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DISSERTATION

Titel der Dissertation

Biochemical aspects to improve treatment of anemia in endstage renal disease and advanced biomarker assessment in iron related disorders

verfasst von

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Your path is hard but

will be amply rewarding. *furtune cookie*

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I. Declaration

"I hereby declare that this dissertation is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this dissertation. Due acknowledgement has been made in the text to all other material used.

Throughout this dissertation and in all related publications I followed the guidelines of Good Scientific Practice published online in July 2012 at the Medical University of Vienna".

II. Publications arising from this thesis

Accepted peer-reviewed publication

- *Praschberger, M., Cornelius, C., Schitegg, M., Goldenberg, H., Scheiber-Mojdehkar, B., & Sturm, B.* (2013).

  Bioavailability and stability of intravenous iron sucrose originator versus generic iron sucrose AZAD. Pharmaceutical Development and Technology. doi:10.3109/10837450.2013.852575

Submitted publications

- *Praschberger, M., Haider, K., Cornelius, C., Schitegg,M., Sturm , B., Goldenberg,H. and Scheiber-Mojdehkar, B***.**

  Iron sucrose and ferric carboxymaltose: No correlation between physicochemical stability and biological activity

- *Eppie M Yiu MBBS[1,2,3], Geneieve Tai BBiomedSci[1], Roger E Peverill PhD[4], Katherine J Lee PhD[1,5], Kevin D Croft PhD[6], Trevor A Mori PhD[6], Barbara Scheiber-Mojdehkar PhD[7], Brigitte Sturm PhD[7], Monika Praschberger MSc[7], Adam P Vogel PhD[1,8], Gary Rance PhD[8], Sarah EM Stephenson BSc[1,3], Joseph P Sarsero PhD[9], Creina Stockley MSc[10], Chung-Yung Lee PhD[11], Andrew Churchyard PhD[12], Marguerite V Evans-Galea PhD[1,3], Monique M Ryan MMed[1,2,3], Paul J Lockhart PhD[1,3], Louise A Corben PhD[1] , Martin B Delatycki PhD[1,3,13].*

  An open-label trial in Friedreich ataxia suggests clinical benefit with high-dose resveratrol

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V. Abbreviations

BSA	Bovine serum albumin
CCD	charge-coupled device
CKD	Chronic kidney disease
CRF	Chronic renal failure
DCF	Dichlorofluorescein
DCFH-DA	2´,7`-dichlorofluorescein diacetate
DCTAF	5-(4,6-dichlorotriazinyl) aminofluorescein
DFO	Desferrioxamin
DMEM	Dulbecco´s minimum essential medium
DMT1	Divalent metal transporter 1
DMSO	Dimethyl sulfoxide
DTPA	N,N-Bis(2-[bis(carboxymethyl)amino]ethyl)glycine
ECLIA	Electrochemiluminescence immunoassay
ECL	Electrochemiluminescence
EDTA	Ethylendiamintetraacetate
ESA	Erythropoiesis stimulating agents
ESRD	End stage renal disease
EPO	Erythropoietin
EPO-R	Erythropoietin receptor
FAS	Ferrous ammonium sulfate
FC	Ferric carboxymaltose
FCS	Fetal calf serum
Fl-Tf	Fluorescein- Transferrin
FRDA	Friedreich´s ataxia
HLB	Hydrophilic-lipophilic balance
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
Hx	Hemopexin
ID	Iron deficiency
IDA	Iron deficiency anemia
IL	Interleukin
IRE	Iron responsive element
IRP	Iron regulatory protein
ISA	Iron sucrose AZAD
ISC	Iron-sulfur cluster
ISCU	Iron-sulfur cluster assembly enzyme
ISD11	Cystein desulfurase enzyme
ISS	Iron sucrose similar
i.v.	intravenous

IVI	Intravenous iron
LIP	Labile iron pool
MCOs	Multi-copper oxidases
MMP8	Matrix metalloproteinase 8
MRM	multiple reaction monitoring
NBIA	Neurodegeneration with brain iron accumulation
NFS1	Cystein desulfurase enzyme
NGAL	Neutrophil gelatinase-associated lipocalin
NTBI	Non-transferrin-bound iron
PBS	Phosphate buffered saline
PMA	Phorbol 12-myristate-13-acetate
PMSF	Phenylmethylsulfonylfluoride
RBC	Red Blood Cells
rhuEpo	recombinant human Erythropoietin
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute
SIH	Salicylaldehyde isonicotinoyl hydrazone
SIS	Stable Isotope Standards
sTrR	soluble Transferrin Receptor
Tf	Transferrin
TfR	Transferrin receptor
TSAT	Transferrin-saturation
Vit. C	Vitamin C

1 Abstract

Impaired iron homeostasis can lead to several serious diseases. For example, anemia of end-stage renal disease is due to decreased iron levels, whereas in hemochromatosis there is extensive body iron burden. However, besides general iron overload, there exists also organelle specific iron overload, such as the mitochondrial iron load in Friedreich´s ataxia or tissue specific iron overload, like the brain in neurodegeneration with brain iron accumulation (NBIA).

This thesis focused on three different pathologies with impaired iron homeostasis:

1. Iron deficiency in chronic kidney disease patients with special emphasis on treatment with intravenous iron formulas
2. Mitochondrial iron overload in Friedreich´s ataxia
3. Neurodegeneration with brain iron accumulation (NBIA)

Iron sucrose is one of several intravenous (i.v.) iron formulas available for clinical use to treat anemia of chronic kidney disease (CKD). This preparation is well studied, safe and effective when given intravenously and is known as the originator product.

Recently new generic iron sucrose preparations have entered the market and several studies have raised concerns as to bioavailability and biostability equivalence because iron sucrose represents the originator nanoparticle iron medicinal product. Producers of the originator drug and the Committee for Medicinal Products for Human Use (CHMP) from the European Medicines Agency (EMA) raised concerns about equivalence of the new generic products compared to the originator product, resulting in the generation of new guidelines for approval of biosimilars (ie. generic biotechnology products).

In the first part of this project we investigated bioavailability and biostability of iron sucrose originator compared to its generic product and performed non-clinical pharmacological and toxicological studies as proposed in a reflection paper by the European Medicines Agency. The results of this extensive analytical characterization demonstrated that the generic sucrose product is similar to the originator product.

Other intravenous iron formulas such as ferric carboxymaltose, a new parenteral iron preparation, have been approved for more rapid administration of large i.v. iron doses,

which may be more favourable and time- and cost-saving for the patients, since the frequency of physician visits is decreased.

Concerning ferric carboxymaltose, limited data are available describing its cellular metabolism and its toxicity. Therefore, in this part of the project we characterized complex stability, toxicity and bioavailability of iron carboxymaltose in comparison to iron sucrose. The expected results will improve our understanding about metabolism and therapeutic profile of this parenteral iron preparation.

The second part of the project focused on the inherited disease Friedreich´s ataxia. Patients display decreased expression of the FXN gene which results in low synthesis of the mitochondrial protein frataxin and show signs of biochemical impairment in iron metabolism due to mitochondrial iron load. Currently, no treatment exists for the disease, only symptomatic therapy is available. However, it was shown that decreased frataxin levels correlate with the severity of the disease. The antioxidant resveratrol was proposed to modify frataxin levels in animal studies, therefore, a clinical study was performed to evoke a new possible treatment. In this thesis an improved method for a more accurate and cost saving way to measure frataxin protein in patient lymphocytes was established.

In the last part of my thesis I focused on neurodegeneration with brain iron accumulation (NBIA). In an earlier clinical study it was shown that the iron chelator Deferiprone has the ability to cross the blood brain barrier and has the ability to lower brain iron levels. Therefore, in a clinical pilot study a patient with PLA2G6 associated neurodegeneration (PLAN) was treated with Deferiprone to lower brain iron content. Multiplex multiple reaction monitoring (MRM) proteomics was used to assess the patient’s systemic state of iron trafficking, oxidative and inflammatory stress prior to and during Deferiprone treatment.

2 Zusammenfassung

Störungen in der Eisen-Homöostase können zu schweren Erkrankungen führen. Beispielsweise ist die Anämie bei chronischer Niereninsuffizienz durch einen verringerten Eisenspiegel verursacht, während es bei hereditäre Hämochromatose zu einer Eisenüberladung kommt. Neben generellen Eisenüberladung, können auch bestimmte Organellen betroffen sein, beispielsweise die Mitochondrien bei Friedreich-Ataxie oder bestimmten Organe, wie Gehirn bei Neurodegeneration mit Eisenakkumulation im Gehirn (NBIA).

Diese Arbeit konzentriert sich auf drei verschiedenen Pathologien mit gestörter Eisenhomöostase:

1. Eisendefizienz bei Patient mit chronischer Niereninsuffizienz mit besondere Berücksichtigung der Behandlung mit Intravenösen Eisenpräparaten.
2. Mitochondriale Eisenüberladung bei Friedreich Ataxie
3. Neurodegeneration mit Eisenablagerung im Gehirn (NBIA)

Eisen-Saccharose ist eines von mehreren intravenösen (iv) Eisen Präparaten, das zur Behandlung der Anämie chronischer Nierenerkrankungen zum Einsatz kommt. Dieses Präparat ist gut untersucht, sicher und wirksam und als das Originalpräparat bekannt.

Seit kurzem sind generische Eisen-Saccharose Präparate auf den Markt und in mehreren Studien wurden hinsichtlich ihrer Bioverfügbarkeit, Biostabilität und Gleichwertigkeit Bedenken geäußert. Die Hersteller des Orginalpräparats, das „Committee for Medicinal Products for Human Use“ (CHMP) und die Europäischen Arzneimittelagentur (EMA) äußerten sich besorgt über die Gleichwertigkeit der neuen generischen Produkte im Vergleich zum Originalpräparat, was in neuen Richtlinien für die Zulassung von Biosimilars (dh generische Biotech-Produkte) resultierte.

Im ersten Teil dieses Projektes wird die Bioverfügbarkeit und biologische Stabilität vom Original Eisen-Saccharose Präparat im Vergleich zu dem generischen Produkt-anhand einer, nicht-klinischen pharmakologischen und toxikologischen Studie untersucht.

Eisencarboxymaltose, ist ein weiteres neues parenterales Eisenpräparat, über welches wenige Daten über den Zellstoffwechsel und dessen Toxizität vorliegen. In dieser Studie wurde die Komplexstabilität, Toxizität und Bioverfügbarkeit mit Eisen-Saccharat verglichen. Die zu erwarteten Ergebnisse werden unser Verständnis über den Stoffwechsel und das therapeutische Profil dieses Eisenpräparats verbessern.

Der zweite Teil des Projekts konzentriert sich auf die Erbkrankheit Friedreich-Ataxie. Patienten zeigen eine verringerte Expression des Gens FXN, welches sich durch eine geringe Synthese des mitochondrialen Protein Frataxin äußert. Dadurch kommt es zu einer Störung der zellulären Eisenhomöostase bedingt durch eine mitochondriale Eisenüberladung. Derzeit gibt es nur symptomatische Therapie. Es wurde gezeigt, dass verringerte Frataxin Spiegel mit dem Schweregrad der Erkrankung korrelieren. Resveratrol, ein Antioxidans, hat im Tierversuch Frataxin erhöht, daher wurde eine klinische Studie durchgeführt. In dieser Arbeit wurde für die klinische Studie ein verbessertes Verfahren für eine genauere und kostensparendere Quantifizierung von Frataxin-Protein in Patienten-Lymphozyten entwickelt und angewendet.

Der letzte Teil der Arbeit beschäftigt sich mit dem Erkrankungsbild der Neurodegeneration mit Eisenakkumulation im Gehirn (NBIA). In einer früheren klinischen Studie wurde gezeigt, dass der Eisen-Chelator Deferipron die Blut-Hirn-Schranke überwinden und dabei den Eisenspiegel im Gehirn senken kann. Daher wurde in einer klinischen Pilotstudie ein Patient mit PLA2G6 assoziierte Neurodegeneration mit Eisenakkumulation im Gehirn (PLAN) mit Deferiprone behandelt. Mittels Multiplex Multiple Reaction Monitoring (MRM) Proteomics wurde der systemische Eisen Zustand, oxidativer Stress und Entzündungsparamter vor und nach der Behandlung mit Deferiprone untersucht.

3 Introduction

3.1 Iron

Iron is an important metal in the organism because of its role as a biological catalyst. It is involved in numerous biological processes including oxygen transport, DNA synthesis and electron transport (W.Willimore, 2011).

However, iron in excess is potentially cytotoxic. Ferrous iron is capable to generate reactive oxygen species (ROS) such as the hydroxyl radical. Free radicals initiate lipid peroxidation of cell membranes and oxidative damage of proteins (Halliwell and Gutteridge 1984). Therefore, the levels of reactive iron must be carefully controlled and limited.

To secure the vital functions of the cell there are various proteins to handle iron homeostasis. This includes proteins for iron uptake (metal transporter, transferrin receptors), its transport in the plasma (transferrin), and its non-toxic storage (in ferritin).

Imbalance in iron metabolism leads to iron related diseases.

3.2 Iron Homeostasis

3.2.1 Erythroblasts and Macrophages

Iron in the body is absorbed from the small intestine, but the major source is recycled from the monocyte–macrophage system via phagocytosis of senescent erythrocytes. The formation of red blood cells takes around 25 days, here time and presence of erythropoietin and iron is very important for the development of healthy erythrocytes.

Erythroblasts mainly take up transferrin-bound iron via transferrin receptor 1 (TfR1) mediated endocytosis. In the acidic endosome, iron is released from transferrin and is exported from the endosome via DMT1, the divalent metal transporter 1 into the cytosol and enters the cytosolic labile iron pool (LIP). Iron from the LIP could either be stored in ferritin or used for synthesis of iron sulfur clusters or heme, which takes place at its last step in the mitochondria. Excess heme is either exported via the heme exporter, FLVCR or catabolised via the heme oxygenase-1 pathway, which is shown in Figure 1.

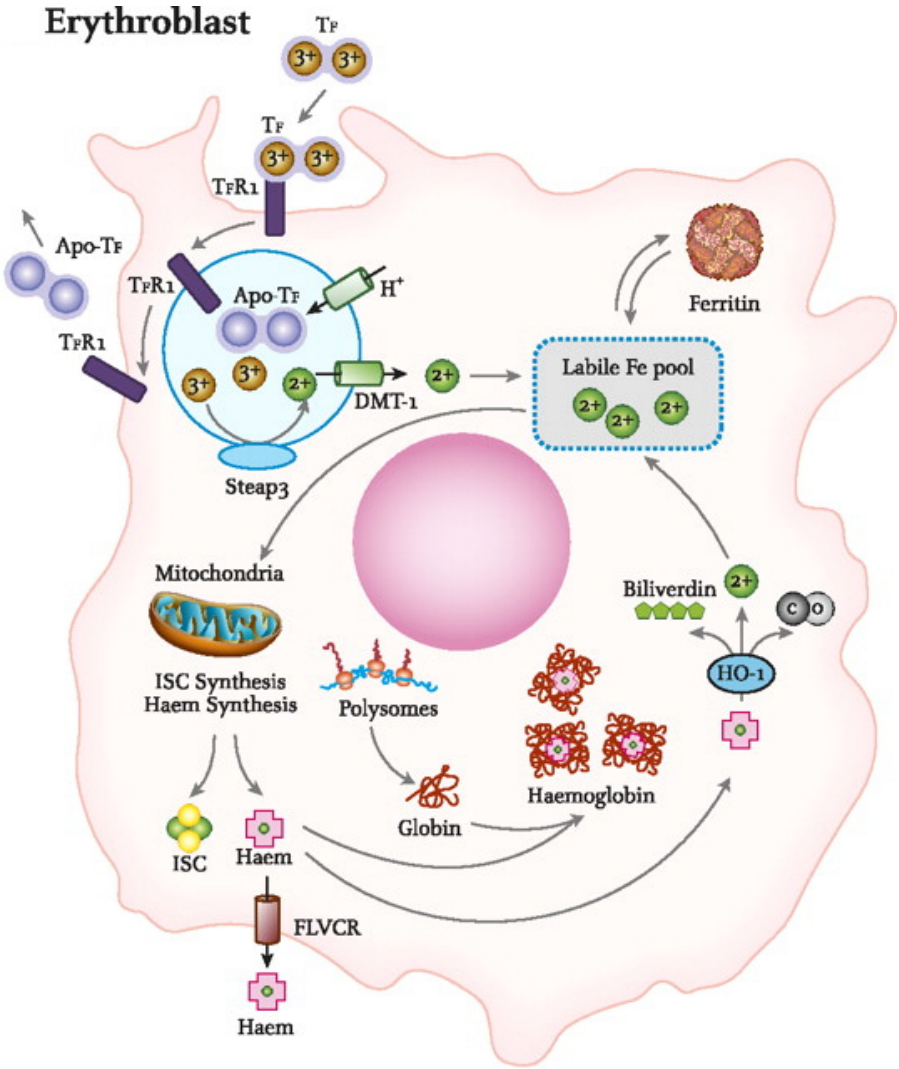


Figure 1: Iron metabolism in erythroblasts modified (Evstatiev and Gasche 2012)

Erythroblasts take up transferrin-bound iron via transferrin receptor 1 (TfR1). The relase of transferrin-bound iron takes place in acidic endosomes. Relased iron (ferric iron) is reduced to ferrous iron by Steap3 and exported into the cytosol via DMT-1, while transferrin and TfR1 recycle to the surface. The majority of intracellular iron in erythroblasts is used for hemoglobin synthesis, which is achieved through the mitochondria. Excess heme is either exported via FLVCR or catabolised via the HO-1 pathway.

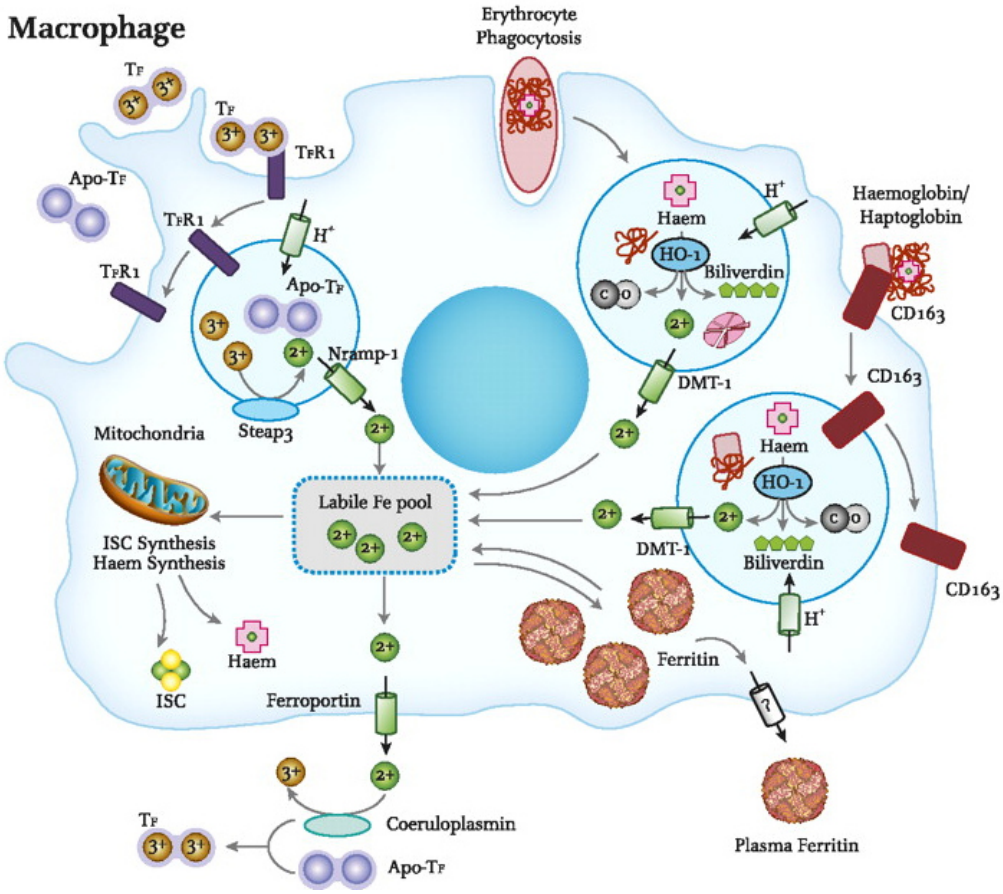


Figure 2: Iron metabolism in macrophages modified (Evstatiev and Gasche 2012)

Macrophages are responsible for iron recycling, phagocytosing senescent erythrocytes and releasing accumulated iron back into the circulation in a regulated manner. Phagocytosed erythrocytes are lysed in lysosomes, and heme-bound iron is released via heme oxigenase 1. Besides phagocytosis other iron import pathways are free hemoglobin scavenging via CD163 and TfR1-mediated transferrin uptake. Some iron from the labile iron pool is utilised for metabolic purposes, some is stored in ferritin, and some exits the macrophage via ferroportin. Exported ferrous iron is subsequently oxidised by ceruloplasmin and bound to transferrin. Most of the plasma ferritin comes from macrophages but the exact export route is still unclear.

Phagocytosis of senescent erythrocytes is one of the main functions of macrophages and the major source of iron in the body (Fig. 2). After 120 days, erythrocytes are internalised by macrophages and degraded in lysosomes. Heme-bound iron is released from heme by Heme oxygenase (HO-1) and exits the lysosomes via the divalent metal transporter, DMT1 where released iron enters the labile iron pool, the LIP.

Some iron from the labile iron pool is used for metabolic purposes, some is stored in ferritin, and some exits the macrophage via the iron exporter ferroportin. Exported ferrous iron is subsequently oxidised by ceruloplasmin and binds to transferrin and is delivered to the bone marrow for erythropoiesis. Other iron import pathways, including free haemoglobin scavenging via CD163 and TfR1-mediated transferrin uptake, play a role.

3.2.2 Transferrin (Tf)

The total amount of iron in adult humans is 3 to 5g (Zhang and Enns 2009), where up to 80% is found in the red blood cells as hemoglobin, 10-15% is present in muscle myoglobin and other iron containing enzymes. Around 0.1% of the iron circulates in the plasma that is bound tightly to transferrin (Tf). The functions of Tf are the transport of iron between sites of absorption, storage, and utilisation. Developing erythroid cells, as most of other cells types, get iron from plasma Tf.

