/-Tfr2-/- mice, although differences of genetic backgrounds made complex the comparison of the different genotypes.
Methods and Materials: To further assess the role of Tmprss6 in Tfr2-/- mice we back-crossed Tfr2-/- and Tmprss6-/- mice and analyzed 3-5 animals for genotype at 10 weeks of age. We analysed hematological parameters, hepatic [LIC] and splenic [SIC] iron content and evaluated the Bmp-Smad-Hamp pathway in the liver by real-time PCR.
Results: Tmprss6-/-Tfr2-/- mice are anemic with Hb levels similar to Tmprss6-/- mice, confirming that TfR2 acts genetically upstream TMPRSS6. However, the phenotype of Tmprss6-/-Tfr2-/- mice is not identical to that of Tmprss6-/- mice. Tmprss6-/-Tfr2-/- mice have higher RBCs and reticulocytes count and MCV and MCH significantly lower than Tmprss6-/- mice. SIC is comparable among all genotypes, while LIC, as expected, is low in Tmprss6-/- and high in Tfr2-/- mice. The homozygous inactivation of Tmprss6 in Tfr2-/- mice reduces LIC to the levels of wt littermates. We found that Bmp6 expression corresponds to LIC in all genotypes analyzed. In Tmprss6-/-Tfr2-/- mice Hamp expression (and Hamp/LIC ratio) is higher than in wt, but lower than in Tmprss6-/- mice. We interpret this result as the effect of an erythroid-mediated Hamp suppression.
Conclusion: Our data suggest that in the double mutants the Bmp-Smad pathway is more active than in wt mice, but less than in Tmprss6-/- mice. Surprisingly, the expression of other target genes of the Bmp-Smad pathway, as Id1 and Smad7, in Tmprss6-/-Tfr2-/- mice is comparable to that of wt animals, proving that the system is activated at the same rate in both genotypes. These results might suggest that in the absence of Tfr2, the activation of the BMP signalling, expected because of the loss of Tmprss6, is somehow attenuated, likely by the expanded erythropoiesis and that perhaps a specific Hamp inhibitor is lacking. Considering the importance of the erythroid signal/s in Hamp regulation, it is possible that the hypothetical inhibitor is related to the erythroid form of TfR2. Comparison of similar studies in liver conditional Tfr2-/- Tmprss6-/- will help to verify this hypothesis.

Poster #313

BRACHIAL ARTERY PSEUDOANEURYSM FOLLOWING PHLEBOTOMY FOR IRON OVERLOAD: FIRST CASE REPORT

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(Presented By: Jean-Michel Davaine)

Introduction: Phlebotomies are widely used for treatment of iron overload and considered as a very well tolerated procedure. Case report. We report the first case of an adult patient treated by phlebotomies for dysmetabolic iron overload and developing an arterial brachial pseudoaneurysm. The outcome after surgery was favorable.
Results: A venipuncture procedure can lead to the development of an arterial pseudo-aneurysm.
Conclusion: This complication must be kept in clinician's mind since it illustrates that a treatment such as phlebotomy, widely considered as a benign and non invasive procedure, can exceptionally be harmful.

Poster #314

DIAGNOSIS OF ATYPICAL IRON DISORDERS: CLINICAL APPLICATION OF NEXT-GENERATION SEQUENCING

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(Presented By: Nathan Subramaniam)

Introduction and Objectives: Most disorders of primary iron overload and iron-refractory anaemia have a genetic origin. Mutations in the *HFE* gene cause hereditary haemochromatosis (HH) and account for about 95% of HH in European populations, however mutations in other genes (termed non-HFE HH) are being increasingly identified in non-European populations. We aim to develop and apply Next-Generation Sequencing technology to diagnose genetic iron overload and deficiency disorders in a systematic fashion.
Methods: Genomic DNA was extracted from patient buffy coat samples. Thirty genes and 10 promoter regions associated with genetic iron overload or anaemia were then simultaneously amplified using a custom designed AmpliSeq panel. Samples had a DNA barcode ligated to allow multiplexing of several patient samples before high coverage sequencing was carried out on an Ion Torrent PGM. Sequences were aligned to the Human Genome version 19, and sequence variants identified and annotated using a custom bioinformatics pipeline. Variants were denoted as novel if they did not appear in the 1000 Genome database, dpSNP database, or the ESP5400 database.
Results: We have applied this technology to identify the genetic basis of atypical iron overload in two patients of Asian origin. In patient A, 161 single nucleotide polymorphisms (SNPs) were identified, with five novel SNPs identified in *HFE*, *TFR2* and three other panel genes. Nineteen SNPs produced non-synonymous changes, 16 of which are uncharacterised, with two predicted to be deleterious by a number of modelling algorithms. In patient B, 105 SNPs were identified, with four novel SNPs occurring in *TFRC*, *TFR2* and two other panel genes. Twelve SNPs produced non-synonymous changes in key genes such as *HFE*, *TFRC* and *TFR2*, seven of which are uncharacterised, three of which are predicted to be deleterious.
Conclusions: Iron overload in Asia may be a more complex disorder than expected, resulting from multiple compounding effects and attributable to genes other than the currently designated non-HFE HH genes of *HAMP*, *HFE2*, *TFR2*, and *SLC40A1*. The ability of our system to identify novel mutations and eliminate the ethnic bias of *HFE* screening allows greater insight into iron regulation in Asian populations. Our novel screening system will provide a valuable resource for clinicians and researchers within the Asia-Pacific region and worldwide.

Poster #315

ASSOCIATION BETWEEN HFE P.(C282Y) AND CORONARY HEART DISEASE IN THE NPHSII COHORT: INTERACTION WITH SMOKING

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UCL
(Presented By: Ann Walker)

Introduction: The *HFE* p.(C282Y) variant (rs1800562) is homozygous in ~90% of well-characterised patients with haemochromatosis in the UK. *HFE* p.(H63D) (rs1799945) is present in ~ 5%.
In genome wide association studies, *HFE* p.(C282Y) is associated with reduced low density lipoprotein cholesterol (LDL-C) levels and *HFE* p.(H63D) is associated with hypertension. Although both LDL-C and hypertension influence the risk of coronary heart disease, neither *HFE* variant has been reproducibly and significantly associated with coronary heart disease.
Heterozygotes for p.(C282Y) have subclinical but significant biochemical evidence of iron accumulation. Iron overload causes oxidative damage to molecules and tissues. Cigarette smoking is associated with increases in circulating markers of oxidative stress and increased risk of cardiovascular disease. We therefore hypothesised that interaction between *HFE* p.(C282Y) and smoking may be associated with increased risk of coronary heart disease.

Methods and Materials: The aims of this study were to investigate in the prospective Northwick Park Heart Study II (NPHSII) the association of *HFE* p.(C282Y) and p.(H63D) with lipid traits, blood pressure (BP) and coronary heart disease and the interaction with smoking.
Results: *HFE* p.(C282Y) and p.(H63D) were genotyped by KASPar competitive allele PCR in the NPHSII cohort of 3012 healthy UK males. Genotypes were in agreement with Hardy Weinberg equilibrium. Due to the low frequency of the minor allele of p.(C282Y) (0.074), homozygotes and heterozygotes were analysed together (CY+YY) in Cox regression models of association with coronary heart disease.
Neither variant was significantly associated with cardiovascular risk traits (LDL-C, HDL-C, triglyceride, total cholesterol, body mass index, systolic and diastolic BP) in the entire cohort. No significant association was seen between any of the p.(C282Y) genotype categories, nor any of the p.(H63D) genotypes, and coronary heart disease, in the entire cohort.
In non-smokers, p.(C282Y) was not significantly associated with coronary heart disease after adjustment for age and GP practice (p=0.06), but was significant after full adjustment for age, GP practice, systolic BP, total cholesterol & triglyceride concentrations (p=0.03; hazard ratio 0.56, 95% confidence intervals 0.34-0.95). In smokers, p.(C282Y) was significantly associated with coronary heart disease after both first and second adjustments (p=0.009, HR 1.91 (1.17-3.13) and p=0.004, HR 2.05 (1.25-3.36), respectively). There was a significant interaction between smoking and p.(C282Y) genotype category (interaction p-value=0.001 after both adjustments). Coronary event-free survival after 15 years was highest in (CY+YY) non-smokers (93%) and lowest in (CY+YY) smokers (71%).
Conclusion: The risk of coronary heart disease associated with the p.(C282Y) genotype categories is lower in non-smokers, possibly by lowering LDL-C, and higher in smokers, possibly by both factors tending to increase oxidative stress. The study will be replicated in additional cohorts.

Poster #316
NRF2 PROTECTS AGAINST DIETARY IRON-INDUCED LIVER INJURY
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(Presented By: Tiago Duarte)

Introduction: Excessive intestinal absorption of iron as seen in hemochromatosis causes its deposition in the parenchymal cells of various organs, which in some individuals may lead to organ failure. The liver, being the major site of iron storage, is particularly susceptible to the toxic effects of iron. Cellular injury is caused by oxygen radical-mediated damage to cellular organelles, leading to hepatocyte necrosis or apoptosis. Transcription factor Nrf2 is critical for protecting the liver against disease by activating the transcription of genes encoding detoxification/antioxidant enzymes and transporters that aid in the elimination of harmful xenobiotics.
Objectives: We aimed to determine if the Nrf2 pathway constitutes a major defense of cells and organisms against iron-induced toxicity.
Methods: Mouse embryonic fibroblasts (MEFs) were maintained under standard culture conditions. Cell viability was determined with the MTT assay. Reactive oxygen species (ROS) were detected by flow cytometry with the probe chloromethyl-2'7'-dichlorodihydrofluorescein diacetate acetyl ester. Gene expression was determined by real-time reverse-transcription PCR and proteins were analyzed by western blot. Experimental animals were manipulated in compliance with the recommendations of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 123) and 86/609/EEC Directive and Portuguese rules (DL 129/92). Routine serum biochemistry analysis was used to measure alanine aminotransferase (ALT) activity, and to quantify serum iron and total iron binding capacity. Iron content in liver samples was measured by the bathophenantroline method. Hepatocytic cell injury was determined by histological analysis of the liver, transferasemediated dUTP nick-end labeling (TUNEL) staining, immunofluorescence staining for caspase 3 and transmission electron microscopy.
Results: Iron toxicity was firstly analyzed in MEFs from wt and *Nrf2-/-* mice. Incubation with iron increased intracellular ROS and dramatically reduced cell viability in *Nrf2-/-* MEFs. Iron induced a set of cytoprotective genes only in cells derived from wt mice, suggesting that the Nrf2 pathway plays an important protective role against iron toxicity. To determine the significance of these findings in an experimental animal model of iron overload, wt and *Nrf2-/-* mice were fed *ad libitum* standard rodent chow or ironrich diet (2.0% carbonyl iron) for 2 weeks. Animals of both strains accumulated similar amounts of dietary iron in the liver and were able to respond by activating key regulators of iron homeostasis. Nevertheless, *Nrf2-/-* mice developed marked liver injury, as judged by the presence of inflammatory infiltrates, hepatocyte necrosis, extensive TUNEL staining, damaged mitochondria and increased serum ALT. Notably, liver injury was prevented when *Nrf2-/-* animals on the iron-rich diet received daily injections of the antioxidant mito-TEMPOL. Activation of the Nrf2 pathway by dietary iron in wild-type animals was illustrated by increased expression of two prototypical Nrf2 target genes, GSTa1 and NQO1.
Conclusions: We demonstrate that Nrf2 protects mouse cells and liver against the toxicity of iron overload. This study identifies Nrf2 as a potential modifier of liver disease in iron overload pathology and shows that the lack of Nrf2-mediated protection can be circumvented with antioxidant therapy.
Acknowledgement: FCT and COMPETE/QREN/UE- project PTDC/SAU-FCF/101177/2008 for financial support.

Poster #317

CLINICAL IMPACT OF HFE MUTATIONS IN PORTUGUESE PATIENTS WITH CHRONIC HEPATITIS C

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(Presented By: Paula Faustino)

Introduction: Chronic hepatitis C (CHC) is often associated with alterations in iron and lipid metabolisms, which may affect the long-term prognosis and the response to antiviral treatment. Some studies suggested that the occurrence of HFE mutations may contribute to modulate these metabolisms in CHC. Here, the prevalence of two HFE mutations (C282Y and H63D) was determined in a group of Portuguese CHC patients and the findings were correlated with clinical, histological and virulogical features.

Materials and Methods: 183 CHC patients (118 males and 65 females), mean age 45.84±11.46 years and IMC 25.45±3.96 Kg/m2. Eighty two (44.8%) were treated with standard antiviral therapy and divided into 3 groups: non response (NR)-25.6%, relapse (RR)-9.8% and sustained response (SR)-64.6%. HCV-RNA and genotype were determined by PCR. Liver steatosis, fibrosis stage and degree of necroinflammation (grading) were assessed by liver biopsy (Peter Scheuer score) and clinical parameters were measured by standard techniques: AST, ALT, GGT, lipid profile (LDL, HDL, total cholesterol and triglycerides), iron metabolism (iron, ferritin, transferrin and transferrin saturation), haptoglobin, ceruloplasmin, insulin, glucose, peptide-C and HOMA. Antioxidant potential (tGSH/GSSG ratio) was evaluated by spectrofluorimetry. HFE_H63D and C282Y polymorphisms were analyzed by PCR-RFLP and statistical analysis was performed with SPSS 16.0 (level of significance of $p<0.05$). Patients' exclusion criteria: other chronic liver diseases, alcohol ingestion >40g/day, HIV infection, metabolic and autoimmune diseases.