Transferrin is an 80-kDa glycoprotein that is synthesized mainly in the liver. Each molecule can bind up to two Fe^{3+} ions and form diferric holo-transferrin (holo-Tf) (Bailey et al. 1988). Depending on the iron loading status of Tf, the affinity to the plasma membrane Tf receptor (TfR1) is different. Diferric transferrin has the highest affinity, monoferric transferrin an intermediate affinity, and apotransferrin (iron free transferrin) has a low affinity to TfR1 (Ponka 1999). Due to the higher association constant to diferric transferrin compared to monoferric and apotransferrin (Young et al. 1984), the cells equire iron predominantly by diferric transferrin. After the binding of iron–loaded holo–Tf with high affinity to TfR1 the complex undergoes endocytosis. In the acidic endosome (pH 5.5), Fe^{3+} is released from Tf, reduced by Steap3, a ferric reductase to Fe^{2+}(Ohgami et al. 2005), and exported via the divalent metal transporter 1 (DMT1) and transported to the cytosol or possibly directly to the mitochondria in

erythroid cells (Richardson et al. 2010). Moreover, macrophages also may use this Tf cycle for iron uptake.

However, under physiologic conditions, plasma Tf is hyposaturated (~30%). In diseases resulting in iron overload or anemia where parenteral iron formulas are required, Tf can become saturated with iron, resulting in the generation of non-transferrin bound iron (NTBI) (Hershko et al. 1978), (Espósito et al. 2002).

3.2.3 Non-transferrin-bound iron (NTBI)

The biochemical structure of NTBI is ill-defined (Breuer et al. 2000). There are speculations that NTBI is a mixture of iron-species composed of iron bound to small organic chelators, such as citrate and organophosphates (Breuer et al. 2000).

However, free iron may trigger formation of toxic hydroxyl radicals and reactive oxygen species (McCord 1998). Under physiological conditions, virtually all iron in plasma is bound to Tf. In conditions that cause iron overload, or in renal anemia of chronic kidney patients receiving parenteral iron preparations, Tf can become oversaturated and NTBI may be found (Lawen and Lane 2013), (Ghoti et al. 2012), (Breuer et al. 2000).

To avoid harmful, weakly bound iron in parenteral iron preparations, the preparations are very stable complexes and the iron release of the product is very low. Further the chemical nature of „released iron“ should be easily bound to apo-transferrin to be in a redox-inactive form (Pierre et al. 2002).

In addition NTBI has also been found in serum, where Tf is not fully saturated, therefore it is supposed, that there is a fraction of NTBI which cannot bind to apo-transferrin (Breuer et al. 2000).

3.2.4 Transferrin receptor (TfR)

Plasma iron is the key to the systemic iron supply and homeostasis in our body. In the circulation, Fe^{3+} is bound to the two binding sites of the glycoprotein transferrin. Holo-transferrin is taken up via transferrin receptor 1 (TfR1) mediated endocytosis into the cells.

The uptake of iron is tightly regulated by transferrin receptor (TfR) expression which allows to response to a number of metabolic needs and prevents excessive accumulation leading to production of toxic free radicals.

3.2.5 The Labile Iron Pool (LIP)

Most iron in the cytosol is stored in ferritin, but there exists a transient cytosolic iron that is loosely bound (i.e., labile) and accessible to iron chelators (Breuer et al. 2008). This iron is known as labile iron pool (LIP). The LIP is, as mentioned before, not a static quantity, so the concentration is regulated by the cellular iron uptake and the iron incorporation and release from ferritin respectively. It can consist of iron (II) and iron (III) (Lane et al. 2010), but there are no convincing data about the exact biochemical nature of the LIP. From a clinical perspective, the labile iron pool is redox-active and can generate oxidative stress in many clinical settings.

Intracellular iron deficiency and a decreased concentration for iron in transit/chelatable iron can be observed following decreased iron-uptake or -supply as well as increased iron demand (Richardson and Ponka 1997),(Santos et al. 2000). That shows, cytosolic LIP mirrors the cellular iron content and influences homeostatic adaptive responses.

3.2.6 Ferritin

Ferritin is the safe and major storage form of iron in the body. It is a water–soluble molecule and consists of 24 subunits that form a hollow sphere, where up to 4500 iron atoms, consisting of ferric-phosphate and -hydroxide ions are stored (Arosio et al. 2009),(Harrison and Arosio 1996).

Following uptake of iron by non-erythroid cells, iron enters the labile iron pool and is subsequently stored in ferritin (Young et al. 1985). The release of iron from the iron storage protein ferritin is mediated via proteolysis (Konijn et al. 1999).

However, iron storage predominantely occurs in hepatocytes and reticuloendothelial macrophages.

3.2.7 Hemopexin

Hemopexin (Hx), a plasma protein, has the highest binding affinity to free haem (Kd $<10^{-9}$ M). The main synthesis is in the liver, where it is released into the blood stream, binds to haem and delivers it back to the liver (Tolosano and Altruda 2002). Due to its high binding affinity to haem it is predestined to protect against haem induced oxidative stress (Gutteridge and Smith 1988), (Vinchi et al. 2008), limits the iron source for potential pathogens (Ascenzi et al. 2005) and contributes to iron homeostasis (Hershko 1975).

Recently, a group demonstrated, that Hx-null mice have increased numbers iron-loaded oligodendrocytes (Morello et al. 2009) and therefore increased brain iron. This suggests that under pathological conditions, hemopexin has the ability to act as a protective factor in the nervous system. However, it is still unclear how hemopexin enters the brain or is synthesised by the brain.

Due to these properties, Hx may have the strength to influence the evolution of neurodegenerative diseases.

3.2.8 Haptoglobin

Haptoglobin is a protein, which binds free hemoglobin, and prevents heme-iron mediated oxidation. Haptoglobin is also an acute phase protein, where its transcription is increased in terms of inflammation. Hemoglobin and haptoglobin together form a complex, which also plays an important role during haemolysis. Here, free hemoglobin levels in the plasma increase, leading to haemoglobin accumulation in the kidney and iron loss in the urine. Haemoglobin binds to haptoglobin and this complex can be cleared by receptor mediated endocytosis in the liver which results in iron recycling and preventing renal damage (Levy et al. 2010),(Goldenstein et al. 2012).

3.2.9 Ceruloplasmin

Ceruloplasmin is an abundant glycoprotein in human plasma and is mainly synthesized by the liver (Healy and Tipton 2007). It has enzymatic activity and belongs to the multi-copper oxidases (MCOs), a small group of enzymes, which oxidize their substrate with the concomitant reduction of dioxygen to two water molecules. So far, there are three

multi-copper oxidases detected in humans: ceruloplasmin, hephaestin and zyklopen. All of them can oxidize ferrous iron and are discussed for their potential roles in human iron homeostasis.

Ceruloplasmin has shown a variety of physiological functions including roles in copper transport and oxidation of biogenic amines. Moreover it has been shown that aceruloplasminaemia patients show massive iron overload in various tissues, including liver, pancreas and the brain (Xu et al. 2004), (Harris et al. 1995). Responsible for iron accumulation in these patients is the absence of enzymatically-active holoceruloplasmin, which confirms the role of ceruloplasmin in iron export (Xu et al. 2004), (Harris et al. 1995). Diabetes, retinal degeneration and neurological symptoms are results of iron accumulation in affected individuals (Xu et al. 2004), (Harris et al. 1995) and can be explained by iron toxicity resulting in free radical damage through the Fenton chemistry (Patel et al. 2002)

3.2.10 Lipocalin 2 (NGAL; neutrophil gelatinase-associated lipocalin)

Lipocalins belong to a heterogenous group of soluble proteins which possess several functions including immune response, cell growth, proliferation, iron transport, and synthesis of prostaglandins (Borregaard and Cowland 2006). In addition it has been shown that lipocalin 2 is significantly decreased in the cerebrospinal fluid of human patients with mild cognitive impairment and Alzheimer's disease (AD) and increased in brain regions associated with AD pathology in human post-mortem brain tissues (Naudé et al. 2012). Further, it is described that iron and iron related proteins favour the formation and stabilization of toxic (amyloid beta) Aβ aggregation (Liu et al. 2011), (Wang et al. 2012) and show to accumulate in amyloid plaques (Leskovjan et al. 2011). Interestingly, lipocalin 2 is also highly produced in response to $A\beta_{1-42}$ by choroid plexus epithelial cells and astrocytes, but not by microglia or neurons (Mesquita et al. 2014).

3.2.11 Hepcidin

Hepcidin is a small antimicrobial peptide synthesized by the liver (Pigeon et al. 2001) and plays a key role in the control of iron metabolism. Hepcidin has the ability to bind to ferroportin (FPN) and induces phosphorylation (Figure 3) of the iron exporter FPN. This leads to lysosomal internalization and degradation of FPN and further blocks iron

transport activity (Nemeth et al. 2004). Internalization of FPN on the basolateral surface of enterocytes causes the retention of absorbed iron with subsequent loss by desquamation. In macrophages and hepatocytes the same process causes the failure to release iron, which results in reduced iron availability in the plasma (Pietrangelo 2007).

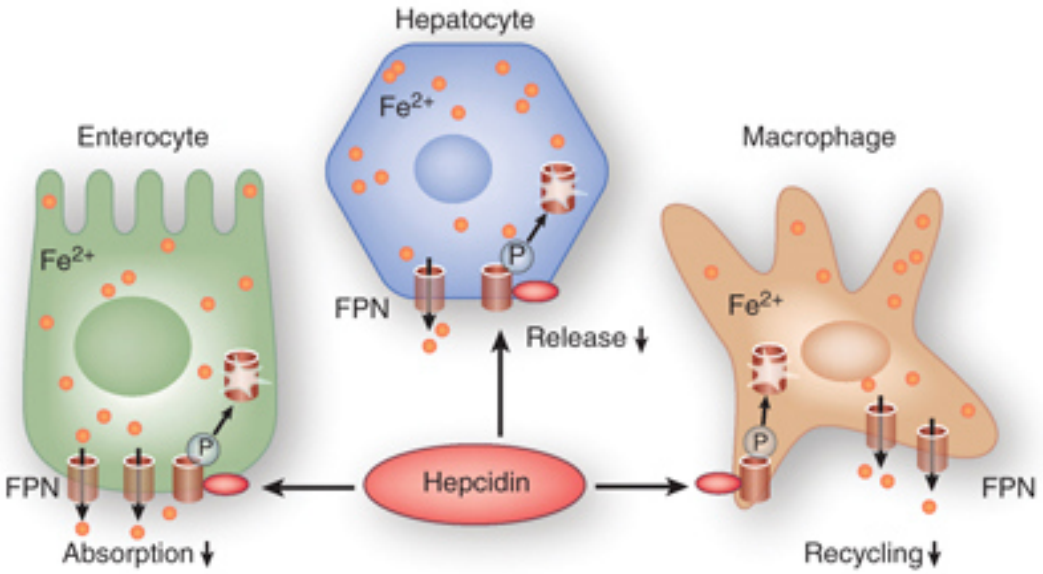


Figure 3: Downregulation of ferroportin expression on the cell surface by hepcidin modified (Cui et al. 2009)

Ferroportin (FPN) is expressed in enterocytes, hepatocytes and tissue macrophages. Hepcidin binds to FPN which lead to its phosphorylation, internalization and degradation. Decreased FPN expression reduces iron absorption in the gut, lowers iron release from the liver, and prevents iron recycling by tissue macrophages.

Genetic studies have shown the importance of hepcidin in iron homeostasis. Disruption of the hepcidin gene results in iron accumulation in the liver, pancreas and heart, increased serum iron levels and reduced iron content in the spleen in mice (Nicolas et al. 2001). In addition, overexpression of hepcidin in transgenic mice resulted in decreased body iron levels and severe microcytic anemia (Nicolas et al. 2002).

In humans, similar findings have been reported. Weinstein and his colleagues showed that patients with large hepatic adenomas had significantly high levels of hepcidin and

decreased serum transferrin saturation and further developed severe anemia due to inadequate response to iron therapy. (Weinstein et al. 2002)

Interestingly, chronic kidney disease and end-stage renal disease patients show high levels of plasma hepcidin, but the mechanism is not clear (Ganz et al. 2008), (Malyszko and Mysliwiec 2007), (Tomosugi et al. 2006).

Beside its role in iron homeostasis, hepcidin may have a direct effect on erythropoiesis. An *in vitro* study showed that hepcidin antagonizes EPO-mediated erythroid colony formation, which could further lead to a possible inhibition of erythroid progenitor growth and/or survival (Dallalio et al. 2006). The assumed function of hepcidin needs to be tested in *in vivo* studies and its interplay with EPO resistance has to be investigated in ESRD patients.

Activation of EPO signalling could be either by exogenous EPO or by endogenous EPO in an autocrine or paracrine way. This opens doors for the development of novel applications for EPO and also for optimization of the use of recombinant EPO for clinical settings including anaemia in chronic kidney disease (Arcasoy 2008).

3.3 Impaired iron homeostasis:

Impaired iron homeostasis in the body can lead to several serious diseases. Decreased iron levels lead to anemia, like in end-stage renal disease, whereas extensive iron in the body is caused but primary iron overload such as in or by hemochromatosis, secondary iron overload such as in beta-thalassemia. Besides general iron overload conditions, there are also partial iron overload conditions e.g. in mitochondria – like in Friedreich´s Ataxia or in special organs, like the brain in neurodegeneration with brain iron accumulation (NBIA). This study was focused on three pathologies with impaired iron homeostasis and will be presented in the next few chapters (see .3.3.1. to 3.3.3.)

1) Iron deficiency/anemia in chronic kidney disease patients with special emphasis on treatment with intravenous iron formulas
2) Mitochondrial iron overload in Friedreich`s ataxia
3) Neurodegeneration with brain iron accumulation (NBIA)

3.3.1 Iron deficiency

Iron deficiency (ID) is one of the most prevalent nutrition deficiencies in the world (McLean et al. 2009). It is caused by various physical problems like blood loss, inadequate nutrient intake, increased iron demand, inadequate gastrointestinal absorption, abnormal iron distribution or iron transport.

Clinically iron deficiency is defined as a decrease of total body iron content.

Disruption of iron availability in the body can lead to inadequate synthesis of iron containing enzymes, which are essential for metabolic processes. This can induce haematologic, metabolic and neurodegenerative diseases (Hentze et al. 2004).

Iron deficiency anemia (IDA) occurs when iron deficiency is severe enough to influence erythropoiesis.

3.3.1.1 Anemia in end-stage renal disease

Anemia wears the number of red blood cells (RBC) and the hemoglobin level are reduced and the volumes of packed RBC´s in the blood are below the normal values. In patients with chronic kidney disease (CKD) renal anemia is the result of inadequate endogenous erythropoietin production by the kidney and leads to reduced erythropoiesis, which can be corrected by administration of erythropoiesis stimulating agents like recombinant human erythropoietin (rhuEPO).

3.3.1.2 Iron deficiency anemia in end-stage renal disease

Response to erythropoiesis stimulating agents is largely dependent on adequacy of iron stores (2006). Therefore administration of erythropoiesis stimulating agents (ESA) in combination with i.v. iron supplementation form the cornerstones in the treatment of CKD patients (Kovesdy and Kalantar-Zadeh 2009).

3.3.1.3 Treatment of anemia in ESRD

3.3.1.4 Parenteral iron

According to the National Kidney Foundation Disease Outcome Quality Initiative (NKF KDOQI) the goal of iron therapy is to achieve and maintain a target haemoglobin level not greater than 13.0 g/dL in CKD patients receiving ESA therapy (2006).

Therefore, the use of parenteral iron supplementation is recommended for patients with CKD, where oral iron is insufficient (2006), (Locatelli et al. 2004).

The degradation of parenteral iron formulations occurs mainly in the reticuloendothelial cells and the iron is delivered by transferrin to the erythroblasts of the bone marrow. However, it has been shown that parenteral iron formulations can also be taken up by liver parenchyma cells, such as the human hepatoma HepG2 cells (Scheiber-Mojdehkar et al. 2003), (Sturm et al. 2003).

The pathophysiological relevance of these *in vitro* findings were corroborated in a recent study, where a substantial number of ESRD patients (84%) receiving erythropoiesis stimulating agents in combination with intravenous iron were found to develop hemosiderosis (Rostoker et al. 2012).

Generally, there are various IVI preparations available, which correct iron deficiency effectively, but there is also a potential risk factor due to the toxicity of ferrous iron compounds, which are associated with IVI therapy. Besides the toxicity, iron has also effects on endothelial cells, leucocytes and cytokines, as well as the occurrence of non-transferrin bound iron (NTBI) in the blood.

However, pharmacological and toxicological profiles can vary between various iron preparations depending on the different carbohydrate moieties.

Multiple parenteral iron preparations exist for administration of iron to patients with end-stage renal disease suffering from anemia (Zager et al. 2002). Today, all i.v. iron products in use are iron-carbohydrate complexes, but they differ from each other by size of the iron core and the identity and density of the surrounding carbohydrate-shell (Auerbach 2010).

The pharmacokinetic characteristics of the i.v. iron formulas depend on the stability of the iron complex. A high release rate of bioactive iron results from a weak iron binding to the complex and results in a high risk for increased free iron when the product is injected. This may lead to transferrin oversaturation (Fishbane 2003), (Ternes et al. 2007), (Toblli et al. 2010). In addition, non-transferrin bound iron (NTBI) can lead to oxidative stress and tissue damage (Andrews and Schmidt 2007), (Evans et al. 2008).

There are three main classes of i.v. iron preparations used in clinic comprise for several years: iron dextran, iron gluconate and iron sucrose.

3.3.1.5 Iron dextran

Iron therapy with i.v. iron dextran is not generally recommended because of the potential risk of anaphylactic reactions (Faich and Strobos 1999), (Chertow et al. 2006), (Locatelli et al. 2004).

3.3.1.6 Iron gluconate

Iron gluconate is approved for use in hemodialysis patients and widely replaced iron dextran as the preferred intravenous iron preparation (Auerbach and Ballard 2010)

3.3.1.7 Iron sucrose

Iron sucrose contains no dextran and therefore the incidence of allergic side effects are rare (Geisser et al. 1992), (Michael et al. 2002). Iron sucrose has been well characterized in terms of physicochemical structure and behaviour (Danielson 2004), (Geisser et al. 1992).
Clinical experience has also shown that iron sucrose is well tolerated (Van Wyck 2004).

3.3.1.8 Generic iron sucrose

Due to the terms of decreased drug acquisition costs, generic iron sucrose preparations have recently been launched onto the market. As the production process itself and adjuvants may have an influence of the final product, comparable to the production of biosimilars, also generic iron sucrose theoretically may differ from the originator. Therefore generic iron sucrose is regarded to be only iron sucrose similar (ISS).

A recently published reflection paper by the European Medicines Agency (EMA) illustrated the importance of non-clinical studies for generic nanoparticle iron medicinal product applications, such as iron sucrose complexes to ensure clinical similarity (Anon.).

Toblli and his colleagues also raised concerns about the equivalence of original iron sucrose (Venofer) and ISS preparations which were marketed in East Asia (Toblli et al. 2010). In addition there are also on-going discussions in terms of batch-to-batch consistency and variability of the manufacturing process of biosimilars as well as iron nanoparticle similars, which may account for changes in quality attributes in original drugs over time (Schiestl et al. 2011).

3.3.1.9 Ferric carboxymaltose

Recently, new generations of i.v. iron preparations came on the market, which allow administration of high doses of iron, which are effective in the treatment of iron-deficiency anemia (Auerbach 2010). Ferric carboxymaltose (FC) is one of these new agents to be approved for more rapid administration of large iron doses (up to 1000mg). The preparation consists of a ferric hydroxide core stabilized by a carbohydrate shell. The structure of the product allows the controlled delivery of iron within the cells, with minimal risk of release of large amounts of ionic iron in the plasma (Geisser 2009), (Lyseng-Williamson and Keating 2009).
In general, more information about cellular metabolism, possible accumulation and toxicity of all parenteral iron preparations are needed, because of the strong connection between life expectancy, used dosages and application frequency of parenteral iron in patients with renal failure (Parkkinen et al. 2000), (Canziani et al. 2001).

3.3.1.10 Recombinant human erythropoietin

A low production of erythropoietin, a shortened life span of erythrocytes, an impaired response of erythrocyte progenitor cells to EPO and disturbances in iron metabolism contribute to the development of anaemia of CRF (Arndt et al. 2005). Therefore therapy with recombinant human erythropoietin (rhuEPO) is necessary to avoid blood transfusions in dialysis patients.

Beside the influence of rhuEPO on iron metabolism in terms of storage, metabolic utilisation and release of iron in the body, it has been show that rhuEPO has pleiotropic effects.

It is proposed that rhuEPO has the ability to deplete iron stores and limit systemic iron availability, which leads to reduced oxidative catalyses and a degree of protection against free radical-mediated vascular damage. There is evidence that rhuEPO could act as a catalytic “iron chelator” (Bailey et al. 2006).

In addition it has been shown that rhuEPO is part of a highly potent endogenous neuroprotective system in the brain and acts as a cardioprotective system in the heart (Burger, Xenocostas, and Feng 2009), (Sirén and Ehrenreich 2001). Other pleiotropic effects of rhuEpo recognized were its anti-apoptotic and immune-modulatory activities in erythroid, parenchymal and immune cells or the increased expression of the small mitochondrial protein frataxin in lymphocytes (Nairz et al. 2012), (B Sturm et al. 2005).

3.3.2 Friedreich´s Ataxia (FRDA)

Friedreich ataxia (FRDA) is a hereditary neuro- and cardio-degenerative disorder, with an estimated incidence in Caucasians of 1 in 30 000 (Epplen et al. 1997) (Pandolfo 2012a). In particular, it is an autosomal recessive disease, which is caused by mutations in the FXN gene. The GAA triplet repeat expansion is in the first intron of the FXN gene on chromosome 9q13-21 which effects the correct transcription and reduces the amount of frataxin within the cell and the mitochondria (Campuzano et al. 1996).

Deficiency of frataxin is related to the pathogenic mechanisms of the disease, where patients develop cardiomyopathy and severe neuropathology (Campuzano et al. 1996), (Schmucker and Puccio 2010), (Martelli et al. 2012). Heart failure is the major cause of death in FRDA patients, but it is not known how frataxin influences the observed cardiomyopathy.

Furthermore, it has been shown that decreased frataxin expression is correlated with the severity of FRDA measured by the Friedreich Ataxia Rating Scale (Evans-Galea et al. 2012). However, a general lack of frataxin is not compatible with life and causes embryonic lethality early in gestation in mouse studies (Cossée et al. 2000).