Results: Sixty two patients (33.9%) carried one or two mutant H63D allele (HD+DD), being 4.4% homozygous (DD). C282Y polymorphism was present in 5.5% of the patients; all were heterozygous. No difference was found comparing HFE_H63D and C282Y polymorphisms with the type of antiviral response. Regarding H63D, we observe a decrease in the degree of necroinflammation (grading) and in tGSH/GSSG ratio and an increase in total cholesterol for carriers of the mutant allele (HD+DD) comparing to HH individuals ($p=0.004$; $p=0.006$; $p=0.042$, respectively). For C282Y, our study revealed that heterozygous CY had higher serum iron and transferrin saturation levels ($p=0.038$; $p=0.006$, respectively) and lower total cholesterol ($p<0.0001$). In the total studied population, this last clinical parameter was found to be increased in patients with less necroinflammation and steatosis ($p=0.023$; $p=0.046$) and patients with higher fibrosis stages (moderate and intense) showed higher serum iron levels.

Conclusions: These data suggest a relevant role of HFE_H63D and C282Y polymorphisms in some clinical and histological features of chronic hepatitis C.

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Poster #318

HEPCIDIN KNOCKOUT MICE DEVELOP CHRONIC LIVER INJURY AND LIVER FIBROSIS AS A CONSEQUENCE OF LYSOSOMAL IRON OVERLOAD

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(Presented By: Mariia Lunova)

Background and Aims: Hepcidin is the central regulator of iron homeostasis. Disrupted hepcidin signalling results in hereditary hemochromatosis and iron overload seen in chronic liver disorders. While the association between iron overload and development of end-stage liver disease is well established, the understanding of the underlying mechanisms is hampered by the lack of a suitable animal model. To circumvent this problem, we analyzed hepcidin-knockout (KO) mice as a model of iron-overload associated liver disease.

Methods: Hepcidin wild-type (WT) and KO animals fed 3 % carbonyl iron-containing chow were compared to mice kept on standard diet. Liver histology and serum parameters were used to assess the extent of liver injury/fibrosis. Iron distribution was determined by subcellular fractionation and electron microscopy.

Results: Among mice kept on iron-rich diet, 6 month old hepcidin KOs (vs. WTs) displayed profound hepatic iron overload (2543±114 vs. 1493±136 $p< 0,005$), elevated liver enzyme (AST: KO 261±15, WT 142±34 $p< 0,05$) and serum iron levels, mild hepatocellular inflammation and apoptosis. 12, but not 6 month old KOs fed iron-rich diet developed moderate liver fibrosis as determined by Sirius red staining and increased hydroxyproline levels. The liver injury was accompanied by a marked lysosomal iron overload and lysosomal fragility with release of cathepsins into the cytoplasm, while no major differences were seen in mitochondrial morphology or injury markers. Increased p62 levels as well as elevated lipofuscin pigment suggested a

defect in protein degradation. As a potential mechanism leading to lysosomal iron overload, the expression of DMT1 and STEAP3, i.e. the molecules needed for lysosomal iron export, was greatly reduced. Finally, hepcidin KOs exposed to iron-rich diet also displayed an elevated oxidative DNA damage.
Conclusions: Hepcidin KOs represent a unique tool to study the mechanism of iron overload-related liver diseases and implicate lysosomal injury as a crucial event in iron toxicity.

Poster #319
TFR2 BETA ISOFORM HAS A ROLE IN CARDIAC IRON METABOLISM
Martina Boero, Francesca Tullio, Rosa Maria Pellegrino, Antonietta Palmieri, Pasquale Pagliaro, Giuseppe Saglio, Claudia Penna and Antonella Roetto
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(Presented By: Martina Boero)

Introduction: Together with liver, duodenum and bone marrow, heart is an organ in which iron metabolism must be tightly regulated. In fact iron overload associated diseases, like haemoglobinopathy and haemochromatosis, can present signs of heart failure. Moreover, iron fluctuations during heart ischemia have been associated with hypoxia injury damage (Berhenstein et al, 2008). TFR2 is a gene involved in iron metabolism transcribed in two main isoforms, the full length (Tfr2 alpha) and a shorter form (Tfr2 beta). The function of the latter form is correlated to iron efflux from splenic reticuloendothelial cells via iron exporter Ferroportin 1 (Roetto et al, 2010). It was previously documented that Tfr2 beta is highly transcribed in heart (Kawabata et al, 1999).
Methods and Materials: In order to understand whether Tfr2 beta has some role in cardiac iron metabolism we induced ischemia reperfusion (IR) in hearts of WT and Tfr2 beta null mice (TFR2 KI and TFR2 LCKO-KI) (Roetto et al 2010).
IR is an experimental technique consisting in rapid ischemia induction on isolated heart followed by reperfusion with oxygenate solution, mimicking the clinical condition of an acute myocardial infarction followed by reperfusion therapy, which is responsible of IR injury (Penna et al, Curr Med Chem, 2012). During IR, cardiac iron is subjected to rapid variations and the iron amount present during reperfusion could influence ROS generation and modulate the reperfusion associated injury entity. Significant protection from reperfusion damage has been experimentally obtained through the ischemic preconditioning phase (IPC) before IR, consisting in very short cycles of ischemia/perfusion before the long infarcting ischemia (Murry et al, 1986). A recent paper showed that a selective and significant L-ferritin (FtL) increase induced by IPC phase could acts as scavenger of the mobilized iron during the ischemic phase (Chevion et al, 2008). The authors concluded that the small amount of iron released during IPC could be the signal for FtL increase.
Results: Cardiac iron content was evaluated through standard methodology in hearts from WT and Tfr2 beta null (KI and LCKO-KI) animals. No significant differences were found in Tfr2 mice hearts vs WT.
Subsequently, animals from the three groups underwent the following IR protocol: 30-min ischemia and 60-min reperfusion. Myocardial infarct area was evaluated by nitroblue-tetrazolium staining. Total infarct size, expressed as a percentage of left ventricle (LV) mass, was 58±3% in hearts of WT group. A significant smaller infarct size was observed in hearts of Tfr2 KI and LCKO-KI (37±6% and 36 ±6% of LV mass, respectively) ($p<0.002$ vs. WT).
Ferritin subunits protein analysis in Tfr2 beta null mice before IR revealed that, while FtH amount remains constant, FtL was significantly increased compared to WT hearts, a scenario similar to that seen in preconditioned WT animals.
Conclusion: In conclusion Tfr2 beta isoform seems to be involved in cardiac iron metabolism and Tfr2 silencing results to have a protective effects on IR induced damages. It is likely that Tfr2 downregulation and FtL upregulation positively affect iron-dependent signals that are involved in preconditioning cardioprotection. Transcriptional analysis of the main iron genes and proteins behavior in Tfr2 targeted hearts will help clarifying the underlying molecular mechanisms.

Poster #320
UNEXPLAINED ISOLATED HYPERFERRITINEMIA IN PATIENTS WITHOUT MUTATIONS IN FERRITIN GENE AND L-FERRITIN IRES REGIONS
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(Presented By: Giulia Ravasi)

Introduction: Ferritin is an ubiquitous protein that stores iron in the cell cytosol and small amount is also secreted in plasma. Although serum ferritin levels does reflect to some degree the amount of iron overload of any cause, it is largely influenced by the presence of metabolic syndrome, inflammatory states and liver disease. Hyperferritinemia due to abnormalities of iron-dependent regulation of ferritin synthesis is observed in Hereditary Hyperferritinemia Cataract Syndrome (HHCS) caused by mutations in the L-ferritin IRE. Recently, a form of benign hyperferritinemia associated with increased glycosylated ferritin in serum and L-ferritin gene mutations in the heterozygous state was described, suggesting a defect in ferritin secretion. Here we describe 14 Italian patients with unexplained hyperferritinemia.
Methods: 9 probands (8 men and 1 woman) and 5 siblings (4 men and 1 woman) with isolated hyperferritinemia were enrolled in study. Inclusion criteria were: transferrin saturation below 45%, absence of known causes of hyperferritinemia (metabolic syndrome, liver disease, high alcohol intake, hyperthyroidism, inflammatory states), absence of iron overload, as assessed by

liver biopsy or Magnetic resonance. DNA samples were extracted by peripheral blood leukocytes using standard kit. Ferroportin and L-Ferritin genes and IREs regions of L-ferritin were directly sequenced. p.Cys282Tyr and p.His63Asp variants of HFE were searched for, despite normal transferrin saturation. Serum iron indices and other biochemical data were measured by standard methods.

Results: The table shows age, serum iron, transferrin saturation and serum ferritin levels in probands and siblings. No at risk genotypes were found in HFE and no causal mutations were identified in Ferroportin and L-Ferritin genes, and in the Iron Responsive Element (IRE) of L-ferritin. Nobody had cataract or family history of early cataract. None of the available probands' parents and offsprings had hyperferritinemia.

Discussion and Conclusion: We described 9 probands with unexplained isolated hyperferritinemia and without iron overload. These patients had no mutations in ferroportin, and IRE and coding regions of L-ferritin gene. Five probands have affected siblings, but no affected parents or offsprings. This findings suggest the existence of a recessive form of benign hyperferritinemia not dependent by mutations in L-ferritin gene. Further studies are needed to clarify this form of hyperferritinemia at both genetic and pathogenetic level.

	Probands (N=9)	Siblings (N=5)
Age (yrs)	47 [21-63]	40 [34-49]
Serum iron (μg/dL)	101.4 [66-155]	95.7 [90-101]
Transferrin saturation (%)	31.1 [20.3-40.6]	29.5 [27-33]
Serum ferritin (μg/L)	1927.1 [927-5090]	1604 [365-2339]

Poster #321

SINGLE AND REPEATED DOSE FIRST-IN-HUMAN STUDY WITH THE ANTI-HEPCIDIN SPIEGELMER NOX-H94

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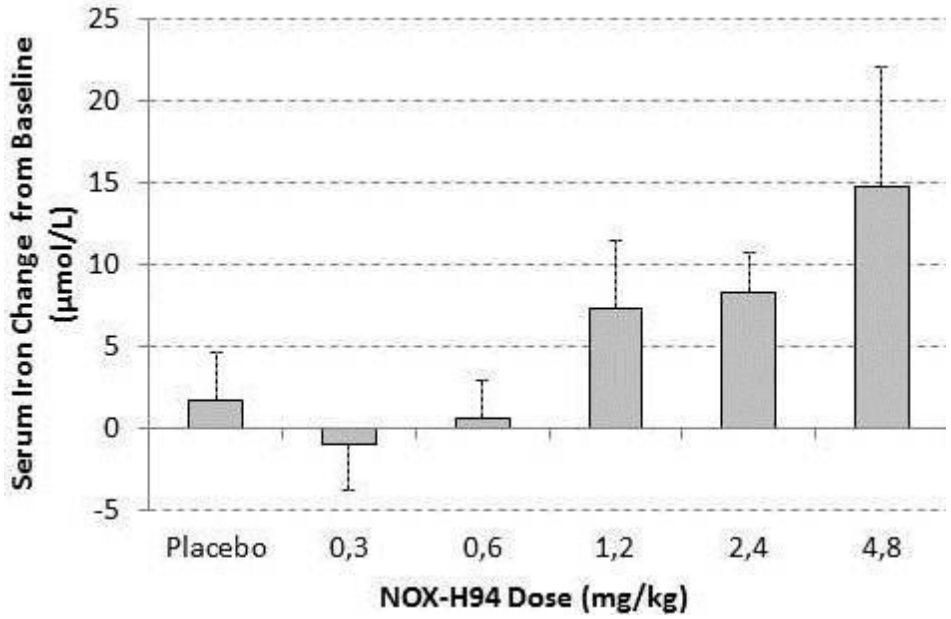
(Presented By: Kai Riecke)

Introduction: NOX-H94, a PEGylated anti−hepcidin L−RNA oligonucleotide, is in development for treatment of anemia of chronic disease (ACD). Currently, treatment of ACD is challenging because many ACD patients do not respond to erythropoiesis stimulating agents and repeated intravenous iron administrations bear a risk of iron overload. Targeting hepcidin may provide a better tolerated and more efficacious alternative.

Methods and Materials: This first-in-human study investigated the safety and tolerability, pharmacokinetics, and pharmacodynamics of escalating single and repeated doses of intravenous NOX-H94 in healthy men and women. The study protocol was approved by an independent ethics committee and conducted in accordance with the Declaration of Helsinki. Five successive cohorts of 8 healthy male and female subjects were randomly assigned to i.v. doses of 0.3, 0.6, 1.2, 2.4, and 4.8 mg/kg of NOX-H94 (n=6) or placebo (n=2). Similarly, 2 cohorts of 8 male subjects randomly received 5 i.v. doses of either 0.6 or 1.2 mg/kg NOX-H94 or placebo every other day. Safety parameters, iron parameters, total hepcidin−25, and drug concentrations were assessed during treatment and follow−up periods of ≥3 weeks.

Results: Treatment with NOX-H94 was safe and well tolerated. Headache and fatigue were the only treatment related events that occurred more than once. Mild and transient increases in hepatic transaminases (<2 × upper limit of the reference range) were noted in subjects treated with NOX-H94 at single doses ≥2.4 mg/kg or with repeated doses of 1.2 mg/kg (4.2 mg/kg weekly). After single i.v. doses of 0.3 to 4.8 mg/kg of NOX-H94, peak plasma concentrations of NOX-H94 and systemic exposure increased dose−proportionally. The elimination was bi−phasic with a terminal plasma half−life ranging from 17 to 26 h. No plasma accumulation of the study drug was observed after repeated i.v. administrations. Plasma concentrations of hepcidin increased dose−dependently without exceeding the concentration of NOX-H94. The rate of hepcidin increase was relatively constant from the time of NOX-H94 administration until maximum hepcidin concentrations were reached, independently from the dose of NOX-H94 administered.

Conclusion: These findings suggest that treatment with NOX-H94 does not induce the production of hepcidin and that instead hepcidin accumulates due to protection from degradation and renal elimination while being bound to NOX-H94. Doses of up to 0.6 mg/kg NOX-H94 had no effect on serum iron, serum ferritin, and transferrin saturation. At doses ≥1.2 mg/kg NOX-H94, serum iron and transferrin saturation increased dose dependently, indicating the inhibition of endogenous hepcidin (figure). However, the effects on serum iron were mild and of limited duration, possibly due to iron−induced feedback mechanisms.
Such feedback mechanisms are unlikely in patients with anemia of chronic disease; therefore the effects on iron regulation may be more prominent in patients than in healthy subjects.



Poster #323

SERUM HEPCIDIN LEVELS IN HOSPITALIZED PATIENTS. A PILOT STUDY

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(Presented By: Alejandro del Castillo-Rueda)

Introduction: Hepcidin synthesis is physiologically increased by elevated plasma iron concentrations and decreased by erythropoietic activity, and pathologically increased by inflammation. Hospitalized patients with chronic conditions generally have high levels of hepcidin, which act as an acute phase reactant and should return to normal after cure or improvement, unless these increased levels are due to other causes.
Methods: We conducted a pilot case−control study in adult hospitalized patients with multiple diseases and comorbid conditions grouped according to serum hepcidin levels (infectious diseases, neoplastic diseases, chronic diseases, and iron−deficiency anemia) that lead to hospital admission. Serum levels of hepcidin were determined in batches using a commercial enzyme−linked immunosorbent kit (DRG Instruments, GmbH, Marburg, Germany). Means were compared using the t test; a 1−way analysis of variance was used to compare variables between the groups.
Results: We had 32 cases and 7 controls; 48% were women. Mean age was 69 y; younger patients (56 y) had chronic conditions and older patients (77 y) had cancer. The largest group was that with infections (48%) and iron deficiency, although this was less marked than in the group with iron deficiency anemia; however, ferritin and mean serum hepcidin levels were higher in patients with infection. Hepcidin levels were lower in patients with chronic diseases admitted for exacerbations but not in those with infections, anemia, or cancer. No correlation was found between ferritin levels and hepcidin. In the rare cases in which hepcidin was determined at admission and at discharge, levels were always lower at discharge.
Discussion: Serum hepcidin levels could be a marker of disease prognosis and outcome. Our study was conducted in a heterogeneous population at very different stages of disease; therefore, correlations are complex. No reference method for determination of hepcidin has been established. In addition, the number of patients in our study was too small for results to be interpreted reliably. In some cases, the results were contradictory or group−specific. Our main finding was that hepcidin levels were significantly lower in patients with chronic diseases exacerbated by non−infectious processes, cancer, or iron deficiency anemia. In the remaining 3 groups, no significant differences in hepcidin levels were observed.
Conclusions: Ours is the first study to investigate the possible role of serum hepcidin in the diagnosis, prognosis, and outcome of hospitalized patients in whom we might expect higher hepcidin levels than in a control group. Our findings suggest that modulation of hepcidin production in hospitalized patients is for circulating factors or therapy. Further investigation and larger samples with more defined profiles are necessary.

Poster #324

SUPPRESSION OF TMPRSS6 USING ANTISENSE TECHNOLOGY AMELIORATES HEREDITARY HEMOCHROMATOSIS AND BETA-THALASSEMIA

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(Presented By: C. Casu)

Introduction: In conditions in which hepcidin is chronically repressed patients suffer from iron overload. Both in hereditary hemochromatosis (Hfe-/-) and thalassemic mice, the Smad signaling is low relative to liver iron concentration (LIC), (Kautz et al, Blood; Corradini et al, Gastroenterology; Parrow et al, Blood). TMPRSS6, a transmembrane serine protease mutated in iron-refractory, iron-deficient anemia, is a major suppressor of hepcidin expression. Reduction of Tmprss6 expression in Hfe-/- mice ameliorates the iron overload (Finberg et al. Nature Genetics; Du et al. Science; Folgueras et al. Blood; Finberg et al. Blood). Hepcidin up-regulation using either a hepcidin transgene or Tmprss6-/- also significantly improves iron overload and anemia in a mouse model of beta-thalassemia intermedia (th3/+ mice; Gardenghi et al. JCI; Nai et al. Blood).
Methods and Materials: In this report, we have examined whether reduction of Tmprss6 expression using antisense technology is an effective approach for the treatment of hereditary hemochromatosis and beta-thalassemia. Second generation antisense oligonucleotides (ASOs) targeting mouse Tmprss6 were identified.
Results: When normal male C57BL/6 mice were treated with 25, 50 and 100mg/kg/week ASO for four weeks, we achieved up to >90% reduction of liver Tmprss6 mRNA levels and up to 5-fold induction of hepcidin mRNA levels in a dose-dependent manner. In Hfe-/- mice (both males and females), ASOs were administrated at 100 mg/kg for six weeks. This treatment normalized transferrin saturation (from 92% in control animals to 26% in treatment group) and significantly reduced serum iron (from >300ug/dl in control group to <150ug/dl in treatment group), as well as liver iron accumulation. Histopathological evaluation and Prussian's Perl Blue staining indicated that iron was sequestered by macrophages, which led to an increase in spleen iron concentration. Th3/+ mice mimic a human condition defined as nontransfusion dependent thalassemia (NTDT), associated with iron overload due to ineffective erythropoiesis and suppression of hepcidin. Th3/+ animals exhibit increased proliferation and decreased differentiation of the erythroid progenitors, apoptosis of erythroblasts, reticulocytosis and shorter lifespan of red cells in circulation, leading to anemia (Libani et al, Blood). Th3/+ mice (both males and females) were treated with Tmprss6-ASO for six weeks. In th3/+ mice, ~85% Tmprss6 reduction led to dramatic reductions of serum transferrin saturation (from 55-63% in control group down to 20-26% in treatment group). LIC was also greatly reduced (40-50%). Moreover, anemia endpoints were significantly improved with ASO treatment, including increases in red blood cells (~30-40%), hemoglobin (~2 g/dl), and hematocrit (~20%); reduction of splenomegaly (~50%); decrease of serum erythropoietin levels (~50%); improved erythroid maturation as indicated by a strong reduction in reticulocyte number (50-70%) and in a normalized proportion between the pool of erythroblasts and enucleated erythroid cells. Hemichrome analysis showed a significant decrease in the formation of toxic alpha-globin/heme aggregates associated with the red cell membrane.
Conclusion: In conclusion, these data demonstrate that targeting TMPRSS6 using antisense technology is a promising novel therapy for the treatment of hereditary hemochromatosis and beta-thalassemia.
Additional studies will focus on how suppression of Tmprss6 in these models influences the expression of other iron regulatory genes and the Smad signaling.

Poster #325

THE BMP'S PATHWAY IN HEPCIDIN REGULATION DURING ENDOPLASMIC RETICULUM STRESS RESPONSE

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(Presented By: Susanna Canali)

Introduction: Hepcidin (HAMP) is a peptide hormone secreted by the liver that appears to be the master regulator of body iron homeostasis. It's overproduction causes anemia of inflammation, whereas HAMP deficiency leads to iron accumulation.
The expression of hepcidin is modulated by different stimulatory and inhibitory signals and the amount of this circulating hormone is finely regulated at the transcriptional level in the hepatocytes. In particular there are three main stimulatory pathways that regulates the transcription of hepcidin: the inflammatory pathway mainly through IL6, the endoplasmic reticulum stress response (ER stress) through the transcription factor CREBH and the BMPs (bone morphogenetic protein) pathway.
Methods and Materials: The aim of this work is to investigate whether BMPs are required to modulate the response of hepcidin in ER stress condition and whether an interplay between these two pathway is required to mediate the induction of hepcidin. To evaluate the role of BMP signaling under ER stress condition *in vivo* and *in vitro* we used a small molecule able to inhibits the BMPs pathway (LDN-193189) and two known ER stressors e.i. tunicamycin (TM) and Brefeldin A (BFA).
C57BL/6 male mice (9 week old) received a single injection of TM (2 mg/kg IP) and LDN-193189 (3 mg/kg IP) or relative vehicles, followed 2 hrs later by a single injection of LDN-193189 (3 mg/kg IP) or vehicle respectively. After 5 hrs from TM injection, livers were harvested, RNA extracted and hepcidin mRNA levels measured by qRT-PCR. In separate experiments, cells were treated with or without LDN-193189 (100 nM) for 5 hrs together with either TM (10 µg/ml) or BFA (20 µg/ml).
Results: We found that hepcidin mRNA induction by ER stress was reduced by inhibiting the BMP signaling pathway with LDN-193189 both *in vivo* and *in vitro*. It's known that HAMP promoter contains both CREBH binding sites and BMP-responsive

elements (BMP-RE). In the second part of the work, we wonder if ER stress induction of HAMP promoter requires the BMP-RE at -84/-79 position in the promoter. HepG2 cells were transfected with wild-type or mutant mHampluciferase vectors and treated with either TM or BFA. The mutation of the BMP-RE in the context of the hepcidin promoter construct abolished the ER stress mediated response. These results demonstrate that the BMP-RE is functionally important for the activation of hepcidin promoter in ER stress condition. Collectively, this study demonstrates that inhibition of BMPs signaling attenuates the induction of hepcidin gene expression in ER stress condition, both *in vitro* and *in vivo*.
Conclusion: Our studies support the concept that BMP and CREBH act together to regulate iron homeostasis and suggest a key role of BMPs also in the ER stress stimulatory pathway.

Poster #326
HEPCIDIN- A USEFUL SERUM MARKER IN ACTIVE CROHN'S DISEASE
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(Presented By: Tariq Iqbal)

Introduction: The iron regulatory peptide hepcidin is expressed both in response to iron stores and systemic inflammation. It is therefore key to the anaemia of chronic disease (ACD) which is common in Crohn's Disease (CD). Hepcidin has also heen Implicated in the pathogenesis of CD in animal models. In this pilot study we investigated serum hepcidin levels in patients with CD in relation to disease activity and serological markers of anaemia and inflammation
Method: Serum hepcidin was measured by mass spectromtery in 12 patients with active (CDa) and 17 patients with inactive CD (CDi). Disease activity was assessed using the Crohn's Disease Activity Index (CDAI) and a cutoff of 150 was used. Haemoglobin (Hb), ferritin and CRP were also measured. In addition serum hepcidin was measured in 48 "controls" with gastro-oesophageal reflux disease.
Results: Median hepcidin was significantly higher in CDa (90ng/ml) than CDi (32ng/ml) or controls (48ng/ml). There was no difference between the latter two groups. CRP was also higher in CDa (19mg/l vs 4mg/l). Taking the CD patient group as a whole, there were significant positive correlations between hepcidin and CRP , hepcidin and ferritin and Hb and ferritin. Although not significant, there was trend for hepcidin to associate negatively with Hb.
Discussion: In this study we have shown, for the first time, that serum hepcidin levels may distinguish active from inactive patients with Crohn's Disease. The correlations between ferrtin, CRP and hepcidin are as expected as these are all acute phase proteins. Similarly the relationship between ferritin and Hb is as expected. However, the trend for hepcidin to negatively associate with Hb suggests that, in the context of CD hepcidin expression is driven more by inflammation than anaemia.

Poster #327
STRATEGIES FOR THE SYNTHESIS OF HEPCIDIN AND ANALOGUES
Anna Adams, Derek Macmillan, Arnold Pizzey and Kaila Srai
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(Presented By: Anna Adams)

Introduction: Iron homeostasis is essential for ensuring the level of iron in the body meets functional requirements without causing toxicity [1]. Hepcidin is a 25 amino acid peptide hormone which acts as a systemic iron regulator. It is secreted from the liver in response to high iron levels in blood and is believed to bind to the iron efflux protein ferroportin, preventing further iron from leaving cells and allowing serum iron levels to return to normal [2]. The mechanism of action of hepcidin is poorly understood, and the need for therapeutic hepcidin agonists and antagonists is evident in cases where hepcidin or hepcidin activity is dysregulated [3]. Native Chemical Ligation (NCL) is the formation of a peptide bond between a peptide possessing cysteine at its N-terminus and a peptide furnished with a C-terminal thioester [4]. Hepcidin is a cysteine-rich peptide, providing multiple sites for NCL. This makes it an interesting and challenging candidate for us to study the use of NCL as a route to analogues.
Methods and Materials: We are using chemical ligation strategies to attach appendages to synthetic peptides comprising part or all of the hepcidin sequence. Our methodology uses peptide acyl hydrazides generated from native hepcidin sequences as precursors for ligations. In this way we have been able to successfully synthesise the full length of hepcidin via the ligation of two halves, and this peptide was folded to give a single isoform. This route to hepcidin could be of interest for the large scale synthesis of hepcidin.
Results: We have found the acyl hydrazide of a truncated hepcidin peptide, comprising residues 1 to 9 to retain biological activity in a cell based assay, this peptide has now been used as a precursor for analogues. We have also been able to generate peptides comprising the native hepcidin sequence, furnished with labels at the C-terminus.
Conclusion: These analogues of hepcidin can be useful as tools for functional studies of hepcidin, and this route can also facilitate the generation of novel hepcidin therapeutics.
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Poster #328
CLINICAL CHARACTERISTICS OF PATIENTS WITH METABOLIC SYNDROME AND IRON OVERLOAD
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(Presented By: Alejandro del Castillo-Rueda)

Introduction/Objective:
To describe the clinical characteristics of patients with hyperferritinemia due to iron overload with no known secondary cause who also presented metabolic syndrome or DIOS (dysmetabolic iron overload syndrome).
Methods: Descriptive observational study conducted over 7 months (November 2011 to May 2012) of patients seen at the iron disorders unit for increased ferritin levels. The inclusion criteria were metabolic syndrome and hepatic iron overload evidenced in MRI (magnetic resonance imaging). Patients in whom this overload could be explained by type I hereditary hemochromatosis or a known secondary cause were excluded.
Results: We studied 112 patients, 14 of whom met the inclusion criteria (13 men and 1 woman). Mean age was 60.36 ± 12.65 years (range, 40-77 years). The mean ferritin values at the first visit were 605.71 ± 173.89 µg/L (range, 341-938), and the mean transferrin saturation index was 43.50 ± 13.40% (range, 23%-62%). Mean iron overload on MRI was 66.43 ± 18.02 µmol/g (range, 40-90 µmol/g). Dyslipidemia was detected in 92.85% of patients, hypertension in 71.43%, glucose >100 mg/dL in 42.86%, and central obesity in 78.57%. Hepatic steatosis was detected in 35.7%.
Discussion: The prevalence of DIOS was 12.5% in patients with hyperferritinemia; therefore, this entity should be included in the differential diagnosis when laboratory results demonstrate its presence. Suspicion is confirmed by measuring ferritin, diagnosis is by hepatic MRI, and treatment is with phlebotomy.
Conclusions: Iron overload can be considered a cardiovascular risk factor and is associated with the development of liver disease.