3.3.2.1 Frataxin

Frataxin is a 17kD small, ubiquitous protein which is expressed at relatively low levels. In adult humans, frataxin is most abundant in the heart, the spinal cord, and the cerebral and cerebellar cortex (Campuzano et al. 1997), (Campuzano et al. 1996). Further, frataxin is also expressed in the liver, skeletal muscle, pancreas and the dorsal root ganglia (Campuzano et al. 1997), (Koeppen et al. 2013), which represent affected tissues in FRDA.

Deutsch et al. also mentioned that levels of frataxin can vary in affected individuals, asymptomatic carriers and controls, with evidence of overlap between these groups (Deutsch et al. 2010). However, individuals with the disease have FXN transcript and protein of only 5-30%, relative to healthy control individuals (Campuzano et al. 1997), (Deutsch et al. 2010), (Pianese et al. 2004).

3.3.2.2 Function of Frataxin

First, the precise function of frataxin remains elusive. Current major hypotheses show that frataxin is involved in assembly of cellular iron-sulfur clusters (ISC) and heme biosynthesis (He et al. 2004),(Lesuisse et al. 2003),(Stemmler et al. 2010) which has a direct impact on mitochondrial function and respiration (González-Cabo et al. 2005). Moreover it was proposed that frataxin can act as an 'iron sensor', 'metabolic switch' and/or iron chaperone for ISC and/or heme biosynthesis (Baur et al. 2006), (Yoon and Cowan 2004), (Lane and Richardson 2010), (Richardson et al. 2010), (Colin et al. 2013).

Just the interaction with components of the ISC machinery is widely accepted and largely supported by the literature (Gerber et al. 2003), (Shan et al. 2007), (Adinolfi et al. 2009), (Layer et al. 2006), (Leidgens et al. 2010), (Li et al. 2009), (Wang and Craig 2008).

3.3.2.3 Frataxin and iron-sulfur cluster (ISC)

ISC assembly is a mitochondrial process in eukaryotes where inorganic iron and sulfur is transferred by scaffold proteins to target apoproteins. Pyridoxal phosphate-dependent cysteine desulfurase and NFS1/ISD11 proteins in interaction with scaffold proteins are responsible for iron sulfur cluster biosynthesis *in vivo* (Raulfs et al. 2008). By using mammalian recombinant proteins, it has been demonstrated that frataxin interacts with complexes consisting of NFS1, ISCU and ISD11. This interaction leads to an increase of cysteine desulfurase activity, resulting in the assumption that frataxin modulates the capacity of NFS1 to provide sulfur for ISC formation (Schmucker et al. 2011).

In vitro studies showed that iron loaded human frataxin has the ability to transport iron to the ISCU (Yoon and Cowan 2003). Beside this proposed function of being an iron donor for Fe-S cluster biosynthesis, frataxin could also act as a regulatory protein that inhibits Fe-S cluster formation (Gerber et al. 2003). Interestingly, Schmucker and her colleagues showed, that the iron concentration does not modulate the interaction between mammalian frataxin and the ternary complex, which is responsible for Fe-S cluster formation. (Schmucker et al. 2011), (Tsai and Barondeau 2010).

This suggests that frataxin is essential for iron–sulfur cluster synthesis as an allosteric switch that activates various iron-sulfur proteins for Fe-S cluster biosynthesis (Tsai and Barondeau 2010). Decreased frataxin levels, like in FRDA patients, reduce the activities of mitochondrial iron–sulfur-dependent respiratory chain enzymes (mitochondrial electron transport complexes I, II, and III) and aconitase further leading to impaired intracellular energy production (Pandolfo 2012b), (Bradley et al. 2000).

All affected enzymes and complexes contain ISC at their active sites. So, free radicals can damage ISC and their inactivation further suggests oxidative stress in FRDA affected tissues (Puccio and Kœnig 2000).

3.3.2.4 Oxidative stress and Frataxin deficiency

Oxidative stress and mitochondrial dysfunction is a feature of FRDA and still a privileged target for therapy (Santos et al. 2010), (Armstrong et al. 2010), (Bayot et al. 2011). Data from different organisms support the hypothesis that frataxin-deficiency causes a dysregulation in the antioxidative defense, which results in oxidative stress. (Santos et al. 2010), (Bayot et al. 2011), (Shan et al. 2013).

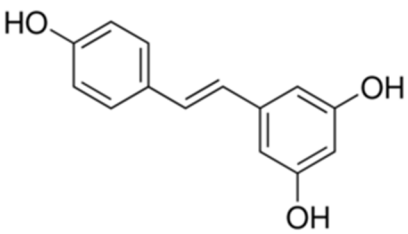
However, much effort has been done to evaluate the potential of antioxidants in preventing mitochondrial damage.

3.3.2.5 Therapeutic approaches

Currently, there exist no viable treatments for patients with Friedreich's ataxia. Patients are only monitored for symptom management. All possible treatments and therapeutic strategies currently under investigation can be divided into four categories: palliative and symptomatic treatments, use of iron chelators and antioxidants, and frataxin level modifiers.

This work will just focus on the antioxidant resveratrol which was proposed to modify frataxin levels *in vivo* (Li et al. 2013).

3.3.2.6 Resveratrol



Resveratrol (3,4′,5-trihydroxy-trans-stilbene) is a polyphenolic compound that is synthesized naturally by several plant species and is commonly found in red grape skin, berries and nuts (Pirola and Fröjdö 2008).

Figure 4: Structure of resveratrol [http://sigmaalldrich.com]

This compound has anti-oxidant properties which exerts its action in different ways: it scavenges reactive oxygen species (ROS), increases the activity of enzymes which metabolize ROS, such as superoxide dismutase (SOD), or decreases the activity of enzymes playing a role in ROS production (Rocha et al. 2009), (Juan et al. 2005), (Kohnen et al. 2007), (Li et al. 2006).

Further, resveratrol was identified to increase frataxin expression in cellular and mouse models of Friedreich´s ataxia (Li et al. 2013).

Therefore, an open label clinical pilot study using resveratrol as a treatment for Friedreich´s ataxia was performed. The target of this clinical study was to evaluate the effect of two different doses of resveratrol on lymphocyte frataxin levels over a 12-week period in individuals with FRDA, which were assessed within this work by an improved quantitative method, developed and used for the first time during this thesis. Secondary aims included the effect of resveratrol on FXN mRNA, oxidative stress markers and clinical measures of disease severity. Safety and tolerability were also evaluated.

3.3.3 Neurodegeneration with Brain Iron Accumulation (NBIA)

Abnormal iron accumulation in specific regions of the brain has been found in diseases with known genetic causes like neurodegeneration with brain iron accumulation (NBIA) (McNeill and Chinnery 2011). In general, NBIA disorders represent a clinically and genetically heterogeneous group of conditions where neurodegeneration is accompanied by elevated levels of brain iron.

PARK14 Parkinsonism, a NBIA disorder, is associated with a mutation in PLA2G6 gene, which encodes a type A2 calcium-independent phospholipase that catalyses the hydrolysis of glycerophospholipids and has been implicated in normal phospholipid remodelling, nitric oxide-induced or vasopressin-induced arachidonic acid release and in leukotriene and prostaglandin production (Rouault 2013).

A lack of PLA2G6 in mouse models results in rupture of the inner mitochondrial membrane (Zachman et al. 2010), (Claypool and Koehler 2012). Besides this, Park 14 shows increased brain iron, primarily in the basal ganglia and is characterized by progressive extrapyramidal dysfunction leading to rigidity, dystonia, dysarthria and sensorimotor impairment and often includes dystonia-parkinsonism. (Kurian and Hayflick, 2013), (Yoshino et al., 2010), (Miller et al., 2009). However, there is no evidence that decreased A2 calcium-independent phospholipase synthesis is responsible for iron accumulation in the brain (Hayflick and Hogarth 2011), and it is not known whether iron accumulation contributes to disease progression or whether accumulation of iron occurs only after widespread neuronal death. Nevertheless, clinical studies showed that decreasing brain iron levels with iron chelators showed significant improvement of neuropathy and ataxic gait in some NBIA patients (Forni et al. 2008), (Zorzi et al. 2011). The challenge for removal of brain iron is that potential drugs have to cross the blood–brain barrier and that the normal quantity of iron in the brain is not defined. So it is even more important that we increase our understanding of pathophysiological background in neurodegenerative disease which further influences therapeutic implications.

Multiplex multiple reaction monitoring (MRM) proteomics was used to investigate patients with PLA2G6 associated neurodegeneration (PLAN) treated with the iron

chelator Deferiprone. The investigation focused on the patient's systemic state of iron trafficking prior to and during Deferiprone treatment. Long-term goals include investigation of the systemic state in other neurodegeneration with brain iron accumulation (NBIA) diseases in order to assess possible biomarkers of disease progression.

3.3.3.1 Deferiprone

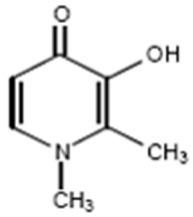


Figure 5: Chemical structure deferiprone [http://sigmaalldrich.com]

Deferiprone (3-Hydroxy-1,2-dimethyl-4(1H)-pyridone) is an oral active synthetic iron chelator, of the main area of application is ß- thalassemia.

Furthermore, the drug also shows the ability to lower iron levels in the brain, detected via MRI after 6 months treatment with deferiprone (Mounsey and Teismann 2012), (Abbruzzese et al. 2011).

4 Aims of this thesis

Iron is a crucial factor for life. Increased body iron stores are potentially toxic, whereas decreased iron levels, like in end stage renal diseases, lead to anemia, To overcome the challenges of anemia, intravenous iron preparations are a key component to handle this issue. Recently, new iron preparations were launched on the market and in addition there is an ongoing discussion about safety and efficacy of generic iron similar products.

Therefore the **first aim** of this thesis was:

- Assessment of bioavailability and stability of intravenous iron sucrose originator versus generic iron sucrose AZAD.

Iron carboxymaltose is a new iron preparation in clinical use. The supposed benefit of this compound is to apply it as a safe intravenous bolus administration up to 1000mg iron. This may be more favourable, time- and cost saving for the patients, since less frequent visits at their treating physicians are needed.

The **second aim** of this thesis was:

- To characterize complex stability, toxicity and bioavailability of iron carboxymaltose and iron sucrose.

In the last part of my thesis I concentrated on two diseases with organ- and organelle-specific iron pathology: Friedreich´s Ataxia with iron overload in mitochondria and neurodegeneration with brain iron accumulation (NBIA) with brain iron overload.

Therefore, the **third and fourth aims** of this thesis were:

- Introduction of a new infrared (IR)-based spectrometry system to overcome challenges to assess frataxin protein levels *in vivo* in Friedreich`s ataxia patients treated with resveratrol.

- Using proteomics to assess potential biomarkers of systemic iron trafficking, inflammation and oxidative stress in a patient with PLA2G6 associated neurodegeneration (PLAN) being treated with deferiprone.

5 Results

5.1 Bioavailability and stability of intravenous iron sucrose originator versus generic iron sucrose AZAD.

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Declaration of contributions to this work

M.P. planed and performed all the experiments, data analysis, data processing and statistics and wrote manuscript. C.C prepared and tested the anti-calcein antibody for the LIP measurements and critically reviewed the manuscript. M.S. prepared and tested fluorescent appotransferrin for the NTBI measurements and critically reviewed the manuscript. H.G. critically reviewed the manuscript. B. S.-M. supervised the experiments, hosted the research, supervised data analysis, data processing and statistics and helped writing and critically reviewed the manuscript and obtained funding for M.S.. B. S. planed and supervised the study, obtained financial support and wrote on the manuscript.

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RESEARCH ARTICLE

Bioavailability and stability of intravenous iron sucrose originator versus generic iron sucrose AZAD

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Abstract

Context: Severe iron deficiency requires intravenous iron supplementation to replenish iron stores. Intravenous iron sucrose has been used for decades for the treatment of anemia. New generic iron sucrose products are now marketed for the use in several countries and there is an ongoing discussion about the safety and efficacy of iron sucrose similars.
Objective: In this study, we compared the iron sucrose originator Venofer® and the generic iron sucrose AZAD (ISA) regarding bioavailability, toxicity and stability in human THP-1 cells and HepG2 cells.
Methods: The bioavailability of Venofer® and ISA was investigated in both cell types by a ferrozin-based assay. The release of incorporated iron was assayed by atomic absorption spectroscopy. Ferritin content was measured by enzyme-linked immunosorbent assay (ELISA). HepG2 cells were used to investigate the intracellular labile iron pool (LIP), which was measured by the fluorescent calcein assay. The amount of redox-active iron within the iron formulations was assayed using fluorescent dichlorofluorescein.
Results: We found no significant differences in all parameters between Venofer® and ISA in regard of bioavailability, toxicity and stability *in vitro*.
Discussion: ISA shows identical physico-chemical features and identical bioavailability *in vitro*. This study is a profound basis for future clinical tests with generic iron sucrose compounds.

Keywords

Bioavailability, biological stability, generic intravenous iron, iron sucrose, iron uptake, oxidative stress

History

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Introduction

The appropriate use of intravenous iron (IVI) is increasingly recognized as fundamental to the optimal management of iron deficiency anaemia in a number of settings including inflammatory bowel disease or patients with mal-absorption of iron[1–5].

Iron deficiency may also be caused by blood loss during dialysis, increased erythropoiesis following administration of erythropoietin and insufficient absorption of iron from the gastrointestinal tract. Most dialysis patients require IVI supplementation to replenish iron stores[6]. Venofer® (Vifor International Inc., St. Gallen, Switzerland) was first introduced in Switzerland in the early 1950s with numerous clinical trials, safety reviews and assessments. Recently generic iron sucrose copies of this iron sucrose originator so-called iron sucrose similars (ISSs) have been approved via the generic approach without the same degree of testing or number of patients exposed to the originator[5–7]. Venofer® and ISSs are complex macromolecules that belong to the class of non-biological complex drugs[7], whose structure is closely dependent on the manufacturing process. Differences in the manufacturing process raises potential concerns because it may lead to subtle structural modifications, which can affect the physicochemical properties of the drug. Such modifications can modify stability and redox properties, which affect its potential to influence cytokine activation and reactive oxygen species (ROS) generation[6,8].

Recent studies in rat models and patients have demonstrated differences between certain ISS preparations and the originator Venofer®[7,10] and also different safety and toxicity profiles of certain ISS versus originator were reported[7,10,12–14]. Therefore it is of great importance that new iron sucrose products are carefully studied to avoid side effects. In patients there is so far only scant information about possible differences in the frequency of side effects[11]. In a clinical study, it was shown that the various parenteral iron preparations (like iron dextran, ferric gluconate, ferric carboxymaltose and iron sucrose) significantly increase ROS production in hemodialysis patients to a similar extent, although the iron preparations are known to have quite different physico-chemical properties and stability characteristics[15]. In the same study, these authors found significant differences when patients were treated with the originator or a ISS—despite their much higher similarity compared to preparations with different carbohydrate shells[15].

Recently, a new iron sucrose generic from Azad Pharma AG has been developed. This generic iron sucrose AZAD (ISA) has identical physico-chemical features as the reference product Venofer® (including the size of the molecules). So far ISA showed *in vivo* no difference in tolerability compared to Venofer®[16]. A recent study in mice showed also that the biodistribution of administered iron is essentially similar for ISA and Venofer®[17].

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In this study, we compared the originator Venofer® and ISA in regard of bioavailability, toxicity and stability *in vitro* using a setting of methods to analyze properties of ISA compared to the originator. Our set up of analytical methods enables the quantification of available iron from iron compounds and its metabolic behaviour *in vitro*. As a cell model we used THP-1 cells as a model for macrophages and HepG2 cells as a model for liver cells.

Methods

Materials

The human hepatoma HepG2 cells and THP-1 cells were obtained from CLS – Cell Lines Service (Eppelheim, Germany). The iron chelator deferiprone (L1) was a generous gift from Dr. Peter Nielsen (UKE, Hamburg, Germany). 2′,7′-Dichlorofluorescein diacetate (DCFH-DA) and Calcein-AM was from Biotium Inc. (Hayward, CA). Isonicotinoyl salicylaldehyde hydrazone (SIH) was a generous gift from P. Ponka (Lady Davis Institute for Medical Research, Montreal, Canada). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was from Carl Roth GmbH + Co.KG (Graz, Austria). Serdolit CHE was from Serva (Vienna, Austria). 1-[3-Di-(ethylamino)-propyl]-3-ethylcarbodiimide was from Aldrich (Vienna, Austria). Nonident P-40 (IPEGAL CA630), ferrozine (3-(2-pyridyl)-5,6-bis(phenyl sulfonic acid)-1,2,4-triazine), and neocuproine (2,9-dimethyl(1,10-phenanthroline)), apotransferrin, thyroglobulin and ferrous ammonium sulfate were from Sigma (Vienna, Austria). Gentamycin was from GERBU Biotechnik GmbH (Wieblingen, Germany). Roswell Park Memorial Institute medium (RPMI) and Dulbecco's Modified Eagle's Medium (DMEM), L-glutamine and trypsin were from PAA (Pasching, Austria). All other chemicals were obtained from Merck (Vienna, Austria). The preparations for testing were iron sucrose originator "Venofer®" (Lotnr. 9920002; Vifor, St. Gallen, Switzerland) and generic iron sucrose AZAD (ISA) (Lotnr. A1129; AZAD Pharma, Toffen, Switzerland).

Calculation of clinically relevant iron concentrations of iron sucrose for non-clinical studies

Frequently used doses which are physiologically active and recommended by the producers are 100 mg iron for Venofer® which, however, is not the maximum clinically used dose. In a clinical study with peritoneal dialysis patients, single doses of 300 mg iron sucrose were used[18]. From pharmacokinetic studies in healthy volunteers it is known that infusion of IVI sucrose leads to rapid high plasma iron levels and that the mean volume of distribution of the central compartment is 3 litres, hence close to the volume of plasma[19]. The expected plasma concentration of IVI after infusion of 100 mg IVI therefore is close to 600 µmol/l plasma and with 300 mg one can expect plasma concentrations close to 1800 µmol/l. The *in vitro* concentrations for the assays were calculated accordingly.

Cultivation of cells

HepG2 cells were cultured in DMEM containing 10% fetal calf serum, 2 mM L-glutamine and gentamycin (50 µg/ml) under standard tissue culture conditions (5% CO_2, 37 °C).

Human monocyte THP-1 cells were grown in suspension in RPMI containing 10% fetal calf serum, 2 mM L-glutamine and gentamycin (50 µg/ml) under standard tissue culture conditions (5% CO_2, 37 °C). For the experiments, THP-1 cells were differentiated to adherent macrophages, with phorbol myristate acetate (PMA) which was added to the RPMI-medium to give a final concentration of 160 nM. After four days of cultivation, the cells were used for the experiments.

Treatment of the cells with IVI preparations

HepG2 cells and THP-1 macrophages cultivated in 6-well or 96-well plates were incubated with various concentrations of IVI preparations (Venofer® or ISA) in medium, supplemented with 10% fetal calf serum, 2 mM L-glutamine, gentamycin (50 µg/ml) for the indicated time at 37 °C. Finally, the cells were washed on ice once with cold medium (4 °C) containing 50 µM DTPA and twice with pure cold medium (4 °C) to remove surface bound iron.

Iron uptake

For the measurement of the total cellular iron content the cells were lysed in NP-40 buffer (150 mM NaCl, 1% IPEGAL CA630, 50 mM Tris, 1 mM phenylmethyl sulfonylfluoride) (700 µl/well) and ultrasonicated. The iron content of the samples was assayed spectrophotometrically by the ferrozine method[18]. A standard curve was generated by using the standard atomic absorption iron solution from Sigma. The standard (20 µg/ml in 0.5 M HCl) and 500 µl of cell lysate were incubated with 5% (w/v) $KMnO_4$ and 1.2 M HCl for 2 h at 60 °C in the dark. Finally, the ferrozine reagent (6.5 mM ferrozine, 13.1 mM neocuproine, 5 M ammonium acetate and 2 M ascorbic acid) was added to the samples, vortexed and 200 µl aliquots were transferred to a 96-well plate and measured photometrically at 540 nm. The iron content was normalized to the protein content of each sample which was assessed by the standard procedure using the protein assay-reagent (Bio-Rad, Vienna, Austria).

Iron release

The cells were loaded with IVI as described above. Finally, the cells were washed and apotransferrin (2.5 mg/ml) was added to the supernatant to initiate the release of iron. Aliquots from the supernatant were taken at different time points (0, 5, 15, 30, 60, 120 min) to determine the iron which was released from the cells into the supernatant. The amount of iron was measured with a graphite furnace atomic absorption spectroscopy Hitachi Z-8200 with a heated graphite tube and longitudinal Zeeman effect background correction was applied. Measurements were performed using a hollow cathode lamp (slit width 0.20 nm) and measured at 248.3 nm. 15 µl sample volume was used. The amount of iron was correlated to the amount of protein of the same sample.

Assessment of the labile iron pool (LIP)

The method was carried out according to Sturm et al.[21]. For the fluorescent calcein-assay, HepG2 cells were loaded with different concentrations of IVI. After the incubation, the cells were washed and finally loaded with 0.25 µM Calcein-AM in 20 mM Hepes buffered medium for 15 min at 37 °C and washed again. Finally, the cells were washed with medium and incubated with medium containing an anti-calcein antibody and 20 mM Hepes.

The plate was measured at Ex 485 nm/Em 535 nm (measurement A) with a fluorescence plate reader (Anthos Zenyth 3100, HVD Vienna, Austria). Two minutes after addition of 100 µM SIH, a strong iron chelator, the plate was measured again (measurement B). The difference between measurement B and measurement A represents the LIP.

Quantification of ferritin by a ferritin-ELISA

The cells were incubated with IVI for 3, 6 and 24 h, washed and finally lysed with NP40 buffer, sonicated and stored at −80 °C. Ferritin was determined by a human ferritin enzyme immunoassay test kit (BioCheck, CA).

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Assessment of transferrin-chelatable iron

Transferrin-chelatable iron was assayed by iron free fluorescent-transferrin (fluorescent apo-transferrin; Fl-aTf), whose fluorescence is stoichiometrically quenched by iron which binds to the protein.

Fluorescent apo-transferrin was prepared according to the method of Breuer and Cabantchik[22].

Transferrin-depleted human serum (by ultrafiltration through a 20 kD cut-off filter, ICON-concentrator, Pierce, FL) was supplemented with 75 µM IVI ± 0.56 mM ascorbic acid for 1 h at 37 °C. To assay for transferrin-chelatable iron, 10 µl of the sample were placed in quadruplicates in black 96-well plates with clear, flat bottoms (Greiner-Bio-One GmbH, Kremsmünster, Austria). Two of the wells were be incubated with 180 µl reagent A (containing 0.6 µM Fl-aTf in HBS), the other two wells were incubated with 180 µl reagent B (containing 0.6 µM Fl-aTf, 5 mM EDTA in Hepes buffered saline (HBS)). HBS consisted of 150 mM NaCl and 20 mM Hepes pH 7.4. After incubation for 1 h, 2 h and 3 h in the dark at 37 °C, the fluorescence (Ex 485 nm/Ex 535 nm) was measured in a fluorescence plate reader (Anthos Zenyth 3100 HVD Vienna).