Poster #329
THE STATIN-IRON NEXUS: ANTI-INFLAMMATORY TREATMENT FOR ARTERIAL DISEASE
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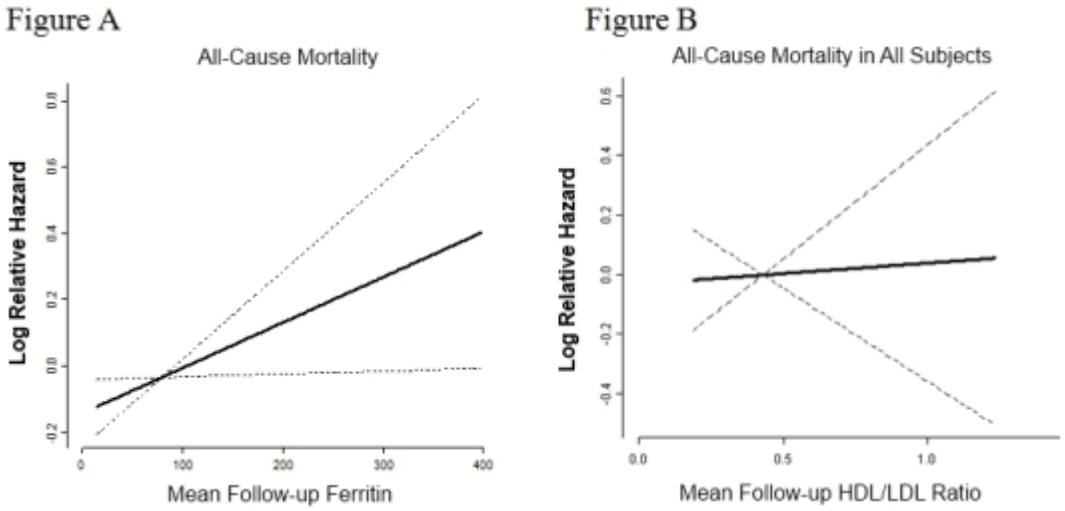
(Presented By: Leo Zacharski)

Introduction: Mechanisms by which statins improve vascular disease outcomes include inhibition of cholesterol synthesis and/or inflammation. In favor of an anti-inflammatory mechanism is the fact that drugs other than statins that lower cholesterol have no effect on vascular outcomes, statins are effective in patients with normal cholesterol levels, and statins are anti-inflammatory. The latter may be due to statin effects on iron homeostasis including induction of heme oxygenase-1; inhibition of hepcidin; inhibition of iron-catalyzed ROS production; and reduction of inflammatory cytokines, macrophage iron and cholesterol, and serum ferritin levels.
Methods: Data from a prospective randomized single-blinded clinical trial of iron (ferritin) reduction by calibrated phlebotomy in advanced peripheral arterial disease (PAD), the FeAST trial (NCT00032357), permitted comparison of effects of ferritin levels versus HDL/LDL ratios (both were randomization variables) on clinical outcomes in participants receiving and not receiving statins; n = 1,277; 6 year duration.
Results: Log relative hazards from the Cox proportional hazards regression analysis showed a significant association between mean follow-up ferritin levels (MFFL) and all-cause mortality for the entire cohort (p=0.037, figure A) and patients randomized to iron reduction (p=0.028). Kaplan-Meier analysis showed significantly better survival for patients having MFFL below compared to above the median of the means (p=0.003). (Amer Heart J. 2011;162:949-57). Statin use at entry (59% of patients) was associated with significantly lower ferritins (p=0.037) and higher HDL/LDL ratios (p<0.0001). No relationship existed between ferritin levels and HDL/LDL ratios at entry in statin users, non-users or the entire cohort. No relationship existed between mean follow-up HDL/LDL ratios and MFFL. Log relative hazards showed no association between mean follow-up HDL/LDL ratios and survival (p=0.840, figure B). Kaplan-Meier analysis comparing patients having mean follow-up HDL/LDL ratios above versus below the median of the means showed no effect of increasing HDL/LDL ratios on mortality.
Discussion: Statins apparently increase HDL/LDL ratios and reduce ferritin levels by non-interacting mechanisms. Improved clinical outcomes were associated with lower ferritin levels but not with improved lipid status.
Conclusions: We postulate the existence of a statin-iron nexus by which statins improve CVD outcomes at least partially by countering pro-inflammatory effects of excess iron stores. This conclusion is supported by basic, epidemiologic, pathophysiologic and clinical trial data showing commonalities between clinical benefits of statins and maintenance of physiologic iron levels. Thus, iron reduction itself may be a safe, effective and low-cost non-pharmacologic alternative to statins.

FIGURE A, p=0.037 FIGURE B, p=0.840



Poster #330

EFFECT OF IRON DEPLETION ON LIVER DAMAGE IN NONALCOHOLIC FATTY LIVER DISEASE: PRELIMINARY RESULTS OF A RANDOMIZED CONTROLLED TRIAL

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(Presented By: Luca Valenti)

I**ntroduction:** Hyperferritinemia and increased iron stores are common and associated with hepatic fibrosis in nonalcoholic fatty liver disease (NAFLD), whereas iron depletion decreased insulin resistance and ALT levels in controlled studies.
Aim: to assess whether iron depletion improves histological liver damage in NAFLD with increased iron stores.
Methods: NAFLD patients with presence of histological iron or ferritin >250 ng/ml and histological inflammation/ballooning despite lifestyle counseling >6 months were randomized to iron depletion by fortnightly phlebotomies until ferritin <100 followed by maintenance or counseling alone, monitored every 24 weeks for anthropometric, metabolic, and hepatic parameters, and control liver biopsy was performed after 96 weeks. The primary outcome was any improvement in NAFLD activity score (NAS) without worsening fibrosis (ClinicalTrials identifier: NCT00658164).
Results: 21 patients were randomized to phlebotomy and 17 to counseling. Basal features were not different between the two groups. Phlebotomy achieved a reduction in biochemical and histological iron parameters ($p<0.001$) without side effects, after 18±13 phlebotomies (5±4g of iron removed). By the end of the study, serum aminotransferases were significantly lower in phlebotomized patients than in controls (ALT: 24±10 vs. 37±10; AST: 21±5 vs. 28±6 IU/ml; $p<0.01$ for both), whereas there was no influence on GGT. Control biopsy was refused by some patients, generally because of normal liver enzymes in the treatment group, or the possibility to undergo phlebotomy in the controls. Nevertheless, in a per-protocol analysis of patients who completed the study, amelioration of histological damage was demonstrated in 7/11 (64%) phlebotomized patients vs. 1/8 (12%) controls ($p=0.025$).
Conclusions: phlebotomy is well tolerated in patients with NAFLD and increased iron, effectively reduces iron stores, and results in an improvement in ALT and AST levels. Iron depletion may possibly improve liver damage in patients with NAFLD and increased iron stores.

Poster #331

ANEMIA AND IRON METABOLISM IN PATIENTS WITH PORTAL THROMBOSES

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(Presented By: Elena Lukina)

Background: patients with prehepatic portal hypertension (PPH) caused by portal thrombosis often have recurrent bleedings from esophageal and gastric varices. Anemia in these patients is considered to be posthemorrhagic iron deficiency anemia, therefore patients often receive iron treatment.
Aim: to study the iron metabolism in patients with prehepatic portal hypertension due to portal thrombosis.
Materials: we studied 77 patients (31 male and 46 female, mediana of age 44 years) with prehepatic portal hypertension caused by portal thrombosis. 60% patients had bleedings from esophageal and gastric varices, 27%- recurrent bleedings.

The period from the first manifestation of portal hypertension (splenomegaly, varices of esophageal and gastric veins) to examination in our Center varied from 4 to 480 months (mediana = 60 months). 58% patients had hypo- or normochromic anemia, other patients had normal hemoglobin level.
Methods: we investigated the serum indices of iron metabolism (iron concentration, total iron binding capacity, transferrin iron saturation, ferritin, transferrin). Hepcidin level and urine iron excretion were examined in the part of the study group. The control group consisted of 35 healthy volunteers.
Results: all patients had low or normal levels of serum iron (Mediana = 10,1 mmol/L), ferritin (M=39 mkg/L, normal=40-200) and transferrin iron saturation (TIS, M= 17,2%, normal = 25-35%). At the same time, serum transferrin level was low or normal in 66% of patients (mediana 2,6 g/L, normal 2,65±0,05) and serum indices of soluble transferrin receptors were not increased in the majority of patients. Positive desferal test revealed increased urine iron excretion in 33 from 35 examined patients. Increased iron concentration in liver tissue (biopsy) was found in 3 from 6 patients. We also did not find any differences in iron metabolism indices in patients with and without anemia.
Conclusion: iron deficiency in patients with portal thrombosis and PPH may represent the failure of iron metabolism regulation in liver cells caused by ischemic injury of the liver. Presumably anemia may be an adaptive response in patients with ischemic liver.

Poster #332
ENDOTHELIAL DELETION OF FLVCR1A ALTERS VASCULAR INTEGRITY
Sara Petrillo, Sonia Mercurio, Deborah Chiabrando, Lorenzo Silengo, Massimo Santoro, Fiorella Altruda and Emanuela Tolosano
Molecular Biotechnology Center, University of Turin
(Presented By: Sara Petrillo)

Background: The Feline Leukemia Virus Subgroup C Receptor 1 (FLVCR1) is an highly conserved heme export protein existing as two different isoforms, FLVCR1a and 1b, respectively expressed at the cell membrane and on the mitochondrion. Previous studies in mice demonstrated that deletion of both FLVCR1 isoforms results in early embryonic lethality, due to a failure of fetal erythropoiesis (Keel *et al.*, Science 2008). On the other hand, mice lacking only the cell surface isoform die midgestionally due to extended haemorrhages, thus suggesting a role for FLVCR1a in maintaining vessels' integrity (Chiabrando *et al.*, J Clin Invest. 2012).
Methods: To address the specific role of FLVCR1a in the vasculature, we generated a conditional knockout mouse model for FLVCR1a (FLVCR1aflox/flox; Tie2-cre+) by crossing mice carrying a floxed FLVCR1a allele with mice expressing the CRE recombinase enzyme under control of Tie2 promoter, which is specifically active in endothelial cells (ECs). Moreover, we also generated a zebrafish model of FLVCR1a deficiency using specific oligomorpholinos.
Results: FLVCR1aflox/flox; Tie2-cre+ embryos died around embryonic day 16.5 (E16.5) due to extended haemorrhages, particularly evident in the developing limbs, where vessels' architecture was completely lost. The extraembryonic vascular network was properly organized in FLVCR1aflox/flox; Tie2-cre+ yolk sacs but vessels appeared dilated. The endothelial layer correctly surrounded blood vessels' boundaries, thus suggesting a functional impairment in knockout endothelium. Severe vascular alterations, primarily affecting intersegmental vessels, were clearly visible also in zebrafish morphants in which FLVCR1a expression was down-regulated.
Discussion: FLVCR1a knockout endothelial cells, lacking their cell's surface heme exporter, likely experienced a heme overload condition. Endothelial cells are highly sensitive to free heme, a prooxidant and proinflammatory agent by virtue of its ability to promote ROS formation and oxidative stress. Hence, knockout endothelial cells could give rise to more fragile vessels that collapse when blood flow increases during development, thus generating the haemorrhages.
Conclusions: Our data definitively demonstrate that FLVCR1a has an essential role in supporting vessels' integrity. Indeed, vasculogenesis and angiogenesis develop normally in knockout embryos but vascular maturation or maintenance of newly formed vessels is impaired since embryonic stage E11.5. These findings could have future implications in diseases characterized by heme-induced oxidative stress in the endothelium like thalassemias and sickle cell disease.