The ratio between reading A and B was calculated and the iron concentration was derived from a calibration curve with freshly prepared ferrous ammonium sulfate (FAS) in doubly deionized water.

Assessment of redox-active iron

Redox-active iron was measured by the method of Esposito et al.[23], with slight modifications as reported by Schaller et al.[18]. To assess for redox-active iron, buffer (plasma like medium) or human serum (20 µl) was supplemented with various concentrations of IVI preparations and transferred in quadruplicates to black, clear bottom 96-well plates (Greiner Bio-one, Austria). Plasma like medium (20 mM Hepes, pH 7.4, 150 mM NaCl, 120 µM sodium citrate, 40 µM ascorbic acid, 1.2 mM Na_2HPO_4, 10 mM $NaHCO_3$ and 40 mg/ml bovine serum albumin) and HBS were rendered iron free before use by treatment with 1 g/100 ml Chelex-100 (Sigma). Two wells were incubated with iron free HBS containing 150 µM ascorbate and 5 µM dichlorofluorescein (DCF) at 37 °C in the dark. The other two wells were incubated with 180 µl of the same solution containing 50 µM of the iron chelator deferiprone (L1). The kinetics of fluorescence increase at Ex 485 nm/Em 530 nm were measured in a fluorescence plate reader (Anthos Zenyth 3100, Perkin Elmer). Measurements between 120 and 375 min were used to calculate slopes of DCF fluorescence intensity over time. The fluorescence increase measured in the presence of L1 represents oxidation of DCF by several other oxidants, e.g. peroxidases or hypochlorous acid generated by myeloperoxidases. Therefore, the difference in the rate of oxidation of DCF with and without addition of the chelator L1 represents the redox-active component of NTBI. The duplicate values of the slopes with and without addition of L1 were averaged, and redox-active iron (in µM) was determined from calibration curves correlating the difference in slopes with and without L1 against the iron concentration.

Statistical analysis

Data were analyzed with the Graph Pad Prism software. Results are presented as means ± standard error of the mean (SEM). Differences were examined for statistical significance using the one-way analysis of variance (ANOVA). Differences with $p < 0.05$ were assumed to be significant. Significant differences are marked with $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

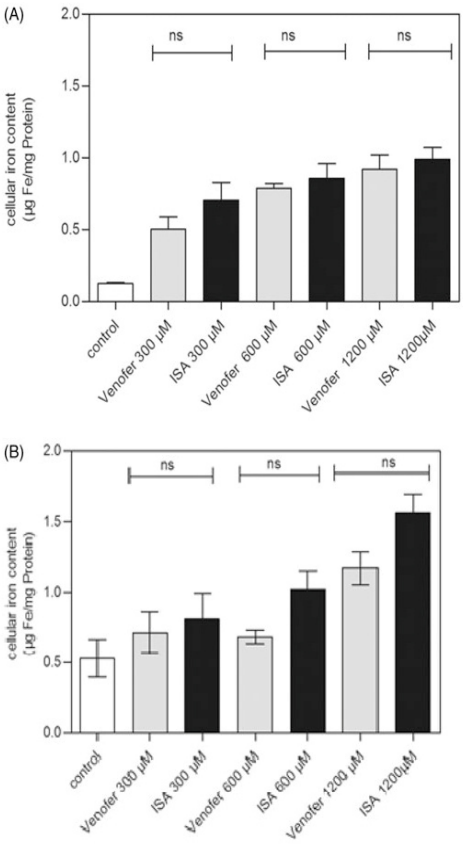

Figure 1. Uptake of intravenous iron by HepG2 cells (A) and THP-1 macrophages (B). HepG2 cells and THP-1 macrophages were incubated with 300, 600 and 1200 µM intravenous iron (Venofer® or ISA) in DMEM (10% fetal calf serum, 2 mM glutamine, gentamycin (50 µg/ml)) for 3 h at 37 °C. Finally, the cells were washed once with 50 µM DTPA, and twice with cold medium to remove surface bound iron. The cells were lysed with NP-40 buffer containing 1 mM of the protease inhibitor PMSF. The amount of iron within the cells was measured with the ferrozin method and then normalized to the amount of protein in the sample using the BioRad protein assay. Data are presented as means ± SEM (HepG2 cells: $n = 7$, THP-1 cells: $n = 6$). Differences were analyzed for statistical significance using one-way ANOVA. Not significant (ns) $p > 0.05$.

Results

Iron uptake

HepG2 cells and THP-1 macrophages were incubated with Venofer® and ISA at iron concentrations ranging from 300 to 1200 µM for 3 h. After the incubation period, the intracellular iron levels were measured using the ferrozine method following digestion of the incorporated iron complexes by high temperature acid permanganate digestion[20]. We found that HepG2 cells took up more iron than THP-1 macrophages, but we could not find significant differences between the uptake rates of Venofer® versus ISA (Figure 1).

Release of iron from iron sucrose loaded cells

To compare the availability of Venofer® and ISA, we loaded HepG2 cells and THP-1 cells with 1200 µM Venofer® or ISA for 3 h at 37 °C. Following removal of the incubation medium and several washings to remove surface bound iron, the release of iron

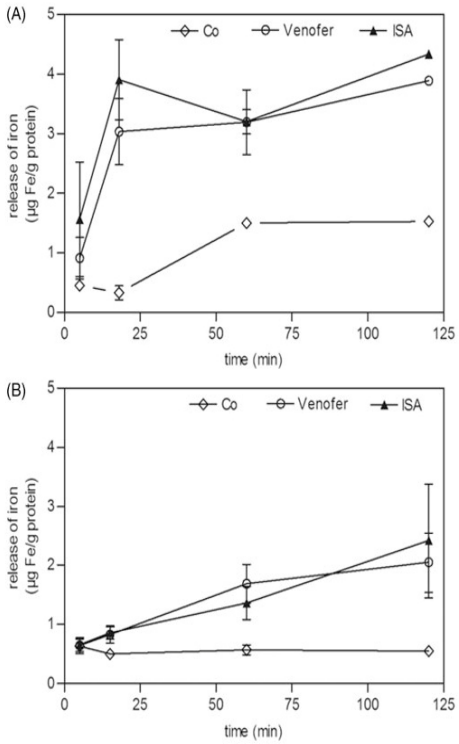

Figure 2. Iron release of intravenous iron by HepG2 cells and THP-1 macrophages. HepG2 cells (A) and THP-1 macrophages (B were incubated with 1200 µM intravenous iron (Venofer® or ISA) in DMEM (10% fetal calf serum, 2 mM glutamine, gentamycin (50 µg/ml)) for 3 h at 37 °C. Finally, the cells were washed once with 50 µM DTPA and twice with cold medium to remove surface bound iron. Then the cells were incubated with medium supplemented with 2.5 mg/ml apo-transferrin to promote cellular iron release. Control cells were not loaded with iron. The release of iron was measured in a time-dependent manner by atomic absorption spectroscopy (AAS). The amount of iron in the release medium was then correlated to the amount of cellular protein in the well using the BioRad protein assay. Data are presented as means ± SEM (HepG2 cells: $n = 4$, THP-1 cells: $n = 4$). Differences for statistical significance were analyzed using one-way ANOVA. Not significant (ns) $p > 0.05$.

was initiated by the addition of apo-transferrin (2.5 mg/ml) to the supernatant. Iron release from the cells was quantified in the release medium by atomic absorption spectroscopy.

In HepG2 cells there was a rapid release of iron within 5 min, whereas in THP-1 macrophages iron release was slower, but showed a constant rate over 2 h (Figure 2). Here again Venofer® and ISA showed the same iron release characteristics.

The intracellular LIP

The cytosolic LIP is a normal part of the total cellular iron, but it is tightly regulated by control mechanisms of cellular iron homeostasis. When this balance gets out of control, free iron can accumulate and cause oxidative damage, mainly by reaction with ROS like superoxide, hydrogen peroxide or organic peroxides[24–26]. The cellular iron pool consists of chelatable and redox-active iron which serves as a crossroad of cellular iron homeostasis, but does also promote the formation of ROS[27–29]. In HepG2 cells, the concentration dependent increase in the LIP with Venofer or ISA was similar (Figure 3). The LIP in THP-1 cells was not measured due to technical reasons.

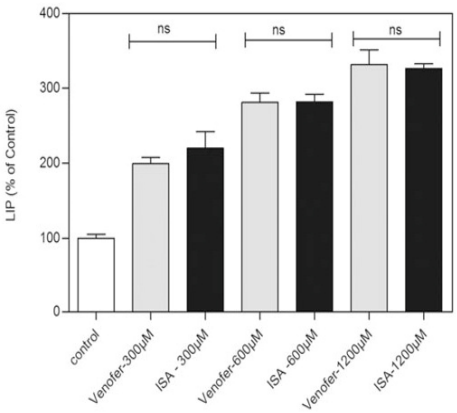

Figure 3. Increase of the labile iron pool following incubation with Venofer® or ISA. HepG2 cells were incubated with 1200, 600 and 300 µM intravenous iron (Venofer® or ISA) in DMEM (10% fetal calf serum, 2 mM glutamine, gentamycin (50 µg/ml)) for 3 h at 37 °C. Finally, the cells were washed with 50 µM DTPA and twice with medium to remove surface bound iron. Then the cells were loaded with 0.25 µM Calcein-AM in 20 mM Hepes-buffered medium for 15 min at 37 °C. Then the cells were washed with medium and incubated with medium containing an anti-calcein antibody and 20 mM Hepes. Fluorescence was measured at Ex 488 nm/Em 517 nm (measurement A) in a fluorescence plate reader. Two minutes after the addition of 100 µM SIH, a strong permeant iron chelator, the plate was measured again (measurement B). The difference between measurement B and measurement A represents the labile iron pool. Cells not loaded with iron were set as 100% fluorescence of control. Data are shown as means ± SEM ($n = 12$). Differences were examined for statistical significance using the one-way ANOVA. Not significant (ns) $p > 0.05$.

Changes in ferritin content

Ferritin synthesis was investigated in HepG-2 cells and THP-1 macrophages after incubation with Venofer® or ISA. Time dependent incubation with 1200 µM Venofer® or ISA for 6, 12 and 24 h showed a comparable stimulatory effect on ferritin synthesis with both products. In HepG2 cells, we found a significant higher increase in ferritin after 6 h of incubation with Venofer®. However, after 24 h of incubation no such difference in ferritin levels between Venofer® and ISA could be observed any more (Figure 4).

Transferrin-chelatable iron

When iron is released from parenteral iron preparations to the plasma, it is potentially harmful when not firmly bound to transferrin. The chemical nature of ''released iron'' should allow that it is easily bound to apo-transferrin and therefore to be in a redox-inactive form[30].

The biostability corresponds to the chemical stability of IVI and can be tested by the ability to transfer iron directly to transferrin[31]. In this assay, fluorescent apo-transferrin was added to transferrin-depleted serum and used as a sensitive fluorescent probe to detect transferrin chelatable iron released from Venofer® and ISA. When iron binds to fluorescent apo-transferrin, its fluorescence is stoichiometrically quenched[22].

Additionally, we added ascorbic acid to Venofer® and ISA because ascorbic acid is considered as an adjuvant therapy to improve efficacy of IVI[32]. Therefore, the amount of transferrin-chelatable iron released from Venofer® or ISA was compared in the presence (Figure 5B) or absence (Figure 5A) of ascorbic acid. We could not find significant differences in the amount of transferrin-chelatable iron between Venofer® and ISA.

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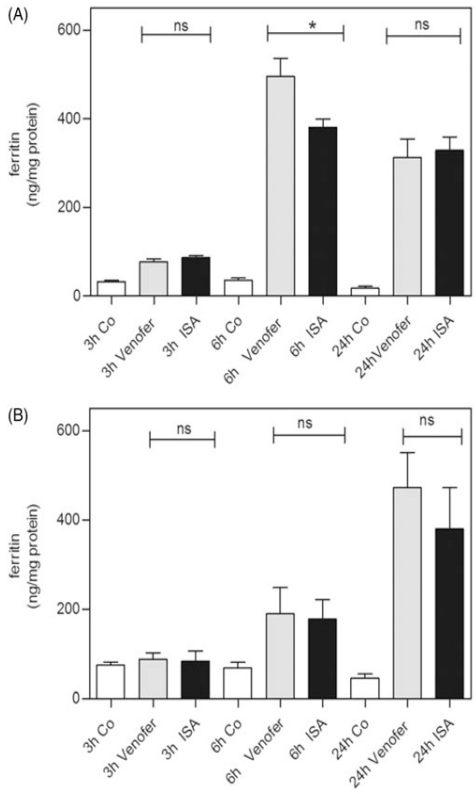

Figure 4. Effect of Venofer® and ISA on cellular ferritin levels in HepG2 cells and THP-1 macrophages HepG2 cells (A) and THP-1 macrophages (B) were washed with pure medium (37 °C) and then incubated with 1200 µM intravenous iron (Venofer® and ISA) in DMEM containing 10% of fetal calf serum, 2 mM glutamine and 10% gentamycin for 3, 6 and 24 h. At the indicated time points the cells were washed once with medium containing 50 µM DTPA and twice with pure medium. The cells were lysed with NP-40 buffer containing 1 mM PMSF. The amount of ferritin in the samples was measured by a human ferritin enzyme-linked immunosorbent assay (ELISA; BioCheck, Foster City, CA). The amount of ferritin was normalized to the amount of protein in the samples. Data are presented as means ± SEM (HepG2 cells: $n = 5$, THP-1 cells: $n = 4$). Differences were examined for statistical significance using the one-way ANOVA. Not significant (ns) $p > 0.05$, *$p < 0.05$.

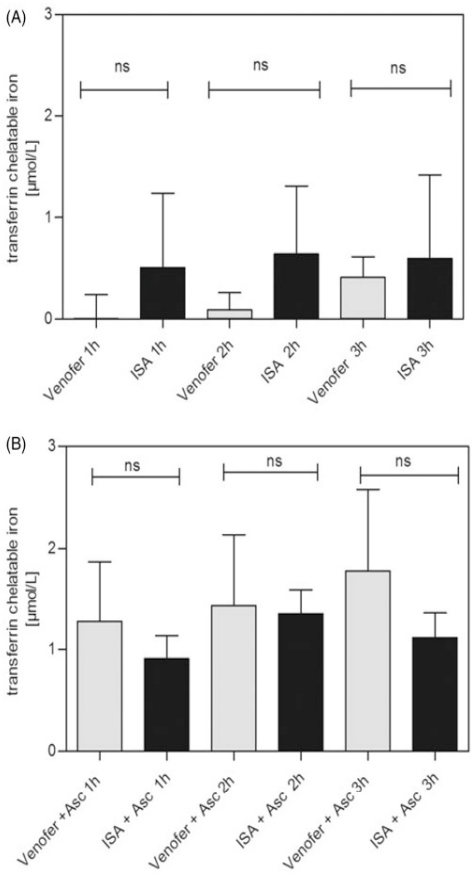

Figure 5. Transferrin-chelatable iron. Transferrin-depleted human serum was incubated with 75 µM intravenous iron (Venofer® or ISA) alone (A) or in the presence of 0.56 mM ascorbic acid (Asc) (B) for 1 h at 37 °C. The samples were then mixed with reagent A (HBS containing 0.6 µM fluorescein-labelled apo-transferrin, Fl-aTf) or reagent B (reagent A containing 5 mM EDTA) and incubated at 37 °C in the dark. After 1, 2 and 3 h the fluorescence was measured at Ex 485 nm/Em 535 nm in a fluorescence plate reader. The ratio between the incubation with and without EDTA (reading B/A) was calculated and correlated to a standard curve generated with ferrous ammonium sulfate at concentrations ranging from 0 to 20 µM. Data are presented as means ± SEM ($n = 4$). Differences were examined for statistical significance using the one-way ANOVA. Not significant (ns) $p > 0.05$.

Redox-active iron

Oxidative stress *in vivo* is the result of an imbalance between the production of oxidants and the respective defence systems of an organism[33]. Free iron presents a dangerous source for the generation of ROS. If the iron within the iron formulations is weakly bound, free redox-active iron can occur.

Therefore, we tested both products for the presence of redox-active iron. In plasma like medium, there was a comparable amount of redox-active iron at all concentrations tested (Figure 6A) while in serum (Figure 6B) no detectable redox-active iron could be found, which reflects the ability of serum proteins to minimise the risk of the generation of ROS mediated by iron.

Discussion

Due to some evidence that certain ISSs differ from the iron sucrose originator in safety and efficacy profiles, it seems prudent for physicians as well as patients who require IVI to have available data on therapeutic equivalence of new ISS preparations versus the originator[34]. It is very important that new IVI products are evaluated regarding bioavailability, stability and potential toxicity *in vitro*. In former studies, we have compared some of the IVI compounds which are available on the market and found significant differences among the different classes of IVI products[21,32,35–37].

The use of intravenous iron for the treatment of anaemia can cause potential danger. First there exists the danger of excess iron after intravenous iron infusion which can increase the potential to generate ROS. Therefore, the quality of parenteral iron preparations is also determined by the presence of redox-active iron. Additionally there is evidence for the occurrence of tissue iron overload (e.g. liver) in long-term treatment with intravenous iron[38]. *In vitro* studies with HepG2 cells can show the uptake rate of various intravenous iron compounds in liver cells. Also the

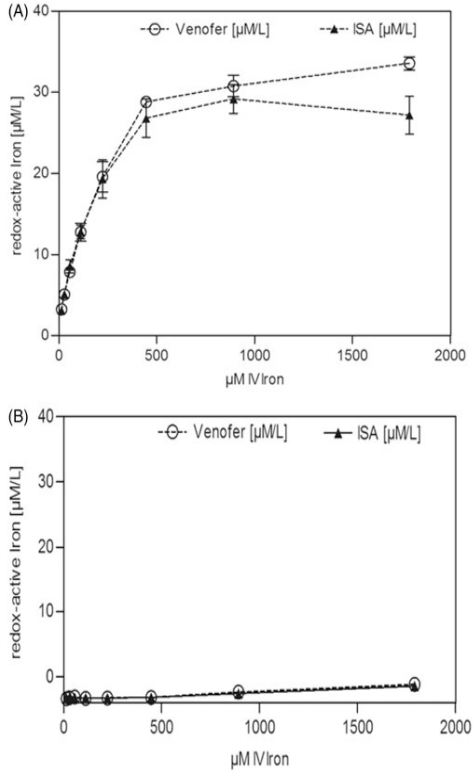

Figure 6. Redox-active labile plasma iron generated from iron sucrose compounds in plasma like medium (A) and human serum (B). Plasma like medium (A) or human serum (B) (20 µl) supplemented with various concentrations of intravenous iron (0–1750 µM) was transferred in duplicates to black, clear bottom 96-well plates. Two wells were incubated with HBS buffer, pH 7.4, containing 150 µM ascorbate and 5 µM DCF at 37 °C in the dark. The two other wells were incubated with the same solution containing 50 µM of the iron chelator deferiprone (L1). The kinetics of fluorescence increase was measured in a fluorescence plate reader (Ex 485 nm/Em 530 nm). Measurements between 120 and 375 min were used to calculate slopes of DCF fluorescence intensity over time. The fluorescence increase measured in the presence of L1 represents oxidation of DCF by oxidants. Data are presented as mean of two experiments.

determination of redox-active iron within the compounds reflects the potential of the compounds to generate ROS *in vivo*. In this study, we compared the iron sucrose originator Venofer® and the iron sucrose generic ''Iron Sucrose AZAD'' (ISA) in a set of different assays designed to find out possible iron related differences regarding the bioactivity, toxicity and biological stability.

First, we measured the uptake rates of iron from the iron sucrose compounds and analyzed whether there is a difference in their efficacy to overcome iron deficiency anaemia. We found no significant difference in the uptake rates between both compounds in HepG2 cells and in THP-1 macrophages.

In vivo after entering the cells the iron should be available to be released into the circulation. Therefore, we measured the release of iron after loading the cells with Venofer® or ISA. We added transferrin to the release medium to initiate the release of iron from the iron loaded cells. HepG2 cells released more iron than THP-1 macrophages. But overall there was no significant difference between Venofer® and ISA.

In HepG2 cells, most of the iron was released within the first 5 min and reached its limit after 30 min. In contrast, THP-1 cells showed a release of iron over a time period of 2 h. This suggests that the iron in HepG2 cells was easier accessible than the iron in THP-1 cells.

Ferritin is the main storage protein for iron in the human body and is regulated by the iron regulatory protein. During high intracellular iron concentrations, when the intracellular labile pool (LIP) is augmented, ferritin levels increase. Under these conditions high iron concentrations can be scavenged by storing iron into ferritin and therefore protect the cells and membranes against oxidative damage. In our study, we could not find differences in ferritin synthesis between the two products.

The LIP is a small part (<5%) of the total iron content and is defined as transient redox-active and labile iron. Uptake of transferrin or non-transferrin bound iron leads to an increase of the LIP, resulting in regulation of iron homeostasis by iron regulatory proteins[39]. The LIP is important for cellular iron homeostasis and associated with the production of ROS. We measured the amount of iron that enters the LIP in HepG2 cells from Venofer® and ISA. The increase in intracellular labile iron was dependent on the iron concentration and was similar with both products.

Therapy with IVI has to be strongly controlled due to the danger of excess iron after iron infusion. Excess iron has the potential to generate ROS and therefore the amount of redox-active free iron within the IVI products is of great importance.

Transferrin can also be considered as an iron buffer in the plasma, keeping redox-active iron low and avoiding adverse reactions. The transferrin binding capacity was tested and no differences were found. We also found that the amount of redox active iron within both products is very low.

Our results are in accordance with the study from Elford et al.[17] where they studied the biodistribution of the two compounds in mice. In general, they also found no significant differences in tissue iron levels (plasma, spleen, bone marrow, liver, heart, stomach, kidneys, liver or lungs).

Conclusion

We found no differences between the two products regarding bioavailability, stability and toxicity *in vitro* which indicate that ISA has a comparable behaviour than the originator Venofer®.

Declaration of interest

The study was funded by Azad Pharma AG. The funders had no role in data collection, analysis and interpretation of the data, decision to publish, or preparation of the manuscript. All authors declare no competing interest. This work was also supported by a FFG grant (Barbara Scheiber-Mojdehkar, TALENTE, No. 2441987-1).