Poster #333
THE ROLE OF IRON IN ATHEROSCLEROSIS
Léon Kautz, PhD, James Onwuzurike, Victoria Gabayan, Bo Qiao, Xuping Wang, Aldons Lusis, PhD, Tomas Ganz, PhD, MD and Elizabeta Nemeth, PhD
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(Presented By: Léon Kautz)

Introduction: The possible role of iron in the promotion of atherosclerosis is an unresolved question. Various studies in animals and humans over the last 30 years assessed the effect of increased body iron on atherosclerosis but have yielded inconsistent results. In the last decade, our understanding of iron biology underwent a radical revision, raising questions about the design and interpretation of numerous studies on the subject. We tested the following conceptual framework. Iron, known as a potent catalyst for generation of reactive oxygen species, likely accelerates atherosclerosis by increasing oxidative stress in the plaque, oxidizing accumulated lipids and promoting inflammation. In atherosclerosis, as in other inflammatory diseases, systemic and local inflammation increases the production of the ironregulatory peptide hormone hepcidin. Hepcidin functions

by inhibiting the release of iron from macrophages, and would have the same effect in the atherosclerotic plaque on macrophages that ingest erythrocytes and apoptotic/necrotic cells. The hepcidin-mediated accumulation of iron in plaque macrophages and the resulting inflammation constitutes a self-amplifying process and is an important promoter of atherosclerosis. We explored the effect of atherosclerosis on systemic (hepatic) hepcidin production and examined the effect of increased macrophage iron on atherosclerosis progression and vascular calcification.
Method: ApoE-deficient mice, the classical mouse model of atherosclerosis, were analyzed on low-fat and high-fat diets to accelerate atherosclerosis development and the mechanism of hepcidin regulation in the liver was explored. To examine the role of macrophages, we used the "flatiron" mouse (ffe) carrying a mutation in ferroportin resulting in inappropriately high macrophage iron accumulation that mimics hepcidin excess. We generated a new mouse model by breeding ffe with ApoE -/- mice to study ApoE/ffe mutants which were expected to have more macrophage iron than ApoE-/- mice. These mice were fed 4 months with either a standard rodent diet or a high lipid diet and we performed an extensive phenotypical characterization of these mice. Finally, we analyzed ApoE-/- and ApoE/ffe mice that were fed 2 months with high lipid diet with and without weekly administration of iron sucrose, another approach that increases macrophage iron content.
Results: Hepatic hepcidin mRNA expression was not increased in any of our atherosclerotic mouse models fed a high-fat diet compared to standard diet, despite severe atherosclerotic phenotypes, although a local production of hepcidin in the plaques environment cannot be excluded. ApoE/ffe mice did not develop larger atherosclerotic lesions than ApoE-/- mice and only had modestly increased vascular calcifications. This small difference between ApoE/ffe and ApoE-/- mice may be related to a relatively small increase in macrophage iron load in ApoE/ffe compared to ApoE-/- mice, possibly because the high lipid content of the diet inhibited iron absorption. To enhance the difference in macrophage iron loading between ApoE/ffe and ApoE-/- mice, we administered parenteral iron. However, after 2 months, ApoE/ffe and ApoE-/- mice treated with iron exhibited smaller atherosclerotic lesions than their respective controls and no significant calcifications were observed.
Conclusion: Neither hepcidin nor macrophage iron play a significant role in the mouse model of atherosclerosis progression.

Poster #334
ACTION OF IRON AND FRUCTOSE ON GENE EXPRESSION IN HUMAN LIVER CELLS
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(Presented By: Tatiana Christides)

Introduction: Evidence suggests that iron and fructose may play a role in the pathogenesis of non-alcoholic fatty liver disease (NAFLD) – the most common liver disorder in the developed world. Furthermore, there is evidence that chemical interactions between iron and fructose generate hepatotoxic metabolites that could contribute to NAFLD progression; however, other studies have shown that fructose sequesters iron and is protective against iron-induced oxidative stress. Previously we showed fructose increased liver iron uptake. The aim of this study was to investigate the effects of iron and fructose - alone and in combination - on gene expression in human liver cells.
Methods: HepG2 hepatoma cells were treated with 1μM ferric ammonium citrate (FAC) and 15 mM fructose for 24 hours, and then harvested for RNA. Gene expression was assessed using Affymetrix Genechip Human Genome microarray and subsequent bioinformatic analysis using GeneGo Metacore software.
Results: Treatment of cells with fructose and FAC together decreased the expression of signalling proteins in two major pathways implicated in development of inflammatory liver disease: transforming growth factor β (TGF β) - as evidenced by lowered gene expression of SMAD2 and SMAD3 - and STAT3/NF-κB. Fructose treatment alone activated complement pathways with increased expression of C3, C5 convertase and the membrane attack complex.
Discussion and Conclusion: Our results suggest that hepatic fructose-iron interactions are anti-inflammatory, possibly via fructose induced iron sequestration; fructose alone, however, may cause inflammation by increasing activity of the innate immune system. The liver is continually exposed to both iron and fructose via the portal circulation system. Further research is essential to assess the roles of iron and fructose metabolism in the development and progression of NAFLD.

Poster #335
NON-TRANSFERRIN-BOUND IRON CORRELATES WITH LDL, AND HAEMOGLOBIN, AND ATTENUATES WITH STATINS: A CASE FOR RBC GLYCATION AND HAEMOLYSIS IN TYPE 2 DIABETES?
Desley White, PhD, BSc Hons, Angela Shore, PhD, Avril Collinson, BSc, PhD and Keith Collard, BSc, MSc, PhD
Plymouth University
(Presented By: Desley White)

Introduction: The glycation of various iron proteins in vitro, by Schiff's base and Amadori reaction between glucose and primary amine groups, alters their ability to interact with iron, and has led to the investigation of non-transferrin-bound iron (NTBI) in people with type 2 diabetes. Such iron may play a role in diabetic atherogenesis. Little agreement is found for NTBI levels in diabetes by other groups using a range of methodologies, and it is not known what form NTBI may take in diabetes, other than that redox active glycated albumin-bound iron has been identified.
Methods and Materials: Using a development of ultrafiltration and HPLC methods using nitrilotriacetic acid (NTA) and deferiprone, a cross-sectional study of NTBI has been conducted in people with type 2 diabetes (n = 54), and controls (n = 24).

NTBI is seen in 79.2% of controls compared with 63.0% of diabetes patients. No significant difference is seen between groups (P = 0.326).
Results: Median level in controls is found to be 0.34 μM (range 0.00 to 1.08 μM); in diabetes, 0.21 μM (range 0.00 to 1.34 μM). No correlation with protein glycation as measured by HbA1c is seen (r = -0.173, P = 0.141). Highly significant correlations are seen between NTBI and haematocrit (r = 0.358, P = 0.002); NTBI and haemoglobin (r = 0.357, P = 0.002); and NTBI and low density lipoprotein (LDL) (r = 0.356, P = 0.002). A correlation is found between NTBI and lipid peroxidation marker, malondialdehyde (r = 0.387, P < 0.001). No direct association is therefore seen between NTBI and protein glycation in type 2 diabetes, by this assay. When cases are pooled and analysed by treatment with statins, a correlation between NTBI and LDL is seen in the without treatment group (n = 29, r = 0.556, P = 0.003), which is not present with treatment (n = 47, r = 0.223, P = 0.137). LDL and haemoglobin correlate increasingly with HbA1c tertile (Tertile 1, r = -0.058, P = 0.789; Tertile 2, r = 0.495, P = 0.014; Tertile 3, r = 0.538, P = 0.007).
To explain these results, a scenario of erythrocyte membrane glycation, oxidation, and haemolysis, is proposed. Erythrocytes are known to have more membrane cholesterol than any other cell, which can be reduced by use of statins. Increased post-translational erythrocyte membrane glycation modifications are known to occur in diabetes, along with increasing membrane lipid peroxidation, and cell fragility, correlating with HbA1c. Plasma haemoglobin from haemolysis oxidizes to methaemoglobin, and can degrade to haem, which is highly pro-oxidant if not complexed by haemopexin or albumin, and is invasive of the sub-endothelium. Iron may be being scavenged from haemoglobin or haem by NTA in our NTBI assay, and tests on lyophilized bovine haemoglobin have found NTA to dose-, and timedependently increase NTBI measurements with our assay. Treatment with statins may therefore reduce erythrocyte membrane lipid peroxidation, resulting in the lessened NTBI vs LDL correlation seen.
Conclusion: Plasma NTBI measured by NTA scavenging and ultrafiltration appears to be closely linked to LDL, haemoglobin, and haematocrit. This may be due to erythrocyte membrane glycation, lipid peroxidation, and haemolysis, causing extracellular haemoglobin or haem.

Poster #336
GENETIC VARIATION OF IRON METABOLISM IN OBESE MICE
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(Presented By: Kathryn Page)

Introduction: Iron deficiency is one of the most common nutrition concerns in the world, with an estimated 4-5 billion affected persons. Debilitating fatigue, altered immune function, decreased work capacity and anemia are among the deleterious consequences of this pervasive disorder. Recent findings have shown that obesity is often associated with iron deficiency, which may contribute to the associated morbidities. Although it is has been suggested that obesity-related iron deficiency could be caused by decreased intestinal uptake of iron, the exact mechanisms are poorly understood at this time. Several genetic disorders of iron metabolism in man, rodents and other vertebrates suggest that multiple loci can contribute to the susceptibility and severity of iron deficiency. Previous studies have shown genetic variation in both iron metabolism and obesity amongst inbred strains of mice. We hypothesize that this genetic variation underlies the differences seen in iron metabolism and obesity between inbred mice.
Methods and Materials: Our aim is to map the quantitative trait loci responsible for the strain specific differences in obesity-related iron metabolism. To do this we will use high resolution SNP analysis to "in silico" map the genetic loci responsible for the divergent iron related phenotypes using the hybrid mouse diversity panel (HMDP) fed on a high fat diet.
Results: Here we show the phenotypic analysis of the variation in iron metabolism for these mice, and how interrelated mineral profiles change for each strain.
Conclusion: This work will contribute to understanding the mechanisms of relationships between obesity and iron metabolism.

Poster #337
HEPATIC IRON GRADE AND PATTERN AFFECTS OXIDATIVE STRESS AND APOPTOSIS IN NONALCOHOLIC FATTY LIVER DISEASE
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(Presented By: James Nelson)

Aim: The goal of this study was to examine the relationship between presence of hepatic iron deposition, apoptosis, histologic features and serum markers of oxidative stress and cell death in nonalcoholic fatty liver disease. We aimed to test the hypothesis that increased NAFLD severity among patients with hepatic RES iron deposition is associated with increased apoptosis and systemic oxidative stress, possibly as a consequence of iron loading in macrophages and other RES cells.

Methods: Clinical, biochemical, metabolic and independent histopathologic assessment was conducted in 83 unselected patients with biopsy-proven nonalcoholic fatty liver disease (NAFLD) from a single center. Apoptosis and necrosis in serum was quantified using serum cytokeratin-18 (CK18) M30 and M65 ELISAs and in liver by TUNEL staining in situ. Serum malondialdehyde (MDA) and thioredoxin-1 (Trx-1) levels were measured to evaluate oxidative stress.
Results: Presence of reticuloendothelial system cell (RES) iron in the liver was associated with nonalcoholic steatohepatitis ($p<0.05$) and increased hepatic TUNEL staining ($p=0.02$), as well as increased serum levels of apoptosis-specific (M30, $p=0.013$) and total (M65, $p=0.006$) CK-18 fragments, higher MDA ($p=0.002$) and lower antioxidant Trx-1 levels ($p=0.012$) compared to patients without stainable hepatic iron. NAFLD patients with a hepatocellular (HC) iron staining pattern also had increased serum MDA ($p=0.006$) but not M30 CK-18 levels or TUNEL staining compared to subjects without stainable hepatic iron. Patients with HC iron deposition limited to hepatocytes had a lower proportion of apoptosis-specific M30 fragments relative to total M65 CK-18 levels (37% vs. ≤ 25%, $p<0.05$), suggesting increased cell death via necrosis in these subjects compared to the other groups.
Conclusions: We found that both HC and RES iron deposition are associated with increased oxidative stress compared to patients without stainable iron. However, RES but not HC iron deposition in this disease is characterized by increased apoptosis in the liver as shown by both in situ TUNEL staining in the liver and circulating CK-18 levels and suggests a mechanism for increased disease severity. By contrast, our data suggest that selective hepatocellular iron deposition in NAFLD may preferentially promote cell necrosis versus apoptosis. Longitudinal studies will also help delineate the dynamic relationship of iron and oxidative stress and the role of these in NAFLD progression.

Poster #338

THE IL1β +3953 C>T POLYMORPHISM MINOR ALLELE IS ASSOCIATED WITH A HIGHER NAFLD ACTIVITY SCORE, LESS PARENCHYMAL IRON, THE PRESENCE OF NONALCOHOLIC STEATOHEPATITIS AND IS AN INDEPENDENT RISK FACTOR FOR ADVANCED FIBROSIS IN SUBJECTS WITH NONALCOHOLIC FATTY

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(Presented By: James Nelson)

Background: Nonalcoholic steatohepatitis (NASH) is a complex, multifactorial disease affected by diet, lifestyle and genetics.
Aim: The goal of this study was to investigate the relationship between IL6 and IL1β cytokine gene polymorphisms and histologic features of NASH in the large, well characterized NASH CRN cohort.
Methods: Real time genotyping for the following SNPs in the IL6 and IL1β genes was performed in 787 adult (≥18 yrs) subjects with biopsy proven NAFLD and hepatic iron staining results: IL6, rs2069849, rs1800795, rs10499563; IL1β, rs1143627, rs16944, rs1143634. Chi2 and ordinal regression was used to determine the association of each genotype with nominal and ordinal variables. Stepwise forward multivariate logistic regression was used to examine the relationship between genotypes and presence of advanced fibrosis or NASH diagnosis after controlling for the following variables selected a priori: age at biopsy, sex, presence of diabetes, BMI and ALT.
Results: The IL1β +3953 C>T (rs1143634, minor allele frequency (MAF)= 22%) TT genotype was associated with the presence of NASH (74 vs 59%, $p=0.05$), grade 2 ballooning (62 vs 41%, $p=0.012$) and advanced stage 3-4 fibrosis (54 vs 24%, $p<0.001$) and was also independently associated with the presence of advanced fibrosis after controlling for age, sex, diabetes, BMI and ALT. Conversely, the IL1β +3953 CC genotype was associated with a decreased prevalence of NASH (56 vs 65%, $p=0.013$) and advanced lobular inflammation (46 vs 54%, $p=0.03$), lower NAFLD Activity Score (NAS) (OR= 0.71, CI=0.55-0.92, $p=0.009$), increased hepatocellular (HC) iron staining (27 vs 19%, $p=0.01$) and higher HC iron grade (OR= 1.55, CI=1.09-2.19, $p=0.015$). The IL1β +3953 CT genotype was associated with a higher NAS (OR= 1.30, CI=1.00-1.69, $p=0.046$) and lobular inflammation score (OR= 1.39, CI=1.04-1.83, $p=0.027$) but decreased prevalence of HC iron (19 vs 26%, $p=0.02$) and lower HC iron grade (OR= 0.67, CI=0.47-0.97, $p=0.032$).
Conclusions: The IL1β +3953 coding region synonymous polymorphism may have significant implications in determining NASH susceptibility; CC genotypes were associated with histologically milder features, while the CT and TT genotypes were associated with more severe disease including advanced fibrosis in subjects with the TT genotype. Further studies are necessary to elucidate the pathogenic mechanism of this polymorphism.

Poster #339

FERRYL HEMOGLOBIN IS A PROINFLAMMATORY AGONIST THAT ACTIVATES ENDOTHELIAL CELLS AND INDUCES MOUSE PERITONITIS

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(Presented By: Viktória Jeney)

Introduction: Several pathologic conditions are associated with hemolysis, i.e. release of ferrous (Fe2+) hemoglobin (Hb) from red blood cells. Oxidation of Hb produces metHb (Fe3+), ferrylHb (Fe4+) and oxoferryl Hb (Fe4+=O) species. Ferryl- and oxoferryl-species are unstable and return to the Fe3+ state by reacting with specific amino acids of the globin chains. In these

reactions globin radicals are produced followed by termination reactions leading to the formation Hb dimers, trimers, tetramers and multimers, referred here as ferrylHb. Products of Hb oxidation including ferrylHb can be found in human blood under physiologic as well as pathological conditions. Although ferrylHb is thought to act in a pro-oxidant and cytotoxic manner its pathophysiological effects remain poorly understood. We reasoned that because of the physical location at the interface between blood and tissues, vascular endothelial cells (EC) might be the main cellular target for putative pathophysiological effects of ferrylHb.
Methods and Materials: Hb solutions at different redox states (Hb, metHb, ferrylHb) were prepared from human erythrocytes. Purity and endotoxin content of the Hb preparations were controlled. Human umbilical vein EC (HUVEC) were used to examine the pathophysiological effects of Hb preparations. Morphological changes in HUVEC monolayers were detected by time-lapse imaging microscopy and immunocytochemistry. EC permeability was assessed using hanging cell culture inserts. Quantitative real time PCR was used to measure mRNA levels of proinflammatory genes i.e. *intercellular adhesion molecule 1* (*Icam-1*), *vascular cell adhesion molecule 1* (*Vcam-1*) and *E-selectin* in EC and in mouse liver. Western blot and cellular ELISA were used to assess expressions of ICAM-1, VCAM-1 and E-selectin in EC. C57BL/6 mice were injected intraperitoneally with Hb solutions at a dose of 2 mg/kg. Peritoneal leukocytes were harvested by peritoneal lavage and cells were identified by flow cytometry.
Results: When exposed in vitro to ferrylHb EC rearrange their actin cytoskeleton and form F-actin stress fibers. This is associated with the formation of intercellular gaps and increased EC permeability. Neither Hb nor metHb led to the formation of F-actin stress fibers or the formation of intercellular gaps. When exposed in vitro to FerrylHb, EC up-regulate the expression of several proinflammatory proteins, i.e. ICAM-1, VCAM-1 and E-selectin. This is accompanied by increased monocyte adhesion. Neither Hb nor metHb induced the expression of these adhesion molecules or increased monocyte adhesion. Upon exposure to ferrylHb, EC up-regulated the expression of mRNAs encoding *Vcam-1*, *Icam-1* and *E-selectin*. When administered to naïve mice intravenously ferrylHb induced the expression of mRNAs encoding *Vcam-1*, *Icam-1* and *E-selectin* in liver. When administered into the peritoneal cavity of naïve C57BL/6 mice, ferrylHb induced a robust inflammatory response revealed by an 19-fold increase in the number of polimorphonuclear cells. This effect was not observed using equimolar amounts of Hb or metHb.
Conclusion: In conclusion, we demonstrate that ferrylHb acts as a proinflammatory agonist in EC, suggesting that oxidation of cell-free Hb and in particular production of ferrylHb might be an important component of the pathogenesis of inflammatory conditions associated with hemolysis and EC activation such as is the case for thrombotic microangiopathies.
Acknowledgement: TÁMOP-4.2.2.A-11/1/KONV-2012-0045.

Poster #340
DOES INSULIN AFFECT EXPRESSION OF PROTEINS INVOLVED IN IRON HOMEOSTASIS? PRELIMINARY RESULTS FROM HEPG2 CELLS AND PRIMARY MOUSE HEPATOCYTES
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(Presented By: Joe Varghese)

Introduction: Mild to moderate iron overload is known to be associated with insulin resistance in type 2 diabetes mellitus, metabolic syndrome and non-alcoholic steatohepatitis. However, the effect of insulin on the expression of hepcidin and other proteins involved in regulation of iron homeostasis in the body is not known. Here we present preliminary results from experiments done on HepG2 cells, a human hepatoma cell line, and primary mouse hepatocytes.
Methods and Materials: HepG2 cells were treated with various concentration of insulin and the expression of genes involved in iron homeostasis was measured by quantitative real-time polymerase chain reaction (qRT-PCR). HepG2 cells transfected with the full-length (2.7 kbp) hepcidin promoter luciferase reporter plasmid and primary mouse hepatocytes isolated from C57BL/6 mice by collagenase perfusion were incubated with varying concentration of insulin. Hepcidin reporter assays were performed and expression of iron-related genes was quantified by qRT-PCR.
Results: There was a significant decrease in hepcidin (*Hamp*) expression in HepG2 cells treated with 10nM insulin. Hepcidin reporter assays also showed a similar trend although results were not statistically significant. Insulin caused a dose-dependent increase in transferrin receptor 1 (*TfR1*) expression in HepG2 cells indicating some changes in iron status after insulin exposure. As previously reported (Lin et al 2007), we found that *Hamp1* expression decreased in primary mouse hepatocytes with time in culture. Although low concentrations of insulin (10nM) tended to increase *Hamp1* expression, this was not statistically significant. In contrast to HepG2 cells, *TfR1* expression tended to be lower in the presence of insulin in primary hepatocytes.
Conclusion: The preliminary results obtained from this study indicate that insulin may affect various proteins associated with iron homeostasis. Further work is required to elucidate whether such effects are implicated in the pathogenesis of iron overload associated with insulin resistance.

Poster Abstracts

Poster #341
MTORC1-MEDIATED PHOSPHORYLATION STABILIZES ISCU PROTEIN: IMPLICATIONS FOR IRON METABOLISM
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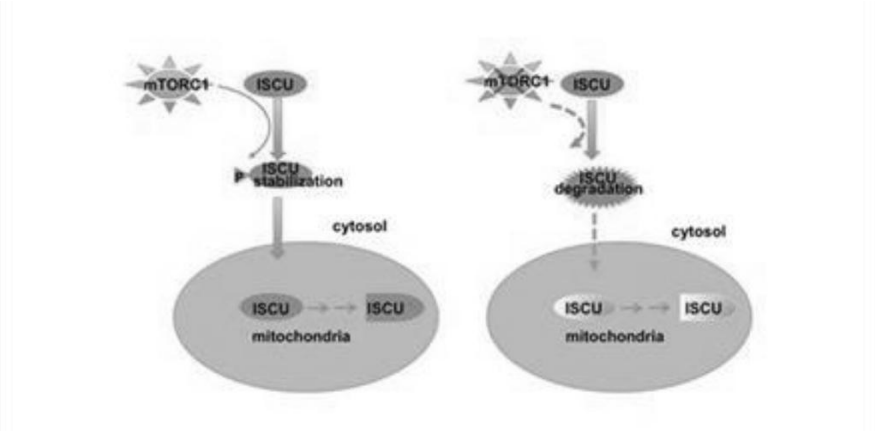
(Presented By: Ping La)

Introduction: The scaffold protein ISCU facilitates the assembly of iron-sulfur clusters (ISCs), which are essential cofactors for many vital metabolic processes. However, it remains largely unknown how ISCs assembly, in particular, how ISCU gene expression and function is regulated by metabolic status. The mammalian target of rapamycin, mTOR, a serine-threonine kinase, plays a central role in nutrient-sensing networks. mTOR can form two distinct complexes with other protein partners, mTORC1 (mTOR complex1) and mTORC2 (mTOR complex 2). The former, mTORC1, is sensitive to rapamycin and responds to amino acids, growth factors or ADP/ATP levels via tuberous sclerosis complex (TSC1/TSC2). Upon nutrient starvation, TSC1/TSC2 inhibits mTORC1 function for survival. However, with nutrient abundance, TSC1/TSC2 complex loses this inhibitory effect; therefore, mTORC1 stimulates cell growth and proliferation, particularly by increasing the biosynthesis including protein translation and lipogenesis.
Method: Site–directed mutagenesis was used to mutate serine to alanine in ISCU. Immunoprecipitation and in vitro kinase assays were applied to testifying mTORC1 phosphorylation of ISCU. Taqman RT-PCR, Western blot and aconitases activity assays were performed to analyze the mRNA and protein levels of ISCU as well as its function respectively. Human embryonic kidney cell line 293T, human cervical cancer cell line HeLa, normal murine lung cell line MLg and murine preadipocyte 3T3-L1 were applied. Due to the inhibitory effect of TSC2 on mTORC1 activity, TSC2-null and wild type mouse embryonic fibroblasts (MEFs), corresponding constitutive and restrained mTORC1 activation, were also used.
Results: We demonstrate that mTORC1 associates with ISCU protein by showing the physical protein interaction of ISCU with mTOR as well as Raptor, a specific subunit of mTORC1. Furthermore, the in vitro kinase assay demonstrated that mTOR phosphorylated ISCU. Compared to varieties of ISCU mutates, the phosphorylation of ISCU by mTORC1 specifically locates at S14 but not other potential phosphorylation sites. Furthermore, the S14 phosphorylation stabilizes ISCU protein in the cytosol and ultimately increases its abundance in the mitochondria and function in ISCs assembly. By applying rapamycin, we demonstrated that insufficiency of ISCU triggered by mTORC1 inhibition prevented ISCs assembly. In contrast, constitutive mTORC1 activation in TSC2-null MEFs enhanced ISCU protein level and ISCs assembly. This further sensitized TSC2-null cells to iron deprivation, due to ISCs biogenesis-triggered iron demand outstripping supply.
Conclusion and Speculation: Responding to metabolic cues, mTORC1 regulates ISCU levels and function, and hence ISCs assembly. Therefore, ISCs assembly is also a part of mTORC1-modulated biosynthesis network which is often dysregulated in cancer. We speculate that it may benefit patients by applying iron deprivation to cancers in the context of mTORC1 activation. As such, Iron deprivation may be more effective in killing the cancer cells where the genetic mutation yields high mTORC1 activities.



Poster #342

CHANGES IN GENE EXPRESSION OF CHOLESTEROL METABOLISM PATHWAYS IN MOUSE MODELS OF HAEMOCHROMATOSIS

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(Presented By: Ross Graham)

Introduction: The liver is central to the metabolism of both iron and cholesterol. Cholesterol is synthesised and further metabolised to bile acids in the liver and the liver plays an important role in regulation of iron metabolism. It is also the organ in which excess iron is stored. Clinically, links have been noted between lipid and iron metabolism, with approximately one-third of patients with non-alcoholic fatty liver disease exhibiting altered iron parameters. On a molecular level, we have previously reported that wild-type mice fed iron-deficient, normal or iron-loaded diets exhibited increased hepatic cholesterol and increased hepatic gene expression of enzymes in the cholesterol biosynthesis pathway with increasing hepatic iron burden. In the genetic disorder, haemochromatosis, the liver can become overloaded with iron; however, clinical studies have suggested that lipid metabolism may not be perturbed in haemochromatosis.

Methods and Materials: We investigated hepatic cholesterol metabolism in three mouse models of hereditary haemochromatosis: $Hfe^{-/-}$, $Tfr2^{Y245X}$ single mutant and $Hfe^{-/-}$ x $Tfr2^{Y245X}$ double mutant animals as well as wild-type controls. Mice were fed normal mouse chow and sacrificed at 10 weeks of age. Hepatic gene expression, total cholesterol and non-haem iron were measured. Liver non-haem iron was similar in $Hfe^{-/-}$ and $Tfr2^{Y245X}$ mice (16.6±0.8 and 17±1 $\mu mol_{Fe}/g_{liver}$, respectively) and significantly higher in the double mutant animals (22.4±0.7 $\mu mol_{Fe}/g_{liver}$; $P<0.004$) than either of the single mutant mice.

Results: Only one group of genes increased significantly with increasing hepatic iron: those involved in cholesterol transport. Gene expression of apolipoproteins A4, C1, C2, C3 and E increased significantly with increasing hepatic iron as did expression of VLDL receptor. In contrast to our findings in wild-type mice, gene expression of cholesterol biosynthetic enzymes did not increase significantly with liver iron burden and there were no differences in hepatic cholesterol between the groups of mutant mice. We also measured expression of genes involved in cholesterol regulation, which similarly, did not increase with increasing hepatic iron. Approximately 50% of cholesterol synthesised in the liver is directed to bile acid synthesis; however, gene expression of bile acid pathway enzymes did not change with respect to hepatic iron burden.

Conclusion: These results suggest that iron-associated cholesterol regulation may be ameliorated by the genetic changes which occur in haemochromatosis.

Poster #343

PROTECTION OF THE DIABETIC HEART: CHANGES IN THE HOMEOSTASIS OF IRON EXPLAINS MYOCARDIAL RESPONSE TO STRESS

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(Presented By: Vladimir Vinokur)

Background: Epidemiological studies have shown that diabetes patients experience increased risk for cardiovascular diseases and acute myocardial infarction (AMI). The clinical outcome of AMI in diabetics is worse than non-diabetics. However, the survival rate of diabetic patients after AMI is higher than that of normoglycemic subjects. Moreover, there are conflicting reports on whether the diabetic heart responds to ischemic preconditioning (IPC). An association between diabetes and tissue Fe overload was reported as well. Based on recently proposed roles for iron and ferritin in IPC, this study focused on the changes in Fe homeostasis in the diabetic heart.