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The number of tables in a publication is limited. Therefore another control experiment (figure 6) regarding viability of HepG2 cells incubation with iron sucrose and generic "Iron Sucrose Azard" is shown here.

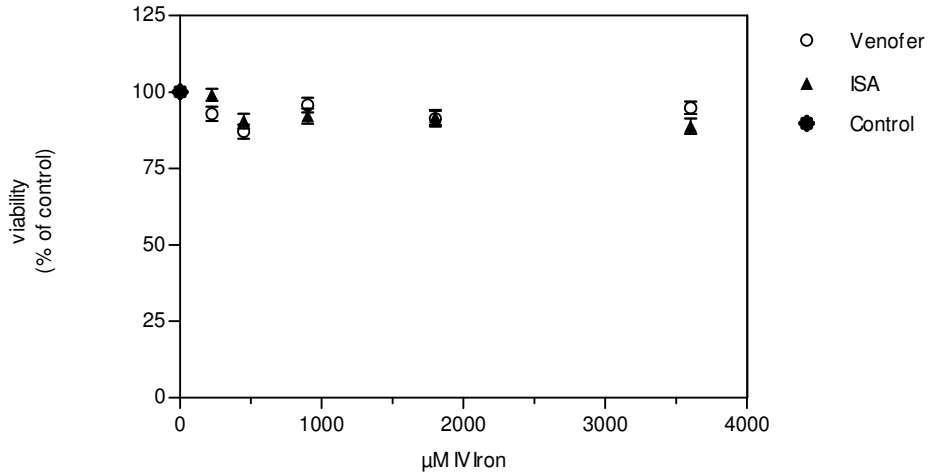

Figure 6: Impact of iron sucrose and the generic "Iron Sucrose AZAD" on HepG2 cell viability assessed with MTT assay.

The cells were incubated for 3h with different concentration (0-3600µM) of i.v. iron preparations. The number of viable cells were assessed by uptake of MTT, which correlates with the number of viable cells. Values are expressed as % of control (control) incubated cells.

Animal studies have indicated that there are different effects on histological, biochemical and physiological behaviour in tissues after i.v. iron administration from different products in their ability to provoke oxidative stress and inflammatory response (Toblli et al. 2012a), (Toblli et al. 2009), (Apopa et al. 2009). In this *in vitro* study with liver parenchyma cells and THP-1 macrophages, we saw no differences between the originator and the generic iron sucrose products in terms of toxicity assessed by the MTT assay.

Therefore, it is from tremendous interest that intravenous iron preparations are evaluated in terms of bioavailability and stability equivalency of the originator vs. ISS. Further it seems prudent also for physicians as well as patients who require intravenous iron to have available data on therapeutic equivalence of new ISS preparations versus

the originator. Also in some former *in vitro* studies we found significant differences in their bioavailability profiles of different classes of intravenous iron preparations compared to the iron sucrose originator (Sturm et al. 2010)

It seems that NBCD (non biological complex drugs) are vulnerable for changing composition, quality and *in vivo* performance but it should be mentioned that "originator" have also their challenge to meet the target qualifications of constituents (Schiestl et al. 2011).

5.2 Iron sucrose and ferric carboxymaltose: No correlation between physicochemical stability and biological activity

Submitted publication

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Iron sucrose and ferric carboxymaltose: No correlation between physicochemical stability and biological activity

Declaration of contributions to this work

M.P. planed and performed all the experiments (excluding figure 8), data analysis, data processing and statistics and wrote the manuscript. K.H. planed and performed experiments leading to figure 8 and critically reviewed the manuscript. C.C prepared and tested the anticalcein antibody for the LIP measurements and critically reviewed the manuscript. M.S. prepared and tested fluorescent apotransferrin for the NTBI measurements and performed pilot experiments for transferrinbound iron and critically reviewed the manuscript. B.S. gave financial support to M.P., critically reviewed the experiments and the manuscript. H.G. critically reviewed the manuscript. B. S.-M. supervised the experiments, hosted the research, supervised data analysis, data processing and statistics and wrote the manuscript and obtained funding for M.S..

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Iron sucrose and ferric carboxymaltose: No correlation between physicochemical stability and biological activity

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Abstract:	Intravenous iron preparations, like iron sucrose (IS) and ferric carboxymaltose (FCM) differ in their physicochemical stability. Thus differences in storage and utilization can be expected and were investigated in a non-clinical study in liver parenchyma HepG2-cells and THP-1 macrophages as models for toxicological and pharmacological target cells. HepG2-cells incorporated significant amounts of IS, elevated the labile iron pool (LIP) and ferritin and stimulated iron release. HepG2-cells had lower basal cellular iron and ferritin content than THP-1 macrophages, which showed only marginal accumulation of IS and FCM. However, FCM increased the LIP up to 2-fold and significantly elevated ferritin within 24h in HepG2-cells. IS and FCM were non-toxic for HepG2-cells and THP-1 macrophages were more sensitive to FCM compared to IS at all concentrations tested. In a cell-free environment redox-active iron was higher with IS than FCM. Biostability testing via assessment of direct transfer to serum transferrin did not reflect the chemical stability of the complexes (i.e. FCM>IS). Effect of vitamin C on mobilisation to transferrin was an increase with IS and interestingly a decrease with FCM. In conclusion, FCM has low bioavailability for liver parenchyma cells, therefore liver iron deposition is unlikely. Ascorbic acid reduces transferrin-chelatable iron from ferric carboxymaltose, thus effects on hepcidin expression should be investigated in clinical studies.

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Iron sucrose and ferric carboxymaltose: No correlation between physicochemical stability and biological activity

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Keywords: Intravenous iron; iron sucrose; ferric carboxymaltose; liver iron overload; macrophages; ascorbic acid.

Abstract: Intravenous iron preparations, like iron sucrose (IS) and ferric carboxymaltose (FCM) differ in their physicochemical stability. Thus differences in storage and utilization can be expected and were investigated in a non-clinical study in liver parenchyma HepG2-cells and THP-1 macrophages as models for toxicological and pharmacological target cells.

HepG2-cells incorporated significant amounts of IS, elevated the labile iron pool (LIP) and ferritin and stimulated iron release. HepG2-cells had lower basal cellular iron and ferritin content than THP-1 macrophages, which showed only marginal accumulation of IS and FCM. However, FCM increased the LIP up to 2-fold and significantly elevated ferritin within 24h in HepG2-cells. IS and FCM were non-toxic for HepG2-cells and THP-1 macrophages were more sensitive to FCM compared to IS at all concentrations tested.

In a cell-free environment redox-active iron was higher with IS than FCM. Biostability testing via assessment of direct transfer to serum transferrin did not reflect the chemical stability of the complexes (i.e. FCM>IS). Effect of vitamin C on mobilisation to transferrin was an increase with IS and interestingly a decrease with FCM.

In conclusion, FCM has low bioavailability for liver parenchyma cells, therefore liver iron deposition is unlikely. Ascorbic acid reduces transferrin-chelatable iron from ferric carboxymaltose, thus effects on hepcidin expression should be investigated in clinical studies.

Introduction:

Intravenous iron (IVI) is used to treat anaemia associated with a variety of medical conditions among them chronic kidney disease (CKD), inflammatory bowel disease, chronic inflammatory arthritis, pregnancy and postpartum (Auerbach et al. 2008)·(Muñoz et al. 2008).

IVI preparations have complex structures with differing physicochemical stabilities (Jahn et al. 2011)·(Geisser et al. 1992)·(Danielson 2004) . Iron sucrose (IS) was thoroughly screened in clinical trials, safety reviews and assessments. High amounts of IVI administration have a potential higher risk of infections (Hoen et al. 1998) and mortality (Feldman et al. 2004· Kalantar-Zadeh et al. 2005) in clinical trials and experimental studies (Parkkinen et al. 2000· Lim and Vaziri 2004· Kuo et al. 2008· Sonnweber et al. 2011). Recent trials suggest that large doses of IS in CKD patients result in radiographic evidence of excess tissue iron, which was not detectable by serum laboratory parameters (Canavese et al. 2004a· Ferrari et al. 2011;Rostoker et al. 2012). Ghoti et al. performed a T2*-MRI study in haemodialysis patients, where extensive IS administration resulted in iron deposition in liver and other tissues (Ghoti et al. 2012). Even low-dose maintenance iron therapy with ferric gluconate could not prevent the risk of iron overload in patients with moderate anaemia (Canavese et al. 2004b). So far no reports exist about possible iron deposition with ferric carboxymaltose (FCM).

IS is mainly degraded in reticuloendothelial cells, delivered to transferrin and further to the erythron. In earlier in vitro studies we showed that in addition non-RES cells, like liver parenchyma HepG2-cells internalise IS (Scheiber-Mojdehkar et al. 2003· Sturm et al. 2003). Recent in vivo results confirmed the concerns arising from our in vitro study about possible liver iron overload in patients receiving IS (Ghoti et al. 2012). This clearly demonstrates that findings from preclinical studies can often predict the in vivo situation and later often prove to be clinically relevant.

Recently, ferric carboxymaltose was launched on the market. Due to its high complex stability up to 1000mg per infusion can be applied and iron stores can be replenished with a very low risk for hypersensibility and other adverse effects without the requirement of a test dose (Kulnigg et al. 2008· Van Wyck et al. 2007· Bailie et al. 2010). Information about metabolic fate of FCM in pharmacological and toxicological target cells is incomplete and there are no reports investigating the possibility of tissue iron deposition.

In this study we aimed to elucidate differences regarding iron utilization and uptake characteristics between IS and FCM in a preclinical in vitro study using clinically relevant concentrations.

More specifically in a cell-based approach we investigated (1) iron uptake in pharmacological and toxicological target tissues; (2) influence on labile iron pool; (3) cellular iron release; (4) generation of oxidative stress in the presence and absence of serum; (5) toxicity profiles in RES and non-RES cellular models.

In cell-free experiments we investigated: (6) direct iron transfer to transferrin; (7) effect of reducing agents (i.e. ascorbic acid) on release of transferrin-chelatable iron and (8) generation of redox-active iron and the ability of human serum to scavenge it.

Results

Uptake of IVI by macrophages and non-reticuloendothelial cells: IVI complexes are mainly degraded by reticuloendothelial cells and delivered via transferrin to the erythron. THP-1 cells, differentiated to macrophages were used as model for reticuloendothelial cells representing the pharmacological target tissue (Tsuchiya et al. 1982). Liver parenchyma cells, which are important for iron storage and homeostasis were shown to incorporate IS (Scheiber-Mojdehkar et al. 2003). Therefore we used HepG2-cells as model for non-RES toxicological target tissues. HepG2-cells incorporated iron in a dose dependent manner from IS, but not from FCM (Fig. 1A). THP-1 macrophages showed no increase in cellular iron with FCM and a slight, but not significant increase (p=0.0655) with IS (Fig. 1B).

Effect of IVI on the labile iron pool: The cytosolic labile iron pool (LIP) is a normal, tightly regulated small part of the total cellular iron. If this balance gets out of control, free iron can cause oxidative damage, mainly by reaction with reactive oxygen species (ROS) (Halliwell and Gutteridge 1990; Rothman et al. 1992; Stäubli and Boelsterli 1998).

Incubation of HepG2-cells with IS increased the LIP in a dose dependent manner (Fig. 2), which is in line with the rise in cellular iron shown in Fig 1A. FCM at 600µM and 1200µM significantly increased the LIP, but to a smaller extent than IS (Fig. 2).

Ferritin expression in response to incubation with IVI: The safe and major storage form of iron in the body is ferritin. Incubation of HepG2-cells (Fig. 3A) with IS (1200µM) resulted in a significant increase in ferritin expression after 6 hours, and less prominent increase after 24 hours. FCM needed 24 hours to stimulate ferritin expression to the same degree. THP-1 macrophages (Fig 3B) had higher baseline ferritin levels than HepG2-cells (Fig. 3A), reflecting their higher basal cellular iron content (Fig 1A and B). IS significantly increased ferritin levels in THP-1 macrophages in a time dependent manner and remained unchanged with FCM.

Iron release: Transferrin-bound iron is important for adequate iron supply for heme synthesis, as it represents the only iron source for erythroblasts. To study iron release from cells loaded with IVI, we used apotransferrin in the release medium at a physiologic concentration (2.5mg/mL) to promote iron release. Iron release from HepG2-cells was significantly higher when loaded with IS (Fig. 4A) which is in line with the observed increase in cellular iron (Fig.1A) and LIP (Fig.2). With FCM there was no evidence of substantial iron accumulation in HepG2-cells (Fig.1A) and release was below untreated cells (Fig. 4A). In accordance with the absence of significant iron uptake in THP-1 macrophages from both IVI`s (Fig 1B), iron release was similar to untreated cells (Fig. 4B).

Cytotoxicity of IVI (MTT-assay): High doses of IVI induced organ damage and oxidative stress in non-anaemic rats (Hemmaplardh and Morgan 1974). Therefore we extended our concentration range of IVI far above clinically relevant concentrations and assessed metabolic activity in HepG2-cells and THP-1 macrophages. In HepG2-cells viability was not affected by both IVI`s (Fig. 5A). In general THP-1 macrophages seemed to be more sensitive to incubation with FCM compared to IS (Fig 5B).

Effect of IVI on oxidative stress in liver parenchyma cells: Oxidative stress is a term denoting an imbalance between production of oxidants and the respective defence systems of an organism (Halliwell and Gutteridge 1999) and can be monitored in vivo by the dichlorofluorescein-assay. HepG2-cells take up several IVI preparations (Scheiber-Mojdehkar et al. 2003; Sturm et al. 2003) and were used in the dichlorofluorescein-assay. Oxidative stress was generally higher with both IVI`s (Fig. 6A), but further increased in a dose dependent manner with IS (Fig. 6A). Similar results were obtained with serum (Fig. 6B), indicating that serum components could not scavenge ROS generation.

Release of redox-active iron from IVI: Transferrin-bound iron is redox-inactive, thereby preventing production of iron-catalysed hydroxyl-radicals. IVI preparations contain low amounts of redox-active iron, which are scavenged to a varying degree by serum components (McCord 1998; Sturm et al. 2010).

In buffer release of redox-active iron from IS was higher compared to FCM (Fig. 7A). With human serum, redox-active iron was generally highly reduced with both IVI`s and practically completely disappeared with FCM (Fig. 7B).

Direct transfer to transferrin: High doses of IVI theoretically can oversaturate plasma transferrin and lead to non-transferrin bound iron, which may participate in deleterious redox-reactions On the other hand transferrin bound iron is the only source for heme producing erythropoietic cells (Pierre et al. 2002). Therefore assessment of direct transfer of IVI to transferrin is of interest.

Visualisation of iron transfer from IVI to transferrin was performed by urea-PAGE allowing the separation of differently iron loaded transferrins and quantification of relative amounts of apo-, holo-, and monoferric transferrin. Human serum was incubated with IVI or ferric ammonium citrate (FAC), which represents soluble low molecular weight iron, for 5 hours at 37°C. With FCM direct transfer to serum transferrin was higher (Fig.8B) compared to IS (Fig.8A). Ferric ammonium citrate was used as positive control and, as expected, iron was immediately bound to transferrin (Fig.8C).

Effect of ascorbic acid on transferrin-chelatable iron: Parenteral iron preparations are stable complexes containing only a very low amount of "free iron". Preferably, the chemistry of the "free iron" should allow that it is readily bound to transferrin to be in a redox-inactive state.

Ascorbic acid had beneficial effects on hematological parameters in haemodialysis patients with elevated or even normal iron stores (Einerson et al. 2011) and increased transferrin-chelatable iron from IS (Sturm et al. 2005). To our knowledge no such reports exist about FCM.

Similar to the results obtained with urea-PAGE (Fig.5), we found in the fluorescent apotransferrin based assay more transferrin-chelatable iron with FCM than IS. With ascorbic acid transferrin-chelatable iron increased with IS, whereas it decreased with FCM within 3 hours. Interestingly, both, the direct binding of transferrin and the ability of reductive iron release from the preparations markedly differed from what we expected from the chemical stability of both IVI preparations (i.e. chemical stability: FCM>IS).

Discussion:

Iron sucrose has a safe profile and has been used for decades. Ferric carboxymaltose represents a new generation of IVI`s formulations and allows a 1000mg bolus administration. Both IVI are iron carbohydrate complexes with differing physicochemical stabilities that may differently affect anaemia management (Jahn et al. 2011; Geisser et al. 1992).

IVI preparations are degraded by reticuloendothelial cells, iron is delivered to transferrin and mainly used by erythroid cell. In vitro studies showed that in addition non-RES cells, like liver parenchyma HepG2-cells, can incorporate some IVI preparations (among them IS) (Scheiber-Mojdehkar et al. 2003; Sturm et al. 2003). This is in line with recent in vivo results from Ghoti et al. where haemodialysis patients receiving IS for a long term developed excessive liver iron overload (Ghoti et al. 2012). This clearly demonstrates that findings from preclinical studies can in many cases predict the in vivo situation and later often prove to be clinically relevant.

Here we investigated uptake and release characteristics of IS and FCM in liver parenchyma HepG2-cells and in THP-1 macrophages. HepG2-cells incorporated significant amounts IS, but not FCM. In THP-1 macrophages we observed a slight, but not significant increase in cellular iron with IS within 3 hours, but not with FCM. This general minimal effect in macrophages may reflect the different terminal half-life of FCM ranging from 7 to 12 hours compared to approximately 6h of IS (ferinject_smpc_2012.pdf. packaging information from http://server-p007.hostpoint.ch/%7Eferinjec/ferinject.com/wp-content/uploads/2011/12/ferinject_smpc_2012.pdf. Accessed 26 Aug 2013).

The cytosolic LIP, a small part of total cellular iron (<5%), is tightly regulated by control mechanisms of cellular iron homeostasis. The LIP consists of a chelatable fraction of iron which may participate in redox-reactions and ROS formation (Breuer et al. 1997; Picard et al. 1996; Lipiński et al. 2000). HepG2-cells showed a dose depended increase in the LIP with IS. Interestingly, the LIP increased up to 4-fold with IS and only 2-fold with FCM.

Ferritin, the major storage form of iron represents the major defence against iron toxicity (Young et al. 1985). Basal ferritin-expression in THP-1 macrophages was higher compared to HepG2-cells, reflecting the higher basal cellular iron content in macrophages.

In line with the effect of IVI on iron content and LIP, ferritin was significantly up-regulated by IS in HepG2-cells. Despite the apparent absence of substantial accumulation of FCM in HepG2-cells, we observed a significant increase in ferritin within 24h. This findings are not contradictory, since very low amounts of iron are sufficient to increase the LIP and in turn up-regulate ferritin-expression via the IRE-IRP system (Rouault 2006).

For adequate bioavailability of incorporated IVI, iron release to the circulation is essential for erythropoiesis in vivo. IS loaded HepG2-cells cell showed significantly higher iron release than untreated cells. Consistent with the lack of substantial accumulation of FCM in HepG2-cells iron release was comparable to untreated cells.

Iron accumulation in THP-1 macrophages was marginal with both iron preparations, and accordingly iron release was similar to untreated cells.

Treatment of non-anaemic rats with non-pharmacological high concentrations of IVI (40mg IVI/kg body weight) was reported to lead to organ damage and oxidative stress (Toblli et al. 2012). Therefore we performed our toxicological in vitro study with IVI far above the clinically relevant concentrations. We found that IS and FCM were non-toxic for HepG2-cells. THP-1 macrophages were more sensitive to FCM compared to IS at all concentrations. As incubation of THP-1 macrophages with FCM failed to increase cellular iron content, presumably damage results from extracellular iron released from FCM.

In the second part of this study we investigated the biological activity of IVI in a cell-free environment. Bioactive iron released from IVI may play a role in oxidative stress and inflammation (Crichton et al. 2006). With IS we found more redox-active iron compared to FCM.

Biostability of IVI can be tested by the ability to transfer iron directly to transferrin by urea-PAGE and was reported to be inversely correlated to the chemical stability of the complex (i.e. chemical stability FCM>IS) (Crichton et al. 2006). Interestingly, when we used a more physiologic approach with human serum instead of transferrin in a buffer to asses iron binding to transferrin, the biostability order was reversed (i.e. biostability IS>FCM). These results were confirmed with a second fluorescence-based method assessing transferrin chelatable iron. This indicates that, besides transferrin, other plasma components influence bioavailabilty of IVI in vivo.

Ascorbic acid enhances iron mobilization to transferrin in serum from IS, but not from other IVI`s (Sturm et al. 2005; Wang et al. 2008) and the order of chemical stability is not predictive for biostability in presence of ascorbic acid. As reported previously, transferrin-chelatable iron from IS increased in presence of ascorbic acid, but unexpectedly declined with FCM. These results are clinically relevant, since ascorbic acid is investigated as a supplement for haemodialysis patients with rhuEPO hyporesponsiveness (Tarng 2007; Attallah et al. 2006; Sezer et al. 2002; Jalalzadeh et al. 2012).

Conclusion:

In conclusion, FCM has low bioavailability for liver parenchyma cells, therefore liver iron deposition is unlikely. Ascorbic acid reduces transferrin-chelatable iron from ferric carboxymaltose, thus effects on hepcidin expression should be investigated in clinical studies

Materials and Methods:

Materials: HepG2-cells and THP-1 cells were from CLS - Cell Lines Service (Eppelheim, Germany). Deferiprone (L1) was a generous gift from Dr. Peter Nielsen (UKE, Hamburg, Germany). 2´,7-dichlorofluorescein diacetate (DCFH-DA) and Calcein-AM were from Biotium Inc., (Hayward, California, USA). Isonicotinoyl salicylaldehyde hydrazone (SIH) was a generous gift from Dr. P.Ponka (Lady Davis Institute for Medical Research, Montreal, Canada). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was from Carl Roth GmbH + Co.KG (Graz, Austria). Serdolit CHE was from Serva (Vienna, Austria). 1-[3-Di-(ethylamino)-propyl]-3-ethylcarbodiimide was from Aldrich (Vienna, Austria). Nonident P-40 (IPEGAL CA630), ferrozine (3-(2-pyridyl)-5,6-bis(phenyl sulfonic acid)-1,2,4-triazine), neocuproine (2,9-dimethyl(1,10-phenanthroline)), apotransferrin, thyroglobulin and ferrous ammonium sulphate were from Sigma (Vienna, Austria). Gentamycin was from GERBU Biotechnik GmbH (Wieblingen, Germany). Roswell Park

Memorial Institute medium (RPMI) and Dulbecco`s Modified Eagle`s Medium (DMEM), L-glutamine and trypsin were from PAA (Pasching, Austria). All other chemicals were purchased from Merck (Vienna, Austria).