Materials and Methods: Two models of the diabetic rat were employed: STZ-treated (type I-like) and Cohen spontaneous diabetic (type II) rats. Hearts from STZtreated animals and their matched controls were subjected ex-vivo to global ischemia and reperfusion, with or without IPC. The perfusion medium contained also a mixture of proteases inhibitors. Ferritin level (mRNAs and protein), ferritin saturation with Fe, and ferritin composition were monitored along the experiments. These parameters (function and biochemistry) were also studied on hearts from the Cohen diabetic animals.

Results: A significant improvement of function was observed in preconditioned control hearts, as expected. In contrast, no functional improvement could be observed in the diabetic hearts when subjected to IPC+I/R, as compared with the I/R protocol. Interestingly, the post-ischemic recovery of diabetic hearts (I/R protocol) was better than the non-diabetic controls under the same protocol. The basal ferritin level in diabetic hearts was 2-fold higher than in non-diabetics. In control hearts IPC caused a 4.2-fold increase in ferritin level, which was conserved during the subsequent prolonged ischemia. During the

reperfusion the level of ferritin dropped back to the basal level. In diabetic hearts subjected to IPC, a 2.1-fold increase over the basal level was obtained, reaching a level similar to that of the controls (after IPC). Consistently with the expectations, ferritin in the diabetic hearts had higher content of Fe. Also, the subunit-composition of the diabetic ferritin has been changed in favor of L-subunit, which is less common (than the H-subunit) in the heart. During the subsequent prolonged ischemia in diabetic hearts, extensive degradation of ferritin, to a level below the baseline occurred. On the other hand, treatment of diabetic hearts with a cocktail of proteases inhibitors led to reduced ferritin degradation, restoring the IPC response. The results of experiments performed on Cohen diabetic rats' hearts were fully consistent with those on STZ-induced diabetic rats.
Conclusions: In diabetic animal of both types, the poor response of the diabetic heart to IPC is associated with alterations in Fe homeostasis, including changes in myocardial ferritin on transcriptional and translational levels.

Poster #344
MITOCHONDRIAL FERRITIN- NULL MICE ARE MORE SENSITIVE TO CARDIOTOXICITY
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(Presented By: Michela Asperti)

Introduction: Mitochondrial ferritin (FtMt) precursor is encoded by an intronless gene targeted to mitochondria, where it is processed in the mature form that has high homology to ferritin H-chain, and retains ferroxidase activity. FtMt expression in various cell lines showed that it accumulates iron efficiently and competes with the cytosolic ferritins for cellular iron sequestration. FtMt was also shown to protect cells from oxidative damage and to modulate cell proliferation, in agreement with a role to protect mitochondria from iron-induced damage. In mice and human FtMt expression is limited to the testis, the heart, neurons and few other cell types characterized by high level of oxidative activity. Although FtMt accumulates in the iron loaded mitochondria of erythroblats from patients with sideroblastic anemia, it biological role has not been established.
Methods and Results: To this aim we produced a novel strain of mice in which the FtMt coding region is substituted with a cassette coding Lac-z as reporter gene. The FtMt+/- and FtMt-/- are viable and apparently healthy with no obvious phenotype. Also the male normal fertility is not evidently affected by the absence of FtMt. In order to verify if FtMt has a protective role in the heart, male mice of 16 weeks were subjected to acute treatments with doxorubicin (15mg/kg, IP). The mortality of FtMt-/- mice was much higher than that of the control ones. The characterization of the damaged hearts is in progress.
Conclusion: In another approach to verify the role of FtMt neurons, the mice were treated with neurotoxic compounds such as Paraquat and Maneb. In conclusion, FtMt seems to have a role in the protection of heart against oxidative insults.

Poster #345
IMMUNE MEDIATED DYSREGULATION OF CELLULAR IRON TRANSPORT PROTEINS AS A POTENTIAL BASIS OF DEFECTIVE IRON HOMEOSTASIS IN CHRONIC HEART FAILURE
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Imperial College London
(Presented By: Karl Norrington)

Introduction: Defective iron homeostasis, characterised by a diminished circulating and functional iron status in the face of seemingly preserved stores, is a major cause of anemia in patients with chronic heart failure (CHF) but its origins are unknown. We hypothesized that disordered iron homeostasis in CHF may be due to the adverse modulation of cellular iron export (ferroportin) and import (divalent metal transporter [DMT] -1) proteins by inflammatory mediators such as hepcidin and tumor necrosis factor (TNF) α.
Methods and Materials: We studied 45 CHF patients (age 68±10 years, 75% New York Heart Association class 3, 78% male) and 13 healthy controls (66±11 years, 77% male). Serum prohepcidin and cytokine levels were measured by ELISA. Healthy peripheral blood mononuclear cells were incubated with or without CHF serum ± anti-TNFα neutralizing antibody. Monocyte ferroportin and DMT-1 expression were quantified by flow cytometry.
Results: Compared to controls and CHF patients with normal circulating iron (transferrin saturation [TSAT] >20%; n=29), CHF patients with reduced circulating iron (TSAT ≤20%; n=16) had lower haemoglobin (P<0.0001) and higher pro-hepcidin (controls: 120±45 mg/mL; TSAT >20%: 132±74 mg/mL; TSAT ≤20%: 192±122 mg/mL; P=0.046), TNFα (P=0.17), soluble TNF receptor-1 (P=0.048), IL-6 (P=0.008) and IL-1β (P=0.09) levels. Ferritin levels did not differ (P=0.29). Sera from patients with TSAT ≤20% (n=8) induced a greater reduction in ferroportin (-2±18% vs. -29±20%, P=0.03) and a greater escalation in DMT-1 (-4±22% vs. 23±48%, P=0.046) expression from baseline than sera from patients with TSAT >20% (n=6). Changes in ferroportin related only to log TNFα (r = - 0.72, P=0.004), log pro-hepcidin (r = - 0.58, P=0.03), and TSAT (r = 0.64, P=0.01). Anti-TNFα antibody diminished the effects of sera from patients with TSAT ≤20% on ferroportin and DMT-1 expression (all P<0.05).
Conclusion: Defective iron homeostasis in CHF is associated with downregulated ferroportin and upregulated DMT-1 expression, a pattern that facilitates monocyte iron retention in vivo. Hepcidin levels relate to such aberrant patterns and TNF-α directly dysregulates monocyte iron transporters in CHF. Unrestricted monocyte iron sequestration may hamper the full utility of iron supplements in this cohort and may be reversed by the targeted ablation of enhanced immune activation.

Poster #346

TELOMERE LENGTH AND IRON OVERLOAD: THE INFLUENCE OF PHENOTYPE AND GENOTYPE

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(Presented By: Gordon D. McLaren)

Introduction: *HFE*–related hereditary hemochromatosis (HH) occurs most often in individuals who are homozygous for the C282Y polymorphism of the *HFE* gene, although some patients are compound heterozygotes, having one copy of C282Y and one copy of the common H63D polymorphism. The excess iron deposition in HH is associated with biomolecular oxidative damage and may lead to age−related conditions. Telomere length has emerged as a marker for biological aging. Shortened telomere length has been associated with shorter life span as well as a variety of diseases related to aging. However, not everyone with an HH−related *HFE* genotype develops iron overload, and not everyone with iron overload has HH. In the current study, we examined the relationship between *HFE* gene mutations, elevated iron levels and telomere length.
Methods: The Hemochromatosis and Iron Overload Screening (HEIRS) Study was designed to evaluate a multicenter, multiethnic sample of 100,000 primary care adults 25 years of age or older. Participants with elevated values of transferrin saturation (TfS) and serum ferritin (SF), and/or homozygosity for *HFE* C282Y, were invited to undergo a comprehensive clinical examination (CCE), as were frequency−matched control subjects. We classified CCE participants by genotype and phenotype as G+ (*HFE* C282Y homozygotes, H63D homozygotes, or compound heterozygotes), G− (homozygous wild type for *HFE* C282Y and H63D), P+ (TfS > 45% and SF > 300 ng/mL in men or SF > 200 ng/mL in women and P− (TfS < 45% and SF < 300 ng/mL in men or SF < 200 ng/mL in women). We defined genotype−phenotype groups as G+P+, G−P+, G+P−, G−P−. Specimens and clinical data were collected from randomly selected participants in the G+P+, G−P+, G−P− groups and all available participants from the small G+P− group. Leukocyte telomere length was measured by quantitative PCR. Values derived for telomere length (T) were normalized for each sample with the corresponding expression of 36b4 gene (S) as T/S ratio. Categorical variables represented gender, age, self−reported Caucasian or non−Caucasian race/ethnicity, health insurance status, education, and telomere length ("short," defined as ≤ 25th percentile, vs. "long," defined as > 75th percentile). Logistic regression was applied to estimate the odds of short telomere length in each genotype−phenotype group, controlling for significant covariates.
Results: The final analytical sample consisted of 1,009 subjects; 257 had short telomeres, and 248 had long telomeres. Genotype−phenotype groups differed significantly by gender, race/ethnicity, and health insurance (p<0.001 for all). Genotype−positive groups were more likely to be Caucasian than genotype−negative groups. Short telomeres were associated with elevated iron phenotype (p<0.001) but not HH genotype (p=0.67). Compared to the G−P− reference group, the adjusted odds of a shortened telomere length in the G+P+ and G−P+ groups was 2.18 (95% confidence interval, 1.22−3.91) and 2.21 (1.49−3.27) times that of the longer telomere group, respectively.
Conclusion: Elevated TfS and SF levels were associated with shorter telomere length in this population. In this first study to examine telomere length in patients with HH or elevated iron test results, the results were independent of *HFE* gene mutations, age, gender, and race−ethnicity. The results suggest that, both among patients with HH-associated HFE genotypes and persons without *HFE* polymorphisms, shorter telomere length is more likely to occur in those with higher iron test results.

Poster #347

TOTAL MORTALITY BY ELEVATED FERRITIN CONCENTRATION - A POPULATION-BASED STUDY

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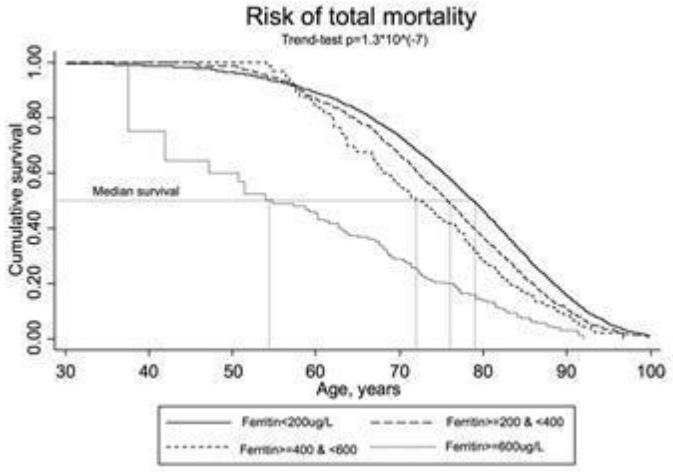
(Presented By: Christina Ellervik)

Introduction: There is evidence for increased mortality in population-based studies in individuals with increased transferrin saturation (TS), as a proxy for iron overload, in a dosedependent manner. Previous population-based studies on risk of total mortality by elevated ferritin concentration have focused on ferritin in either quartiles or tertiles and have not found any association; however, their highest percentiles have included the reference range of ferritin and thus, we speculate if more extreme values of ferritin concentration increases risk of total mortality.
Methods: We examined total mortality according to baseline ferritin concentration in a Danish population-based follow-up study (the Copenhagen City Heart Study) comprising a total of 9005 individuals of which 6014 died during a median follow-up of 20 years (interquartile range 12-28 years).
Results: Cumulative survival was reduced in those with ferritin≥200µg/L versus <200µg/L with a multifactorially adjusted hazard ratio of 1.1(95% CI: 1.1-1.2); p=0.0001) overall, 1.1(1.0-1.2; p=0.009) in men, and 1.2(1.1-1.4; p=0.008) in women. A stepwise increased risk of total mortality was observed for stepwise increasing levels of ferritin concentration (p-trend=1.3*10^(-7)) (figure). The highest risk was conferred by ferritin≥600µg/L vs. ferritin<200µg/L with a hazard ratio of 1.6(1.3-1.9; p=0.00001) and a median survival of 54 years - 25 years earlier than individuals with ferritin below 200 µg/L; results were similar in men and women. In a meta-analysis, odds ratio for total mortality for ferritin upper xtile (quartile or tertile) vs. reference xtile (quartile or tertile) was 1.0(0.9-1.1; p=0.3; p (heterogeneity, Q-statistic)=0.003) under the random effects model.

Discussion: We showed that in a Danish population-based follow-up study, individuals with the threshold level of ferritin≥200µg/L versus <200µg/L have an increased risk of premature death overall and in men and women separately. Moreover, a stepwise increased risk of total mortality was observed for stepwise increasing levels of ferritin, with the highest risk conferred for ferritin≥600µg/L overall and in men and women separately. This study is the largest and most comprehensive study to date estimating risk of total mortality by extreme values of ferritin. Our study confirmed previous findings that increased transferrin saturation is associated with increased total mortality. Furthermore, these data underline the hidden risk of increased ferritin concentration that is concealed in the analyses of total mortality by quartiles or tertiles.
Conclusions: Individuals in the general population with ferritin≥200 µg/L versus <200µg/L have an increased risk of premature death.