Intravenous iron preparations: Iron sucrose (IS) "Venofer" and ferric carboxymaltose (FCM) "Ferinject" were from Vifor, St. Gallen, Switzerland.

Calculation of clinically relevant iron concentrations of IVI for non-clinical studies: In healthy volunteers infusion of IS leads to rapid high plasma iron levels with a mean volume of distribution of the central compartment of 3L, hence close to the volume of plasma (Danielson et al.). The expected plasma iron concentration after infusion of 100mg IVI is therefore close to 600µM, and with 400mg IVI one can expect plasma concentrations close to 2400µM Venofer (Iron sucrose injection, USP, packaging information; http://www.venofer.com/HCP/Index.aspx. Accessed 26 Aug 2013) Administration of ferric carboxymaltose is recommended up to 1000mg per single dose (corresponding to an expected plasma concentration of ~6000µM), (ferinject_smpc_2012.pdf; packaging information; from http://server-p007.hostpoint.ch/%7Eferinjec/ferinject.com/wp-content/uploads/2011/12/ferinject_smpc_2012.pdf. Accessed 26 Aug 2013). The in vitro concentrations for the assays were calculated accordingly.

Cultivation of cells: HepG2-cells were cultured in DMEM, 10% FCS, 2mM L-glutamine and gentamycin (50µg/ml) under standard tissue culture conditions (5% CO_2, 37°C).

Human monocyte THP1-cells were grown in suspension in RPMI, 10% FCS, 2mM L-glutamine and gentamycin (50µg/ml) under standard tissue culture conditions. THP1-cells were differentiated to adherent macrophages by 160nM phorbol myristate acetate (PMA) for four days prior to the experiments.

Treatment of the cells with intravenous iron preparations: HepG2-cells and THP-1 macrophages were incubated with IVI (IS or FCM) in either DMEM (HepG2-cells) or RPMI-medium (THP-1 cells), supplemented with 10% FCS, 2mM L-glutamine, gentamycin (50µg/ml) for the indicated time at 37°C and 5% CO_2. Finally the cells were washed with medium containing 50µM DTPA, and twice with medium alone to remove surface bound iron.

Cellular iron content: To assess total cellular iron content the cells were lysed with NP-40 buffer (150mM NaCl, 1%IPEGAL CA630, 50mM Tris, 1mM phenylmethyl sulfonylfluoride) and sonicated. The iron content of the lysates was assayed spectrophotometrically by the ferrozine-method(Fish 1988). 500µL of cell lysate were incubated with 250µL 5% (w/v) $KMnO_4$ and 1.2M HCL for 2 hours at 60°C in the dark. Finally 50µL ferrozine-reagent (6.5mM ferrozine, 13.1mM neocuproine, 5M ammonium acetate and 2M ascorbic acid) were added to the samples and measured photometrically at 540nm. A standard curve was generated with a standard atomic absorption iron solution from Sigma. The iron content was normalized to the protein content which was assessed with Bio-Rad Protein assay-reagent (Bio-Rad, Vienna, Austria).

Assessment of released iron by AAS: The cells were loaded with IVI as described above. Then the cells were washed with medium containing 50 µM DTPA and twice with medium alone and incubated with apotransferrin (2.5mg/ml) in Hepes (20mM) buffered medium (DMEM/HepG2-cells and RPMI/THP-1 cells) to initiate iron release. Aliquots of the supernatant were taken at different time points and iron was measured with AAS as reported previously (Praschberger et al. 2013).

Assessment of the labile iron pool (fluorescent calcein-assay): The assay was carried out according to Sturm et al. (Sturm et al. 2003). Anti-calcein antibody was prepared according to a protocol of William Breuer (Hebrew University of Jerusalem, Israel) by coupling 50mg calcein (Sigma, Vienna, Austria) and 20mg thyroglobulin (Sigma, Austria) in 10ml 20mM MES buffer, pH 5, followed by addition of 96mg (1-[3-Di-(ethylamino)propyl]-3-ethylcarbodiimide) (Aldrich, Vienna, Austria) under gentle stirring at 4°C over night in the dark. Following dialysis against PBS, pH 7.4 the antigen solution was stored at -20°C until antibody production. Three biweekly subcutaneous injections of the following solutions to a rabbit were used: once 0.5ml antigen solution mixed with 0.5ml complete adjuvant, and twice 0.5ml antigen solution mixed with 0.5ml incomplete adjuvant. Two weeks following the last injection the first bleeding was carried out. The IgG-fraction was obtained by a 77.7% ammonium sulphate precipitation at room temperature for 1h at constant stirring(Abelson J. et al.). Following centrifugation at 5000 x g at 25°C for 30min the pellet was resuspended in 300µl distilled water and dialyzed against PBS, pH 7.4. The antibody solution was stored at 4°C in the presence of 0.02% Na-azide. The calcein-fluorescence-quenching activity was confirmed by recording fluorescence of an iron-free calcein in HBS, pH 7.4.at Ex485nm/Em535nm before and following addition of the antibody solution.

For the fluorescent calcein-assay HepG2-cells were incubated with IVI, and then washed with medium containing 50µM DTPA and twice with medium alone to remove surface bound iron. Following loading the cells with 0.25 µM Calcein-AM in 20mM Hepes buffered medium for 15 minutes at 37°C and washings, they were incubated with medium with anti-calcein antibody and 20mM Hepes. The anti-calcein antibody was used to quench fluorescence of extracellular calcein. Measurement was performed at Ex485nm/Em535nm (measurement A) in a fluorescence plate reader (Anthos Zenyth 3100, HVD Vienna). Three minutes after addition of 100µM SIH, a strong iron chelator, the plate was measured again (measurement B). The difference between measurement B and measurement A represents the LIP.

Quantification of ferritin: The cells were washed with medium (37°C) and then incubated with IVI in DMEM (HepG2-cells) or RPMI (THP-1 cells) containing 10% of FCS and 2mM glutamine, washed on ice once with 50µM DTPA and twice with medium alone and finally lysed with NP40-buffer, sonicated and stored at -80°C. Ferritin was quantified by a human ferritin ELISA (BioCheck, California, USA).

Quantification of apo-, monoferric- and holotransferrin in human serum: Human serum was incubated with IVI and iron loading of transferrin was investigated by urea–PAGE and densitometry (Davy et al. 1982) according to Makey and Seal (Makey and Seal 1976) with slight modifications by using a Tris-borate-electrophoresis buffer without EDTA to avoid removal of iron from transferrin by this chelator (Baldwin 1980)·(Pollack et al. 1977). Serum was separated on 6% polyacrylamide gels containing 6M urea with Tris-borate electrophoresis buffer at 120V (const.) for 2.5h at 4°C. Proteins were stained with 0.1% Amidoblack in 44% methanol and 11% acetic acid. Densitometry was performed in a Fusion Fx7 Imager (Peqlab Biotechnology, Vienna, Austria) and analysed with AlphaEase FC, Version 3.2.1 software from Alpha Innotech Corporation (USA). Total density of all bands representing apo-, holo and monoferric transferrin was set as 100%.

Assessment of transferrin-chelatable iron: Transferrin-chelatable iron was assessed with iron-free fluorescent-transferrin (fluorescent apo-transferrin; Fl-aTf) whose fluorescence is stoichiometrically quenched when iron

binds to the protein as reported previousely (Praschberger et al. 2013). Fluorescent apo-transferrin was prepared according to the method of Breuer et.al. (Breuer and Cabantchik 2001).

Assessment of redox-active iron: Redox-active iron was measured by the method of Esposito et al. (Esposito et al. 2003), with modifications reported by Schaller et al. (Schaller et al. 2005). In this assay dichlorofluorescein (DCF) is converted from its non-fluorescent precursor to the fluorescent form by several oxidants.

DCF was prepared from 2´,7`-dichlorodihydrofluorescein diacetate (DCFH-DA) by hydrolysis before use. 0.5ml 1mM DCFH-DA was incubated with 2ml 0.01n NaOH at 25°C for 30min in the dark and neutralised with 10ml 25mM Na-phosphate buffer (pH 7.2).

Buffer (plasma like medium) or human serum (20µl) were supplemented with IVI and transferred in quadruplicates to black, clear bottom 96 well plates (Greiner, Bio-one, Kremsmünster, Austria). Plasma like medium (20mM Hepes, pH7.4, 150mM NaCl, 120µM sodium citrate, 40µM ascobic acid, 1.2mM Na_2HPO_4, 10mM $NaHCO_3$ and 40mg/ml bovine serum albumin) was rendered iron-free before use by treatment with 1g/100ml Chelex-100. Two wells were incubated with 180µl iron-free HBS (20mM Hepes, 150mM NaCl, pH 7.4) containing 150µM ascorbic acid and 5µM DCF at 37°C in the dark. The other two wells were incubated with the same solution containing 50µM of the iron chelator Deferiprone (L1). The kinetics of fluorescence increase at 485nm/530nm was measured in a fluorescence plate reader (Anthos Zenyth 3100, Perkin Elmer). Slopes of DCF fluorescence intensity over time (between 120 and 375 minutes) were calculated. The difference in the rate of oxidation of DCF with and without L1 represents the redox-active component of NTBI. Duplicate values of the slopes with and without L1 were averaged, and redox-active iron (in µM) was determined from calibration curves correlating the difference in slopes with and without L1 against the iron concentration.

Assessment of reactive oxygen species in HepG2-cells: In the dichlorofluorescein-assay the rate of ROS production in living HepG2-cells was assessed as described earlier. Briefly, cells were treated with non-fluorescent 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), which is deacetylated by cytosolic esterases to form non-fluorescent dichlorodihydrofluorescein (DCFH). By reacting with ROS, fluorescent dichlorofluorescein (DCF) is generated. DCFH-DA (103 mM) was dissolved in DMSO and stored at –20°C. 10^5 cells/well were cultured for 48 hours in 96-well plates and then incubated with IVI in DMEM medium (without methyl red (MR)) alone or supplemented with 10% FCS for 4.5 hours. Cells were washed and loaded with 10 µM DCFH-DA in DMEM (without MR) for 30 min at 37°C, washed again to remove non-incorporated DCFH-DA and further incubated with MR-free DMEM medium. Fluorescence intensity was measured at Ex485nm/Em535nm for 60 minutes at 37°C in a fluorescence plate reader (Anthos Zenyth (HVD, Vienna, Austria). Slopes of fluorescence changes with time were referred to the slope of control and expressed in % of control (set as 100%).

Statistical Analysis: Statistical analysis was performed with the Graph Pad Prism software. Results are presented as means ± SEM. Differences were examined for statistical significance using the paired t-test. Differences with $p<0.05$ were assumed to be significant. Significant differences are marked with * $P<0.05$, ** $p<0.01$ and *** $p<0.001$.

Disclosure: All the authors declared no competing interests.

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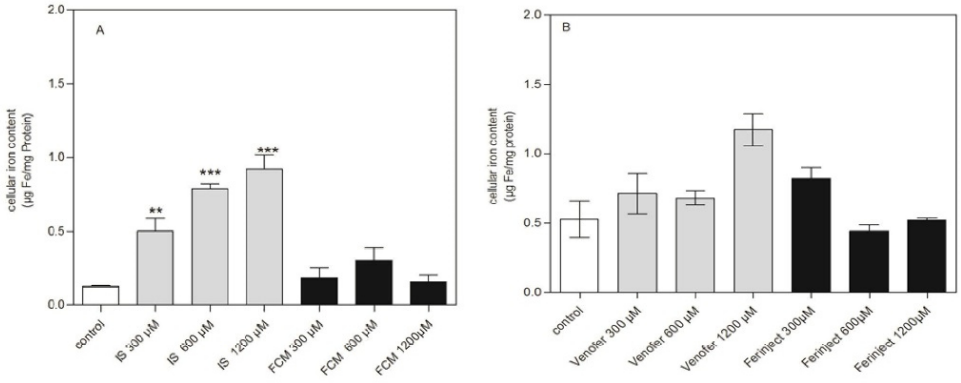


Fig. 1: Uptake of IVI preparations by macrophages and non-reticuloendothelial cells

HepG2-cells (A) and THP-1 macrophages (B) were incubated with intravenous iron preparations (IS or ferric carboxymaltose) in medium (DMEM for HepG2-cells and RPMI for THP-1 macrophages), supplemented with 10% FCS, 2mM glutamine and gentamycin (50µg/ml), for 3h at 37°C. Then the cells were washed once with ice cold medium containing 50 µM DTPA, a strong impermanent iron chelator, and twice with pure ice cold medium to remove surface bound iron. The cells were lysed with NP-40 buffer containing 1mM protease inhibitor PMSF. Iron in the cell lysates was quantified with the ferrozine-method and normalized to protein content of the sample using the BioRad protein assay. All data are presented as means ± SEM (HepG2-cells: n=7, THP-1 macrophages: n=6). Differences among iron uptake from IS or FCM compared to control were examined for statistical significance using the paired t-test. Statistically significant differences of the means vs. control are marked with ** ($p<0.01$) and *** ($p<0.001$).

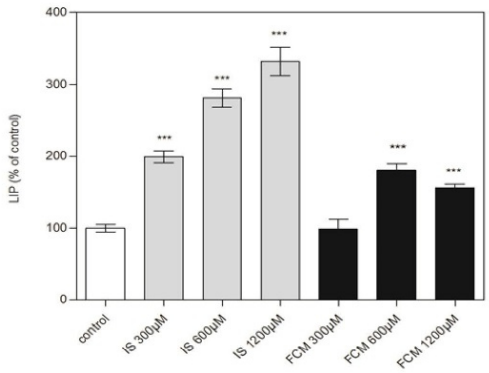


Fig. 2: Effect of iron sucrose and ferric carboxymaltose on the cytosolic labile iron pool

HepG2-cells were seeded in 96-well plates and incubated with iron sucrose (IS) or ferric carboxymaltose (FCM) for 3 hours at 37°C. The cells were washed and loaded with calcein-AM (0.25µM) for 15 min at 37°C in 20mM Hepes-bufferd FCS-free DMEM medium. Then the incubation medium was removed, the cells were washed with FCS-free medium and incubated with DMEM containing 20mM Hepes and anti-calcein antibody. After registration of the baseline fluorescence, the amount of intracellular metal, bound to calcein (Ca-Fe) was assessed by addition of 100 µM of the fast permeating chelator SIH. Calcein fluorescence was measured when the signal reached full fluorescence and remained stable (after 3min). The LIP of the untreated control cells was set as 100%. Data are shown as means ± SEM (n=12). Differences were examined for statistical significance by the paired t-test. Statistically significant differences of the means vs. control were marked with *** ($p<0.001$).

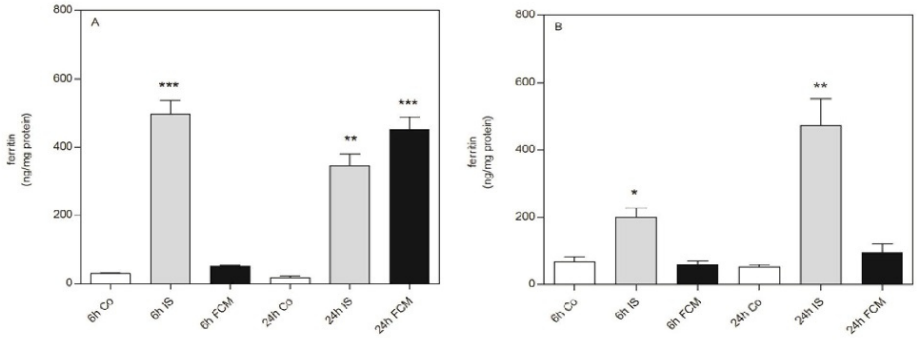


Fig. 3: Ferritin expression in HepG2-cells and THP-1 macrophages in response to IVI preparations

HepG2-cells (A) and THP-1 macrophages (B) were seeded in 6 well plates and incubated with 1200µM iron sucrose or ferric carboxymaltose in medium (HepG2-cells: DMEM; THP-1 macrophages: RPMI), supplemented with 10% FCS, 2mM glutamine and gentamycin (50µg/ml), for 6 and 24 hours at 37°C. Following incubation, the wells were washed, lysed with NP-40 buffer with 1mM PMSF and sonicated. In the lysate ferritin was quantified by ferritin-ELISA and normalized to the protein content of the sample. Data are presented as means ± SEM (HepG2-cells: n=5, THP-1 macrophages: n=4). Differences of the means were examined for statistical significance using the paired t-test. Statistically significant differences of the means vs. control are marked with * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).

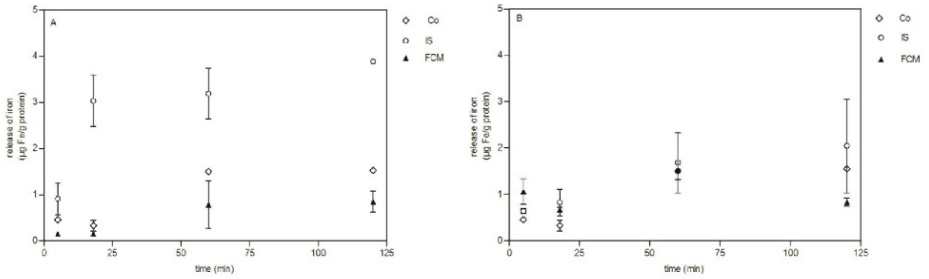


Fig. 4: Release of iron from cells previously loaded with IVI preparations

HepG2-cells (A) and THP-1 macrophages (B), cultivated in 6-well plates, were loaded with IVI (1200µM) in DMEM (HepG2-cells) or RPMI (THP-1 macrophages) medium, supplemented with 10% FCS, 2mM glutamine and gentamycin (50µg/ml), for 3 hours at 37°C and 5%CO_2. After removal of the incubation medium, cells were washed and incubated with release medium (DMEM for HepG2-cells or RPMI medium for THP-1 macrophages) containing apotransferrin (2.5mg/ml) to initiate iron-release. At different time points aliquots of the incubation medium were taken to determine cellular iron release into the medium.

Iron in the release medium was quantified by AAS and correlated to the amount of cells in the well (by quantification of total protein content in the well). Data are presented as means ± SEM (HepG2-cells: n=4, THP-1 cells: n=4). Differences of the means were examined by the paired t-test. In HepG2-cells p= 0.0475 for FCM vs. contol and p=0.0001 for IS vs. contol within 120 minutes.

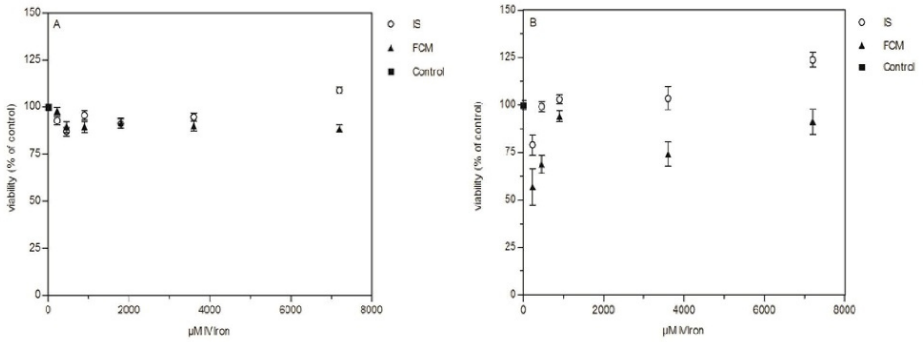


Fig. 5: Cytotoxicity of intravenous iron (MTT-assay)

HepG2-cells (A) and THP-1 macrophages (B) cultivated in 96-well plates, were incubated with intravenous iron preparations in DMEM (HepG2-cells) or RPMI medium (THP-1 macrophages), supplemented with 10% FCS, 2mM glutamine and gentamycin (50µg/ml), for 24 hours at 37°C and 5% CO_2. After removal of the incubation medium and washing of the cells, 100µL fresh medium and 20µL MTT-solution were added to each well and incubated for 30min at 37°C. After removal of the incubation medium, 150µL/well MTT-solvent were added to lyse the cells and for dissolution of the coloured crystals. Absorption was recorded in a plate reader (see Materials and Methods). Shown are the means ±SEM (n=4).

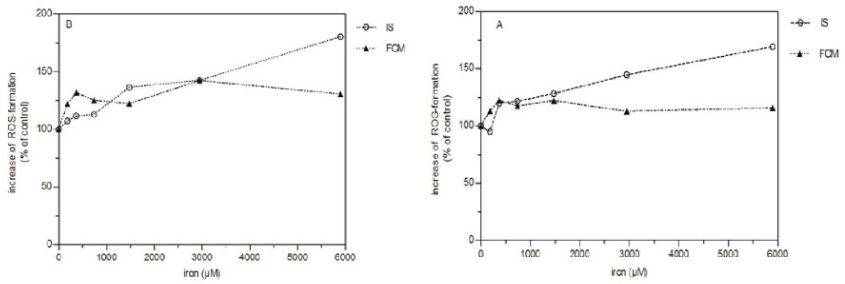


Figure 6: Effect of IVI on oxidative stress in HepG2-cells:

HepG2-cells were incubated with different concentration of IVI in DMEM medium alone (A) or in DMEM medium supplemented with 10% FCS (B) for 4.5 hours at 37°C and 5% CO_2. After removal of the incubation medium, ROS-formation was measured by the DCF-method (see Materials and Methods). The averaged slopes of fluorescence changes with time were referred to the slope of control and are expressed in % of control (set as 100%). Shown are the results of one typical experiment in triplicates.

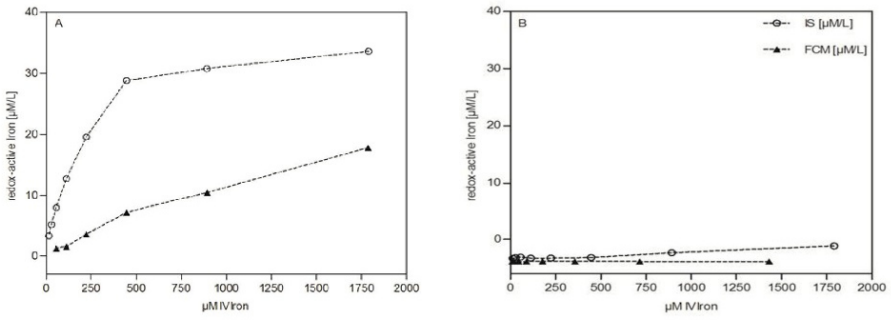
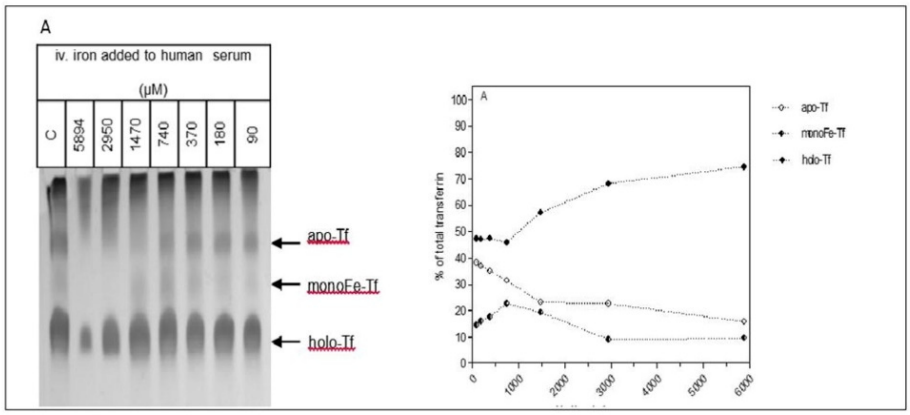


Fig. 7: Release of redox-active iron from IVI preparations in buffer and human serum

Buffer (plasma like medium, see Materials and Methods) (A) or human serum (B) was supplemented with different concentrations of IVI and incubated with 5µM dichlorofluorescein (DCF), in iron free HBS (20mM Hepes, 150mM NaCl, pH7.4) containing 150µM ascorbate, and incubated at 37°C for 3 hours in the dark. In parallel experiments the samples were incubated with the same solution but contained 50µM of the iron chelator deferiprone (L1). The fluorescence increase was measured with a fluorescence plate reader (Ex 485nm/ Em 530nm). The fluorescence increase in presence of the iron chelator L1 represents oxidation of DCF by several other oxidants (peroxides, hypochlorous acid generated by myeloperoxidases). Measurements between 120 and 375 minutes were used to calculate slopes of DCF fluorescence intensity over time. Shown are the results of one typical experiment carried out in duplicate (out of three independent experiments with similar results).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(µM)
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1470
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370
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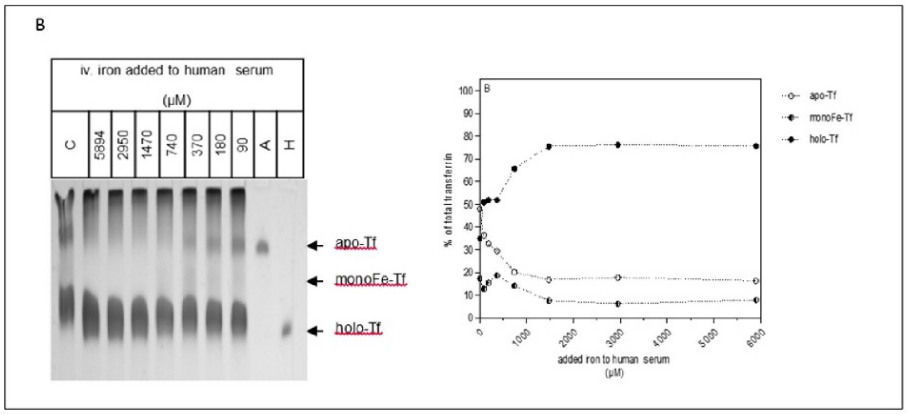
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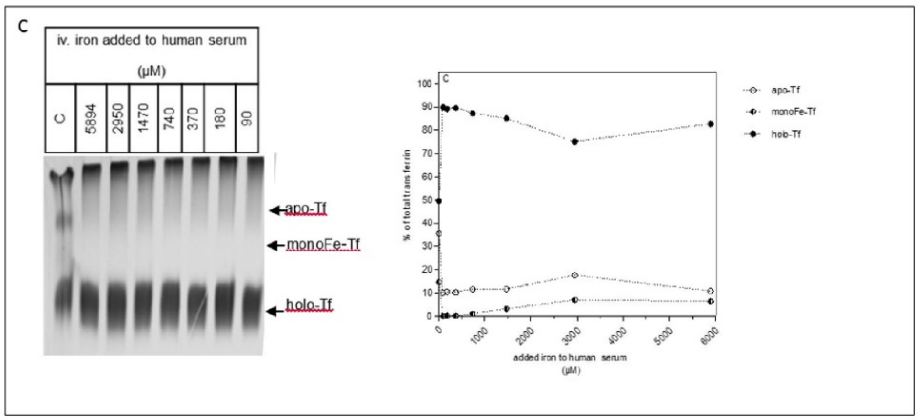

Fig. 8: Direct transfer of IVI to transferrin in human serum

Fig. 8: Direct transfer of IVI to transferrin in human serum

Human serum was incubated with different concentrations of IVI or ferric ammonium citrate (FAC, which represents soluble low molecular weight iron and was used as positive control) for 5 hours at 37°C. To visualize iron transfer from the iron preparations to serum transferrin, at first, most serum proteins (except beta- and γ-globulins) were removed by rivanol precipitation, followed by separation of the differently iron loaded transferrins by urea-PAGE and amidoblack stain. Via densitometry of the stained gels the relative amounts of apo-, holo-, and monoferric transferrin were calculated as percentages of total transferrin in the sample (see Materials and Methods). Incubation of serum was performed with iron sucrose (A), ferric carboxymaltose (B) and ferric ammonium citrate (C). Shown are representative gels stained with amidoblack (on the left side) and quantification of the differently iron loaded transferrins assessed by densitometry (on the right side).The total density of the bands in each sample representing apo-, holo- and monoferric transferrin was set as 100%. The relative amount of differently iron loaded transferrin was calculated as percentage of total transferrin in the sample.

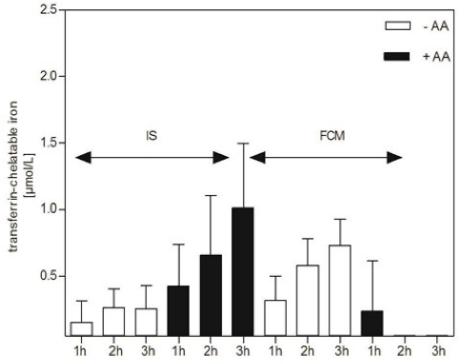

Fig. 9: Ascorbic acid and intravenous iron:

Transferrin-depleted human serum was incubated with 75µM intravenous iron (iron sucrose or ferric carboxymaltose) alone or in the presence of 0,56mM ascorbic acid (AA) for 1 hour at 37°C. The samples were then mixed with reagent A (HBS containing 0.6µM fluorescein-labeled apotransferrin, Fl-aTf) or reagent B (same as reagent A but containing 5mM EDTA) and incubated at 37°C in the dark. After 1h, 2h and 3h the fluorescence was measured in a plate reader Ex 485nm/ Em 535nm. The ratio between the incubation with and without EDTA (reading B/A) was calculated and correlated to a standard curve generated with ferrous ammonium sulphate at concentrations ranging from 0-20µM. Data are presented as means±SEM (n=8). With FCM+AA the bars at 2h and 3h are invisibly small.

The number of tables in a publication is limited. Therefore another control experiment (figure 7) regarding cytotoxicity of intravenous iron (MTT-assay) is shown here.

Incubation of liver parenchyma cells (page 58, figure 5) and THP-1 macrophages (page 58, figure 5) with different concentration of iron sucrose and ferric carboxymaltose showed no differences in viability in liver parenchyma cells. Although ferric carboxymaltose had a lower amount of redox-active iron, macrophages were more sensitive to this iron preparation in the MTT-assay (Fig 7).

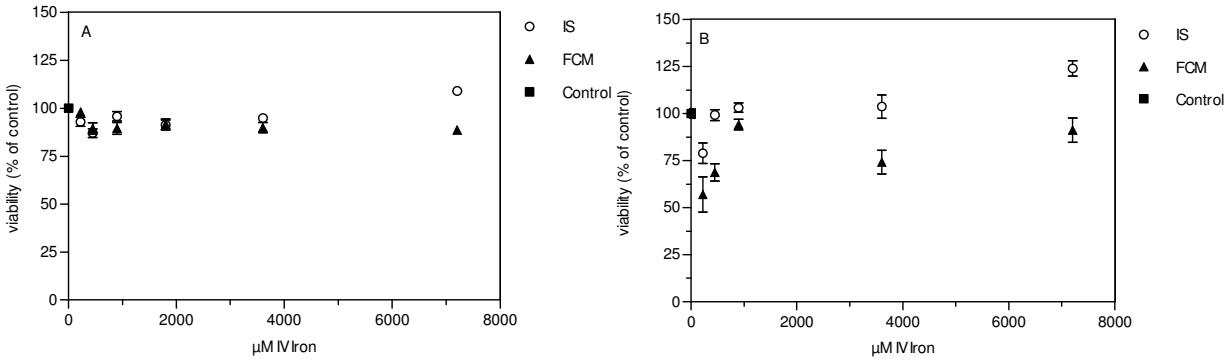

Figure 7: Cytotoxicity of intravenous iron (MTT-assay)

HepG2-cells (A) and THP-1 macrophages (B), cultivated in 96-well plates, were incubated with intravenous iron preparations in DMEM (HepG2-cells) or RPMI medium (THP-1 macrophages), supplemented with 10% fetal calf serum, 2mM glutamine and gentamycin (50µg/mL), for 24 hours at 37°C and 5% CO_2. After removal of the incubation medium and washing of the cells, 100µL fresh medium and 20µL MTT-solution were added to each well and incubated for 30min at 37°C. After removal of the incubation medium, 150µL/well MTT-solvent were added to lyse the cells and for dissolution of the coloured crystals. Absorption was recorded in a plate reader (see Materials and Methods). Shown are the means ±SEM (n=4).

5.3 Using proteomics to assess potential biomarkers of systemic iron trafficking, inflammation and oxidative stress in a patient with PLA2G6 associated neurodegeneration (PLAN) being treated with deferiprone.

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Declaration of contributions to this work

Research Funding was provided by the Rare Disease Foundation of Canada. The patient provided written consent to this research trial via a joint UVic and VIHA ethics approved consent form (CREB # J2013-009).

M.P. assessed all proteins and the related peptidesequences for MRM-measurements, performed the MRM experiments and reviewed the literature. M.M. assessed malondialdehyde (MDA) and 4-hydroxynonanal (4-HN) in plasma, helped with the data analysis and reviewed the literature. J.A., supervised the experiments, supervised data analysis, data processing associated with MRM analysis. S.D. assisted with this project and helped with the data assessment. B.Ch. provided laboratory facilities and assisted with this project. V.E., provided access to PKAN patients and deferiprone.P.M. provided supervision and scientific support. P.B.W. provided supervision, scientific support and obtained funding.

This work was presented as a poster at the International Parkinson and Movement Disorder Society Congress *8-12 June 2014 Stockhom, Sweden.*

Neurodegeneration with brain iron accumulation belongs to a group of degenerative extrapyramidal monogenic disorders with radiological evidence of iron accumulation in the brain (Yoshida et al. 1995), (Gregory et al. 2009), (Levi and Finazzi 2014).

The pathobiochemical mechanism of brain iron accumulation in the different forms of NBIA is not clear. Interestingly is, that there are genes associated with the different NBIA forms that code for proteins directly involved in iron metabolism and those that encode proteins responsible for other functions such as fatty acid metabolism and lysosomal activity (Levi and Finazzi 2014)

One of the differently grouped diseases belongs to the category phospholipase 2 group VI-associated neurodegeneration (PLAN). The best recognized function of this enzyme is the regulation of membranes, by modulating fatty acid recycling and phospholipid amount in the membranes (Balsinde and Dennis 1997), (Winstead et al. 2000). How defects in pathways that underlie phospholipid turnover lead to iron accumulation and, whether the observed brain iron is necessarily toxic is currently unknown. Currently the therapeutic option for these patients is only symptomatic treatment.

However, preliminary clinical trials to treat brain iron accumulation with Deferiprone (Devos et al. 2014), (Mounsey and Teismann 2012) indicate a potential therapy to relieve iron accumulation and the associated increased oxidative stress to dopaminergic neurons in NBIA disorders.

A PLAN patient showed in an axial gradient echo (T2*) bilateral pallidal hypointensities indicative of iron accumulation, without an eye-of-the-tiger sign. (Agarwal u. a., 2012).

Therefore, a clinical pilot study in a patient with PLA2G6 associated neurodegeneration (PLAN) with Deferiprone was performed. Plasma samples from a healthy control and a PLAN patient were collected at baseline and during the 37 week treatment of the PLAN patient with Deferiprone. During this pilot study a total of 4 blood draws was scheduled (Data from the final blood draw were **not** yet available at the time of submission of this thesis). Potential systemic iron parameters, oxidative stress and inflammation biomarkers were reviewed from the literature, selection of the proteins that should be validated by MRM, selection of the peptides for all selected proteins (accession number

(uniprot), protein sequence, plasma concentration) were assessed during this thesis and shown in table 1. From these results a panel of 9 proteins was analysed in the plasma samples, including markers involved in iron metabolism (eg. transferrin, ceruloplasmin and haptoglobin), and inflammation (eg. Lipocalin-2). All samples were run on an Agilent 6490 with ion funnel and analysed as described by Domanski (Domanski et al. 2012). Results are summarized in table 2.

5.3.1 Copper transport protein Ceruloplasmin and inflammatory protein Lipocalin-2

Ceruloplasmin belongs to a small group of Multi-Copper Oxidases (MCOs) in the human body. Ceruloplasmin has shown a variety of physiological functions. Moreover it has been shown that aceruloplasminaemia patients show massive iron overload in various tissues, including liver, pancreas and the brain (Xu et al. 2004), (Harris et al. 1995). The absence of enzymatically-active holoceruloplasmin which confirms the role of ceruloplasmin in iron export seems to be responsible for the iron accumulation in these patients (Xu et al. 2004), (Harris et al. 1995). Interestingly, a murine model with ceruloplasmin/hephaestin double-knockout show protection against increased oxidative stress and retinal degeneration by using the oral iron chelator deferiprone (Hadziahmetovic et al. 2011), (Wolkow et al. 2012).

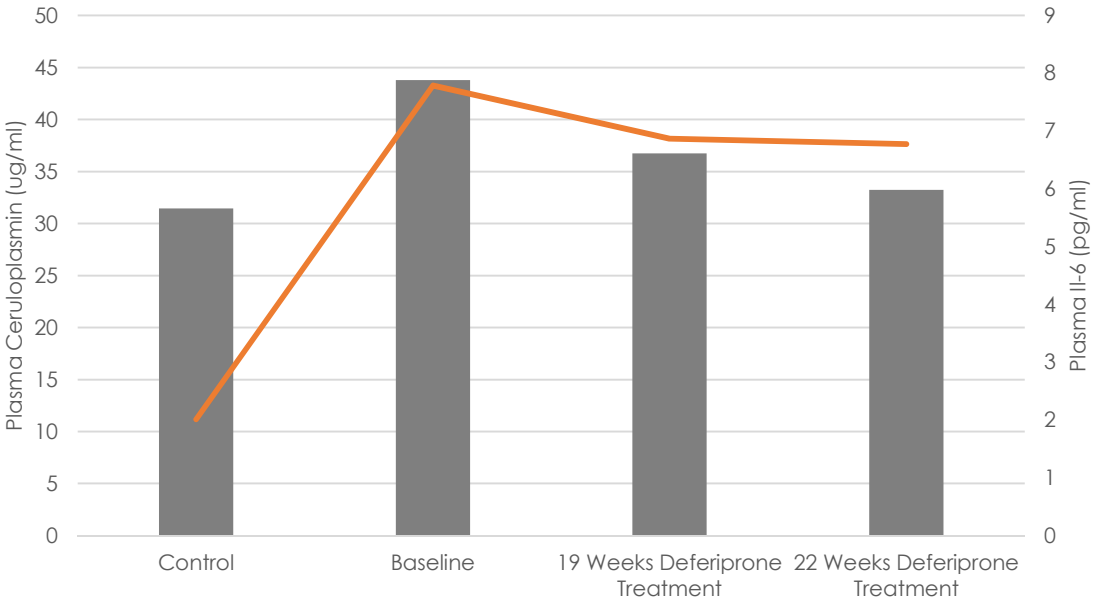

Figure 8: Ceruloplasmin concentration and IL-6

Patient Plasma IL-6 levels over the course of Deferiprone treatment (orange) measured by ELISA. Ceruloplasmin concentration (blue) in PLAN patient plasma was analyzed using MRM proteomics on an Agilent 6490 at the University of Victoria Genome B.C. Proteomics Center.

Assessment of patient plasma using MRM proteomics shows increased ceruloplasmin levels in the PLG2G6 patient compared to control, but turns to normal levels after 19 weeks of treatment. Inflammation parameter IL-6 was increased in the patient, but declined during treatment with Deferiprone.

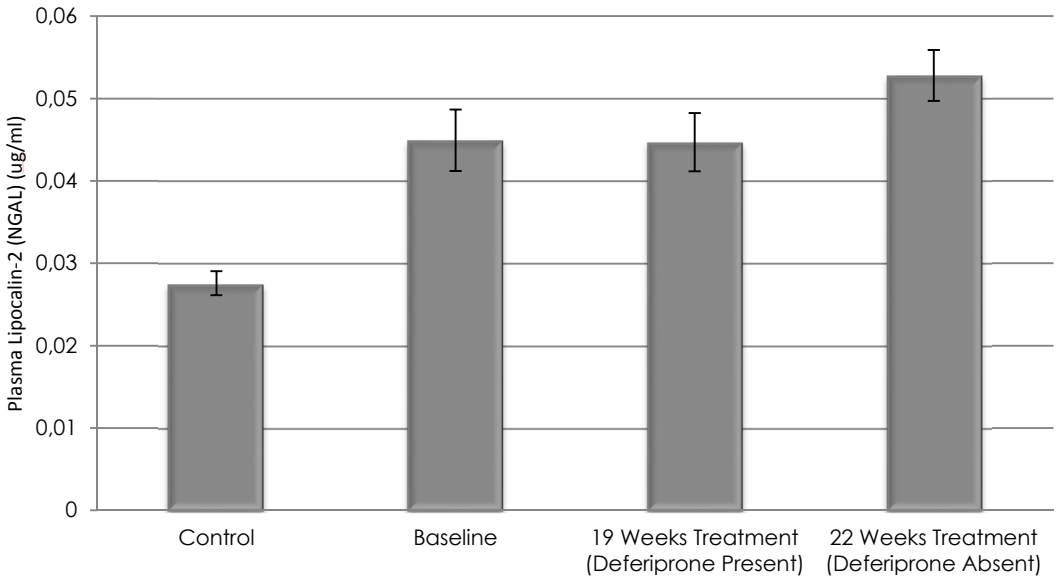

Figure 9: Plasma Lipocalin-2 (NGAL)

Plasma Lipocalein-2 in PLAN patient plasma was analyzed as described by Domanski (Domanski et al. 2012) using MRM proteomics on an Agilent 6490 at the University of Victoria Genome B.C. Proteomics Center. In week 22, deferiprone treatment was dispensed 48 hours prior to the blood draw (resumed immediately following blood draw).

Lipocalin-2 baseline levels were increased in the PLA2G6 patient in comparison to control until the 22 weeks of treatment.

MRM demonstrated increased levels of lipocalin-2, an acute phase protein and ceruloplasmin, an iron trafficking protein in the patient.

5.3.2 Markers of iron trafficking

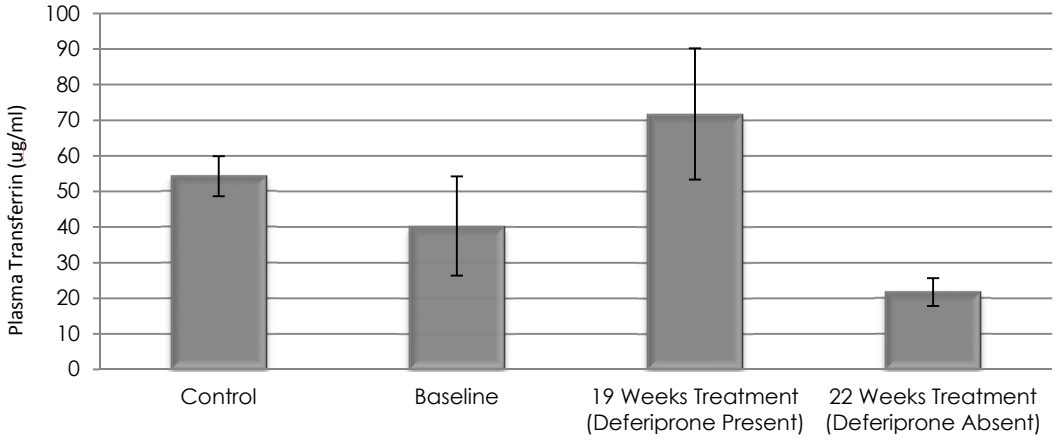

Figure 10: Plasma Transferrin

Plasma Transferrin concentration in PLAN patient plasma analyzed as previously described (Domanski et al. 2012) using MRM proteomics on an Agilent 6490 at the University of Victoria Genome B.C. Proteomics Center. For week 22 blood draw deferiprone was held for 48hours prior to draw (resumed immediately following blood draw).

Transferrin concentrations were in a normal range at baseline and increase during treatment with deferiprone and decline at week 22 when deferiprone is absent.

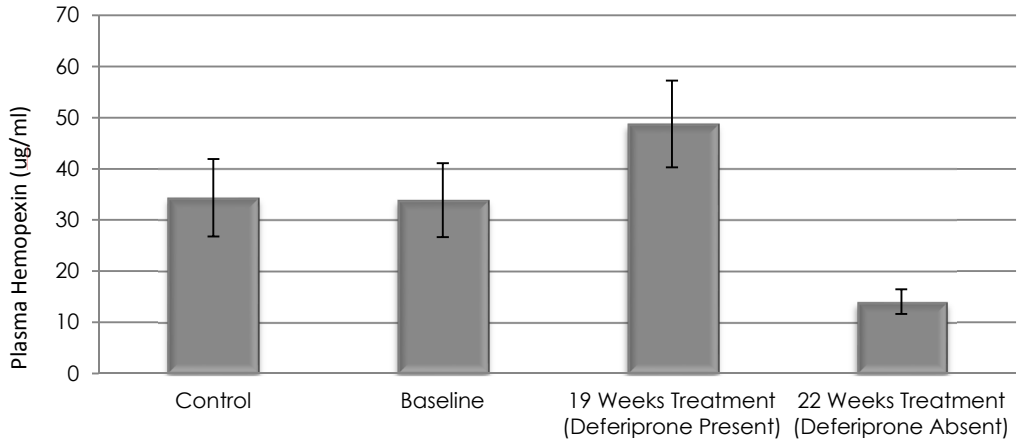

Figure 11: Plasma Hemopexin

Plasma hemopexin concentration in PLAN patient plasma analyzed as previously described (Domanski et al. 2012) using MRM proteomics on an Agilent 6490 at the University of Victoria Genome B.C. Proteomics Center. For week 22 blood draw deferiprone was held for 48hours prior to draw (resumed immediately following blood draw).