Poster #348
THE SOLUTION STRUCTURE, BINDING PROPERTIES, AND DYNAMICS OF THE 34 KDA BACTERIAL SIDEROPHORE BINDING PROTEIN FEPB
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(Presented By: H.J. Vogel)

Introduction: The periplasmic binding protein (PBP) FepB plays an integral role in transporting the catecholate siderophore ferric-enterobactin from the outer to the inner membrane in Gram-negative bacteria.
Methods and Materials: The solution structures of the 34 kDa apo- and holo-FepB were solved by NMR starting from a homology model and refined using dihedral angle, NOE and RDC restraints.
Results: These are the first solution structures for the type III class of PBPs and comparison of the two structures reveals that both forms of FepB maintain similar overall folds. However, binding of the ligand gallium-enterobactin (GaEnt) is accompanied by movement of loop regions that line the ligand binding pocket. As well, large backbone and methyl chemical shift perturbations (CSPs) are observed in regions surrounding the ligand binding cleft and the inter-domain α-helix, including purported ligand binding residues. Reverse methyl cross-saturation experiments corroborate the CSP results and define the GaEnt binding pocket. NMR relaxation experiments indicate that the latter portion of a flexible loop (residues 227 – 242) adopts a more rigid and extended conformation upon ligand binding that helps position residues for interaction with the ligand.
Conclusion: This solution NMR study highlights the critical role that localized structural dynamics have in ligand binding by type III PBPs, which differs from the canonical “Venus-flytrap” motions seen in related type I and II PBPs.

Poster #349
KNOCK-DOWN OF THE MENKES COPPER-TRANSPORTING ATPASE (ATP7A) IN RAT INTESTINAL EPITHELIAL (IEC-6) CELLS INCREASES FERROPORTIN 1 (FPN1) EXPRESSION AND ENHANCES IRON TRANSPORT
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Introduction: Iron homeostasis is determined principally by intestinal transport as no active excretory mechanisms exist. Recent studies demonstrated that copper content and expression of a copper transporter (the Menke’s Copper-Transporting ATPase; Atp7a) increase in duodenal enterocytes during iron deprivation. Hepatic copper content also increases in many mammalian species during iron deprivation. Atp7a delivers copper to the *trans*-Golgi for cuproenzyme synthesis and exports copper from enterocytes; it is thus uniquely positioned to potentially influence most aspects of copper homeostasis. Hephaestin (Heph), also expressed in enterocytes, is a multi-copper ferroxidase which oxidizes iron exported by ferrorportin

(Fpn1) to promote binding to transferring in the interstitial fluid. Heph represents the best characterized link between intestinal iron and copper homeostasis. Whether the copper transport function of Atp7a is necessary for Heph biosynthesis was tested in this study.
Methods: Studies were performed in IEC-6 cells in which Atp7a expression was silenced (>90%) by siRNA (stable shRNA transfection). qRT-PCR, western blotting and iron transport studies were performed by standard methods. **Results**: Metallothionein mRNA expression was induced in Atp7a knockdown (KD) cells >8-fold), likely reflecting increased copper content. Moreover, Heph mRNA expression was reduced ~80% in the KD cells, and ferroxidase activity (measured by a spectrophotometric transferrin assay) in membrane and cytosolic fractions was reduced 50 and 35%, respectively (p<0.001). Importantly, 59Fe transport in the KD cells was unexpectedly increased (as compared to negative siRNA-transfected cells), which coincided with increased Fpn1 mRNA expression (~3.5-fold).
Discussion: This investigation suggests that: 1) Atp7a function influences the expression and activity of Heph, 2) Atp7a KD increases Fpn1 expression and activity in intestinal epithelial cells, and 3) Heph is not essential for iron transport in this model and another FOX exists. These data thus support our previous report of a cytosolic ferroxidase in rodent enterocytes that may complement Heph activity (Ranganathan et al., *Proc. Nat. Acad. Sci. USA*, 2011).
Conclusion: Overall, the experiments presented here provide further mechanistic details regarding the influence of copper on iron homeostasis. Supported by NIH Grant R01-DK074867 (JFC).

Poster #350
EFFECTS OF CHRONIC LEAD EXPOSURE ON THE BRAIN IRON HOMEOSTASIS AND IRON TRANSPORTER EXPRESSIONS IN RATS
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(Presented By: Jayong Chung)

Introduction: Lead and iron metabolisms are closely inter-related. The aim of this study was to examine the effects of chronic Pb exposure during post-weaning period on the iron metabolism in the brain.
Methods and Materials: Sprague-Dawley rats (4 week-old) were provided either 0% (CON) or 0.25% Pb acetate (CP) in drinking water for 4 weeks. Fe and Pb concentrations in different subregions (cortex, cerebellum, hippocampus, striatum) of brain tissues were determined by ICP-AES and ICP-MS, respectively. The protein levels of ferroportin 1(FPN1), divalent metal transporter 1(DMT1), transferin receptor (TfR), and ferritin in the cerebellum were measured by Western blot analyses.
Results: Results showed that chronic Pb exposure significantly increased the levels of Pb in all subregions of the brain that were examined, but the Pb treatment did not change Fe concentrations in any of the brain subregions. On the other hand, Pb exposure significantly increased the levels of FPN1 and DMT1 in the cerebellum. The levels of TfR and ferritin in the cerebellum, however, were not significantly different between the CON and the CP groups. We also examined the effects of Pb on the level of FPN1 expression in SH-SY5Y cells *in vitro*, and found that the Pb treatment induced FPN1 expression in a dose- and time-dependent manner.
Conclusion: Our study results demonstrate that Pb exposure can up-regulate iron transporter expressions in the brain and in the neuronal cells, independent of changes in the iron concentrations.
(This work was supported by the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology, 2012-0004932).

Poster #351
ALTERED IRON METABOLISM EFFECTS AND ANTI-LEUKEMIA ACTIVITY OF A-TYPE PROANTHOCYANIDINS FROM VACCINIUM MACROCARPON (CRANBERRY)
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(Presented By: Laura Bystrom)

Background: Iron is crucial to normal cell metabolism and plays a role in multiple cellular activities. However, iron is also important for maintenance of malignancy, such as for oxidative phosphorylation and nucleotide synthesis. Several studies have indicated that iron chelators have anti−proliferative and anti−tumor effects against several tumor cell lines. Moreover, research has indicated that certain types of iron chelators are able to induce reactive oxygen species (ROS) and thereby selectively target cancer cells. Leukemia cells may be especially vulnerable to iron depletion and elevated ROS level effects. To this end, we investigated the anti-leukemia activity of known natural products with iron chelation activity, and found that A-type proanthocyanidins (A-PACs) from cranberry extracts to be efficacious. Many of the reported health benefits of cranberries, including their anti-tumor and anti-microbial effects, are associated with A-PACs. This information prompted us to investigate the effects of A-PACs on iron metabolism and leukemia cell survival.
Methods: Isolation techniques were developed to obtain A-PAC fractions from cranberry cultivars of *Vaccinium macrocarpon* Ait., or from the cranberry powder CystiCran-40 (CYS), which contains high amounts of A-PACs. Human leukemia cells lines were treated with CYS, individual A-PACs (e.g., dimers, trimers, tetramers), or fractions consisting of a mixture of A−PACs. Furthermore, cell lines were treated with A-PACs and compared with cell lines pre-treated with an antioxidant or holo-

transferrin before treatment with A-PACs. Moreover, A-PACs were assessed by qPCR for gene expression effects on iron metabolism genes or antioxidant genes. Flow cytometry (FACS) was also used to assess A-PACs for their effects on the transferrin receptor 1 (CD71), ROS, cell death (7-AAD) or apoptosis (annexin V).
Results: Pre-treatment with antioxidants or holo-transferrin partially protected leukemia cells from A-PAC induced cell death ($p<0.01$). Additionally, qPCR results indicated A-PAC treatment induced changes in cellular iron metabolism genes (transferrin receptor 1, ferroportin) and genes associated with antioxidant effects (nuclear factor erythroid 2-related factor-2, glutamate-cysteine ligase regulatory subunit, and quinone oxidoreductase) in K562 and Kg-1 leukemia cell lines. These results were consistent with iron chelation effects and indicated sensitivity to A-PACs may be due to a reduction in antioxidant-relevant gene expression. A-PAC fractions from CYS were found to be more effective than individual A-PAC dimers, trimers or tetramers in K562 cell lines. Interestingly, basal ROS levels were higher in K562 leukemia cell lines compared to Kg-1 leukemia cell lines.
Discussion/Conclusions: Results of this study suggest the anti−leukemia activity of A−PACs are not exclusively associated to iron depletion effects but may also involve the production of toxic ROS. A-PACs are redox reactive and therefore when bound to iron may induce ROS as well as sequester iron away from the cell. Moreover, the size and structure of A-PACs may play a role in their anti-leukemia activity. Exploring the iron metabolism and ROS effects of A-PACs may provide new insight into the mechanistic effects of these compounds and potentially identify new vulnerabilities of leukemia cells. Future studies will assess A-PACs alone or in combination with other therapeutic compounds in vitro and in vivo in order to identify optimal and novel therapies for leukemia.

Poster #352
INTERPLAY BETWEEN INNATE IMMUNITY AND IRON HOMEOSTASIS
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(Presented By: Catherine Mura)

Objective: Iron homeostasis in mammalian plays a critical role in mammalian metabolism, but it is also involved in the defense mechanisms against environmental microbes. Hepcidin peptide which acts as an hormone to regulate iron homeostasis by binding to the ferroportin iron cell exporter is induced by both iron and by exposure to pathogen-associated molecules. Hepcidin is thus part of the innate immunity mechanism in restricting the availability of iron to microbial invaders and may affect microbe infection susceptibility. More detailed studies are needed to establish the relationship between iron homeostasis and innate immunity. Here, we analyzed inflammatory response in mice under various iron conditions.
Materials and methods: Wild type and genetically deficient mice for signaling pathways of inflammation, submitted to iron overload or deficient diet were analysed for iron genes and inflammatory responses after LPS induced inflammation. Real-time PCR on hepatic RNAs as well as ELISA were used to measure the expression of hepcidin and several cytokines (IL-6, TNF□, IFN□/□, ..).
Results: The absence of functional TLR4, as well MyD88 transduction pathway, abolishes the hepcidin response to LPS. The absence of functional IL1R1 modifies the hepcidin response to LPS. Some modifications of the inflammatory response were observed in mice either fed with iron enriched diet or iron deficient diet.
Conclusion: The results revealed complex interactions between iron homeostasis and inflammatory response.

Poster #353
DEVELOPMENT AND EARLY VALIDATION OF A NEW COMPETITIVE ELISA HIGHLY SPECIFIC FOR MURINE HEPCIDIN-1 IN SERUM AND URINE
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(Presented By: Mark Westerman)

Background: Studies involving hepcidin in murine models are limited by reliance on liver hepcidin expression analysis. The absence of data on circulating hepcidin-1 concentrations and the inability to sample individuals longitudinally significantly limits basic and pre-clinical studies. Additionally, there is a clear effect of blood sampling itself to access iron markers on hepcidin concentrations and experimental bias can result. To address these issues, we developed a robust C-ELISA that is specific for hepcidin-1 in serum and urine which will allow non-invasive, longitudinal sampling of individual mice in basic and pre-clinical research of novel therapeutics.
Methods: We produced polyclonal antibodies to synthetic murine hepcidin-1 conjugated to a carrier using a modified immunization strategy. Serum from one rabbit (ILS114) was affinity purified by Protein A chromatography. We synthesized and purified a biotinylated murine hepcidin-1 competitor peptide and evaluated purity and antibody binding to both reagents on Coomasie stained non-denaturing Tricine gels and their Western blots probed with ILS114 or streptavidin-HRP. We optimized hepcidin-1 antibody and competitor concentrations, competition buffer, and performed validation studies to assess accuracy, precision, intra- and inter-assay CVs. We tested murine hepcidin-2, rat hepcidin, and human hepcidin by C-ELISA and measured hepcidin-1 in serum collected from three groups of 5 week old C57BL/6 male and female mice (n=8; n=48 total) fed

low iron (4ppm Fe2SO4), normal iron (300ppm), and high iron diets (30,000ppm) over the previous 4 weeks (3 weeks, high iron diet).

Results: ILS114 murine hepcidin antibodies are highly specific for hepcidin-1 in serum and urine. Western analysis shows excellent binding to hepcidin-1 and biotinylated hepcidin-1 but not human hepcidin-25. Westerns probed with streptavidin-HRP revealed a single band at ~4 kD and predicted biotin content. The current (wet) C-ELISA for murine hepcidin-1 is sensitive (EC50=7.1 ng/ml) requiring only 2-5 µl of serum/urine. LLOD and LLOQ was 2.4 and 7.3 ng/ml respectively, with intra-assay CV=2.7% and inter-assay CV=7.3%. Recovery of hepcidin-1 spiked into 2% serum was ±104.2% (0-2500 ng/ml). No competition was observed when equimolar hepcidin-2 was added to hepcidin-1 standard curves. Standard curves in 2% or 5% human urine were indistinguishable with buffer controls. Mean serum hepcidin-1 in iron deficient (4ppm), iron replete (300ppm), and iron overloaded (30,000ppm) C57BL/6 mice was 8.6±8.4, 188.4±52.9, and 609.9±138.7 ng/ml respectively, and are highly concordant with data presented by Hod et al. 2012 (54th Annual ASH Meetings, Atlanta, GA; abstract #2100). Female mice fed iron replete diets only, had significantly elevated hepcidin-1 in serum compared to males cohorts (226.0 vs. 136.1; P=0.006; Student's *t*-test). Regression of hepcidin-1 protein and liver expression levels measured in individuals show liver expression and circulating hepcidin-1 are highly correlated (R2=0.79; Figure 1A, B).

Discussion and Conclusion: We developed a sensitive robust C-ELISA specific for murine hepcidin-1 that insures minimally or non-invasive longitudinal hepcidin-1 measurements in serum or urine from murine models of disease. Data from prototype kits are highly correlated with wet ILS114 C-ELISA (R2=0.96). These kits show great promise for use in basic and pre-clinical studies in murine models.

Figure 1: Linear regression (Panel A) between serum hepcidin-1 concentrations measured by ILS114 C-ELISA and liver hepcidin expression measured by qPCR in 5 week old male and female cohorts from the 3 groups included in iron diet study (n=46). Panel B shows the same regression depicted on a log-log plot.