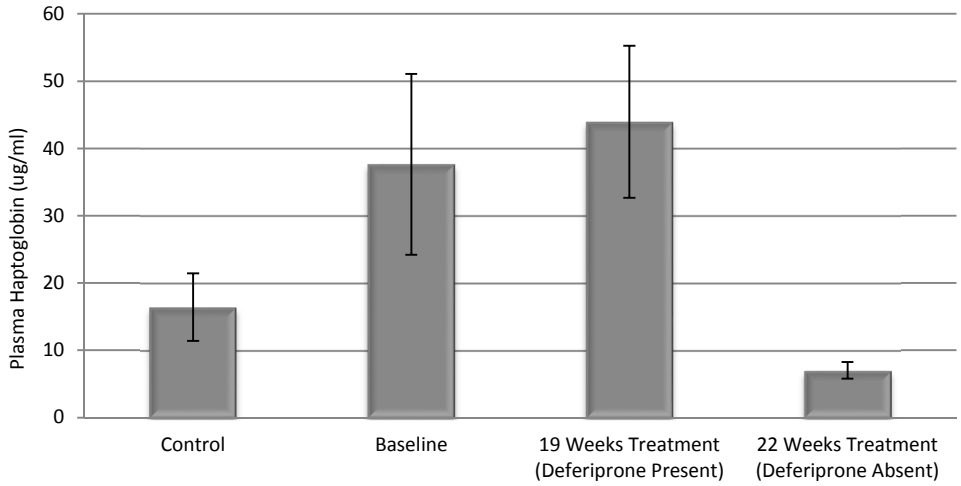

Figure 12: Haptaglobin

Plasma hemopexin concentration in PLAN patient plasma analyzed as previously described (Domanski et al. 2012) using MRM proteomics on an Agilent 6490 at the University of Victoria Genome B.C. Proteomics Center. For week 22 blood draw deferiprone was held for 48hours prior to draw (resumed immediately following blood draw).

Haptoglobin, which binds free hemoglobin, and also prevents from heme-iron mediated oxidation, increased during treatment with deferiprone but significantly decreased in week 22.

These results show that NBIA has multiple effects on systemic iron parameters. A summary of the influcne of the disease on systemic iron parameters in the PLAN patient is shown in table 2.

Analyt	Uniprot Acession No.:	Sequence	Reported plasma concentration (ng/mL)	references (reporting the listed plasma concentrations)
Ceruloplasmin	P00450	DNEDFQESNR	400351,0	(Percy et al. 2013)
		DLYSGLIGPLIVCR		
		NNEGTYYSPNYNPQSR		
		GEFYIGSK		
		DIFTGLIGPMK		
		GAYPLSIEPIGVR		
		HYYIGIIETTWDYASDHGEK		
		ELHHLQEQNVSNAFLDK		
c-reactive Protein	P02741	APLTKPLK	1466,0	(Percy et al. 2013)
		GYSIFSYATK		
		AFVFPK		
		YEVQGEVFTK		
Ferritin -L	P02792	KPAEDEWGK	50,0	(Polanski and Anderson 2007)
		LGGPEAGLGEYLFER		
Gluthatione peroxidase 3	P22352	QEPGENSEILPTLK	17919.0	(Percy et al. 2013)
		TTVSNVK		
		PGGGFVPNFQLFEK		
Habtaglobin	P00738	DIAPTLTLYVGK	1337995.0	(Percy et al. 2013)
		VGYVSGWGR (+10)		
		HYEGSTVPEK		
		VVLHPNYSQVDIGLIK		
Hemoglobin subunit alpha	P69905	TYFPHFDLSHGSAQVK	4898.0	(Percy u. a. 2013)
		VGAHAGEYGAEALER		
		FLASVSTVLTSK		
Hemopexin	P02790	LYLVQGTQVYVFLTK	399702.0	(Percy et al. 2013)
		SGAQATWTELPWPHEK		
IL-18	Q14116	ISTLSCENK	0.059	(Anderson 2005)
IL-1beta	P01584	ISDHHYSK	0.0050	(Polanski and Anderson 2007)
IL-8	P10145	TYSKPFHPK	0.083	(Polanski and Anderson 2007)
		ENWVQR		
Insulin (beta chain)	P01308	GFFYTPK	2.0	(Anderson 2005)
MMP8	P22894	ISQGEADINIAFYQR		
		VDAVFQQEHFFHVFSGPR		

Analyt	Uniprot Acession No.:	Sequence	Reported plasma concentration (ng/mL)	references (reporting the listed plasma concentrations)
NGAL	P80188	ITLYGR	87.0	(Anderson 2005)
		VPLQQNFQDNQFQGK		
		SYPGLTSYLVR		
		WYVVGLAGNAILR		
sTfR	P02786	LAVDEEENADNNTK (+8)	1012.0	(Percy et al. 2013)
		VEYHFLSPYVSPK		
		VSASPLLYTLIEK		
TGF-alpha	P01375	TGGPQGSR (+10)	0.0059	(Polanski and Anderson 2007)
TGF-beta	P01137	VAGESAEPEPEPEADYYAK	14.0	(Polanski and Anderson 2007)
		VEQHVELYQK		
Transferrin (serotransferrin)	P02787	HSTIFENLANK	623834.0	(Percy et al. 2013)
		EFQLFSSPHGK		
		DGAGDVAFVK		

Table 1: Proteins and related peptidesequences for MRM-measurements

Marker	Role	Comparison	Effect
Haptoglobin	Hemoglobin Transport	Patient Baseline vs Control	Elevated (129%)
Lipocalin-2 (NGAL)	Marker of Inflammation	Patient Baseline vs Control	Elevated (62%)
Ceruloplasmin	Ferroxidase and Copper Transport	Patient Baseline vs Control	Elevated (39%)
Transferrin	Iron Transport	Patient Baseline vs Control	Decreased (26%)
Hemopexin	Heme Transport	Deferiprone Present vs Deferiprone Absent	Elevated (43%)
Transferrin	Iron Transport	Deferiprone Present vs Deferiprone Absent	Elevated (78%)
Haptoglobin	Hemoglobin Transport	Deferiprone Present vs Deferiprone Absent	Elevated (17%)
Transferrin	Iron Transport	Patient Baseline vs 22 Weeks of Treatment	Decreased (46%)
Glutathione Peroxidase	Prevention of Oxidative Damage	Patient Baseline vs 22 Weeks of Treatment	Decreased (49%)

Table 2: Summary of the results of MRM analysis

Control and patient plasma were analyzed as previously described (Domanski et al. 2012) using MRM proteomics on an Agilent 6490 at the University of Victoria Genome B.C. Proteomics Center.

5.3.3 Discussion

Neurodegeneration with Brain Iron Accumulation (NBIA) is a group of neurodegenerative disorders that share a common hallmark of iron accumulation inside the Substantia Nigra. Excess iron is potentially cytotoxic due to the reactive Fenton chemistry and is therfore associated with increased oxidative neuronal cell death and parkinsonism (Miller et al. 2009),(Mounsey and Teismann 2012). It has been shown that the degree of iron accumulation is associated with the progression and severity of neurodegeneration (Adibhatla and Hatcher 2010). Additionally, it may also be associated with increased systemic oxidative stress and inflammation as well as altered systemic iron metabolism. PARK14 Parkinsonism is a form of NBIA involving mutations in the PLA2G6 gene which encodes the group VI calcium independent phospholipase A2, which is involved in phospholipid remodeling and membrane homeostasis (Morgan et al. 2006).

We were using proteomics to assess potential biomarkers of systemic iron trafficking, inflammation and oxidative stress in a patient with PLA2G6 associated neurodegeneration (PLAN) being treated with Deferiprone. The patient showed reduced systemic transferrin expression at baseline, which is also present in other diseases of iron overload such as hemochromatosis. Further, increased inflammation parameters like IL-6 and NGAL were detected but declined during Deferiprone treatment. Ceruloplamsin, an acute phase protein was increased in the patient's plasma at baseline, which can also be induced by IL-6 but was decreased during Deferiprone treatment. Iron trafficking proteins (haptaglobin, transferrin and hemopexin) may be elevated due to the response to the iron chelator Deferiprone. MRM based proteomics was successful in measuring a preliminary panel of markers in patient plasma at baseline and during deferiprone treatment.

A possible action of Deferiprone was to bind excess iron leading to reduced systemic oxidative stress accompagnied by a reduction of gluthatione peroxidase.

5.4 Introduction of a new infrared (IR)-based spectrometry system to overcome challenges to assess frataxin protein levels *in vivo* in Friedreich`s ataxia patients treated with resveratrol.

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An open-label trial in Friedreich ataxia suggests clinical benefit with high-dose resveratrol

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EMY, GT, REP, KJL, KDC, TAM, BS-M, BS, MP, APV, GR, SEMS, CS, CYL, MVE-G, PJL and MBD contributed to data collection, analysis and interpretation.
All authors contributed critical review and/or writing of the manuscript.

Friedreichs ataxia is an autosomal recessive inherited disease. It is cause by an expansion of GAA trinucleotides in the region of chromosome 9 resulting in reduced expression of the mitochondrial protein frataxin. Furthermore a clinical study showed a correlation of frataxin content in blood lymphocytes and skeletal muscle. In addition muscle frataxin levels were shown to correlate with the Score for Assessment and Rating of Ataxia (SARA) (Nachbauer et al. 2011).

Decreased frataxin levels are associated with increased oxidative stress in Friedreichs ataxia patients. To lower oxidative stress, resveratrol, a natural antioxidant was proposed to have beneficial effects in FRDA patients. Mouse studies showed that application of resveratrol resulted in a 2-4 fold increase in frataxin expression. Therefore, a clinical study with two different doses of resveratrol in individuals with FRDA over a 12-week period was performed to investigate the effect of resveratrol on lymphocyte frataxin levels *in vivo.*

Participants were recruited from the FRDA clinic at Monash Health, Australia. A randomly not blinded assigned treatment arm of a low dose or high dose was performend. The low dose group received 500mg resveratrol twice daily (BD) (99.5% pure trans-resveratrol, 500mg capsules, Megaresveratrol, Danbury, CT). 2.5g BD resveratrol were given to the group of the high dose. Assessment of adverse events by the Data Safety Committee (DSC) occurred after participants in the low dose group (Stage 1) had completed at least 4 weeks of treatment on the study drug. Enrolment of participants into the high dose group (Stage 2) commenced only after approval by the DSC.

Lymphocyte frataxin levels were assessed at baseline and at week 12 of treatment with resveratrol. Frataxin levels of lymphocytes were assessed by an improved frataxin ECLIA and were normalized to the protein contents of the samples which were assessed by a new infrared (IR)-based spectrometry system (Direct Detect® (Millipore, Austria).

5.4.1 Frataxin expression in low and high dose resveratrol treatment groups

Resveratrol dose	1 g daily (n=12)		5 g daily (n=12)	
Outcome measure	**Difference Mean (95% CI)**	**p-value**	**Difference Mean (95% CI)**	**p-value**
Frataxin (pg/µg protein)	0.08 (-0.5, 0.21)	0.21	0.03 (-0.10, 0.15)	0.62

Figure 13: Effect of resveratrol on frataxin expression in low and high dose treatment groups

Levels of lymphocyte frataxin did not change either with low dose [change in frataxin: 0.08 pg/µg protein (95% CI -0.05, 0.21, p=0.21)] or high dose resveratrol [change in frataxin: 0.03 pg/µg protein (95% CI -0.10, 0.15, p=0.62)] after 12 weeks of treatment.

5.4.2 Discussion

In vitro and *in vivo* animal models of FRDA treated with resveratrol show an increased frataxin protein and *FXN* mRNA expression (Li et al. 2013). Therefore this open-label human clinical pilot trial in FRDA was conducted. The primary outcome of this study was to assess the effect of resveratrol on lymphocyte frataxin protein levels after 12 weeks of treatment with either low (1 g) or high dose (5 g) resveratrol daily. There were no significant changes in frataxin protein expression prior resveratrol treatment either in the low or high dose treatment group. The improved method for quantification of frataxin with the frataxin ECLIA in combination with the infrared (IR)-based spectrometry system (Direct Detect®) for accurate protein quantification showed higher accuracy and time savings in sample preparation and test execution.

6 Discussion

6.1 General discussion

Intravenous iron preparations

are widely used to overcome anemia where oral iron administration is inefficient. Recently, new ISS preparations entered the market and concerns about safety and equivalency with the originator arise (Committee for Medicinal Products for Human Use, 2011). In addition, a study shows some evidence that certain ISS have different safety and efficacy profiles compared to the originator (Toblli et al. 2009).

However, intravenous iron preparations are also known as non-biological complex drugs (NBCD). "This term describes a medicinal product, not being a biological medicine, where the active substance is not a homo-molecular structure, but consists of different (closely related and often nanoparticulate) structures that cannot be isolated and fully quantitated, characterized and/or described by state of the art physicochemical analytical means and where the clinical meaning of the differences is not known. The composition, quality and *in vivo* performance of NBCD are highly dependent on manufacturing processes of both the active ingredient as well as in most cases the formulation" (Schellekens et al. 2014).

Therefore, it is from tremendous interest that intravenous iron preparations are evaluated in terms of bioavailability and stability equivalency of the originator vs. ISS. Further it seems prudent also for physicians as well as patients who require intravenous iron to have available data on therapeutic equivalency of new ISS preparations versus the originator. Also in some former *in vitro* studies we found significant differences in the bioavailability profiles of different classes of intravenous iron preparations compared to the iron sucrose originator (Sturm et al. 2010).

It seems that NBCD are vulnerable for changing composition, quality and *in vivo* performance, but it should be mentioned that "originator" have also their challenge to meet the target qualifications of consistent analytical quality as seen in biological drugs (Schiestl et al. 2011).

Rottembourg et. al. conducted a clinical study to assess the equivalency of ISS and originator in presence of EPO and conclude that ISS do not behave as efficient as the originator. Further, due to higher administration policy of EPO and ISA, to reach target Hb levels, they saw no cost benefits for the health care system (Rottembourg et al. 2011).

EPO is known to influence erythropoiesis and it is very likely that renal patients show comorbidities like EPO hyporesponsiveness (Gilbertson et al. 2013),(Eschbach et al. 1992). Therefore it seems rather unsophisticated to draw conclusions about equivalency of intravenous iron sucrose products where such a dominant confounder was not taken into account. In this regard also other pathobiochemical mechanisms like the iron regulatory protein hepcidin, which strongly influences iron metabolism independent of the iron preparation used, should be mentioned here.

The study outcome and evaluation parameters of this head-to-head patient's population study show higher Hb levels saturation of patients treated with the originator. Interestingly, there was no discussion regarding the strong relationship between hemoglobin (Hb) levels and mortality in patients with chronic kidney disease (CKD). To clarify, it is generally accepted that high Hb levels are associated with increased risk of hypertension, stroke and hospitalizations (Jing et al. 2012). Also guidelines for clinical practice recommend Hb targets in the range of 10.0—12.0 g/dL (Mactier et al. 2011).

In addition, it has been shown that there was a significantly higher transferrin saturation by using the originator, but there was no biochemical discussion about these results. Although it is recognized, that oversaturation of Tf represents a dangerous source for the formation of toxic hydroxyl radicals and reactive oxygen species. (Hershko et al. 1978),(Espósito et al. 2002). (McCord 1998).

To address this, the quality of intravenous iron formulations were determined by the presence of redox-active iron. Kakhlon and colleagues showed that there is evidence for occurrence of tissue iron overload (eg. liver) in long-term treatment with intravenous iron (Kakhlon and Cabantchik 2002a). *In vitro* studies with liver parenchyma cells, as a medical representative of toxicological target cells, show that various intravenous iron

compounds are incorporated by liver cells, which mirrors the results of the clinical studies.

In this *in vitro* study we compared the iron sucrose originator Venofer® and the iron sucrose generic "Iron Sucrose AZAD" (ISA) in terms of iron related differences regarding bioavailability by using a set of different assays.

First, uptake of iron from iron sucrose compounds were assessed in HepG2 cells and THP-1 macrophages to evaluate possible difference in their efficacy to overcome iron deficiency anaemia. No significant difference in the uptake rates between both compounds in HepG2 cells and in THP-1 macrophages were found.

After administration of intravenous iron and entering the cells the iron should be available for the body. Therefore, the ability of iron release from iron-loaded cells was assessed. Liver parenchyma cells released more iron than THP-1 macrophages, but no significant difference between Venofer® and ISA was observed. In particular HepG2 cells released iron within 30 minutes, whereas THP-1 cells need a time period of 2 hours. It seems that iron in HepG2 cells was more easily accessible than the iron in THP-1 cells.

The LIP represents a small part (<5%) of the chelatable total iron content and is defined as transient redox-active and labile iron that rapidly passes through the cell. The iron uptake of transferrin or non-transferrin bound iron is the major source of the LIP, after sequestration by the target proteins. LIP level is midway between the cellular need for iron and the hazard of excessive generation of hydroxyl radical (Kruszewski 2003). Further it has been proposed that cellular source of iron ions of the LIP play a role for Fenton reaction (Breuer et al. 1997). When assessing the amount of iron which enters the LIP in HepG2 cells from Venofer® and ISA, we found a similar dose depended increase of cytosolic iron concentration with both products.

When the cellular LIP rises, the iron regulatory proteins (IRPs) lose their ability to bind to iron responsive elements (IRE) in several mRNAs. This, among other effects, leads to an increase in the synthesis of ferritin, the major iron storage protein. Iron bound to ferritin is harmless; synthesis of ferritin results in incorporation of cytosolic iron and therefore a decrease of the labile iron pool. Thus, ferritin is the major defence against

iron toxicity. In our study, we could not find differences in ferritin synthesis between the two products.

As mentioned before, intravenous iron treatment has to be strongly controlled due to the danger of excess iron that generates ROS after iron infusion. Therefore, the amount of redox-active iron within the intravenous iron products was assessed. The iron transport protein transferrin is an important defence system that acts as an iron buffer in the plasma, which keeps the intracellular concentration of redox-active iron low and further avoids adverse reactions. Therefore, we tested transferrin binding capacity and found that the scavenging of redox-active iron by human serum is completely the same with both iron sucrose products at all concentrations tested.

Tobllie et all showed organ damage and oxidative stress in rats following administration of high concentration of intravenous iron (Toblli et al. 2012b). This is in contrast to our *in vitro* study which showed no difference in cytotoxicity parameters in liver parenchyma cells with both products.

Taken together no differences were seen between the stability of the two products regarding transferrin chelatable iron, the effect of ascorbic acid on transferrin chelatable iron and on redox-active iron generated in cell free experiments. In human serum, the amount of redox-active iron at clinically effective concentrations of both products was equally low. Our results indicate that there are no significant differences in uptake, increase in their labile iron pool and storage in ferritin as well as release of intravenous iron from human liver parenchyma HepG2-cells nor THP-1 macrophages.

These results are in line with Elford et al. who assessed the distribution of the two compounds in mice and in general saw no significant differences in tissue iron levels (in plasma, spleen, bone marrow, liver, heart, stomach, kidneys, liver or lungs) (Elford et al. 2013). More studies are needed to address the question about safety and equivalency of intravenous iron sucrose products with respect to possible confounders."

Other intravenous iron formulas, such as ferric carboxymaltose, a new parenteral iron preparation, have been approved for more rapid administration of large i.v. iron doses, which may be more favourable and time- and cost-saving for the patients, since physician visit-frequencies are decreased. Both products are iron carbohydrate

complexes, but possess different physicochemical stabilities that may differently affect anaemia management (Geisser u. a., 1992b), (Jahn et al. 2011).

Iron sucrose (IS) and ferric carboxymaltose (FCM) were not cytotoxic for HepG2-cells. THP-1 macrophages were more sensitive to FCM compared to IS at all concentrations. It is possible that the higher damage results from extracellular iron released from FCM. FCM releases more iron directly than IS.

Released bioactive iron from iron preparations may also play a role in oxidative stress and inflammation (Crichton et al. 2008). With IS we found higher concentrations of redox-active iron in comparison to FCM .

Biostability of an iron preparation can be tested by the ability to transfer iron directly to transferrin with urea-PAGE and was reported to correspond to the chemical stability of the IVI complexes (i.e. FCM>IS) (Crichton et al. 2008). Interestingly, by using a more realistic approach and assessing direct transfer to transferrin in human serum instead of transferrin in buffer, the biostability order was reversed (i.e. IS>FCM) (Haider 2010). These results were confirmed by assessment of transferrin-chelatable iron by a fluorescence-based method. It should always be kept in mind that other plasma components besides transferrin influence bioavailabilty of the preparations *in vivo*.

Ascorbic acid enhances iron mobilization to transferrin in serum from IS, but not from other iron preparations (Sturm et al. 2005),(Wang et al. 2008) and the order of chemical stability is not predictive for biostability in presence of ascorbic acid. In our experiments, as expected, transferrin-chelatable iron from IS increased in presence of ascorbic acid, but unexpectedly declined with FCM. This is clinically relevant, since ascorbic acid is investigated as a supplement for haemodialysis patients with rhuEPO hyporesponsiveness (Tarng 2007),(Attallah et al. 2006),(Sezer et al. 2002),(Jalalzadeh et al. 2012).

In conclusion, ferric carboxymaltose has low iron–bioavailability for liver parenchyma cells, therefore liver iron overload is very unlikely. Ascorbic acid reduced transferrin-

chelatable iron from ferric carboxymaltose, therefore effects on hepcidin expression should be investigated in clinical studies.

Friedreich´s Ataxia

is an inherited disease with a prevalence of 1 in 30.000 people. The mutated gene of this disease encodes a small mitochondrial protein called frataxin (Campuzano et al. 1997), (Campuzano et al. 1996). Mice studies have shown that a complete knockout of frataxin leads to early embryonic death and is not compatible with life (Cossée et al. 2000). Further it has been shown that decreased frataxin expression in Friedreich´s ataxia patient lymphocytes correlate inversely with age of onset and the severity of FRDA measured by the Friedreich Ataxia Rating Scale (Evans-Galea et al. 2012). However, it is known that frataxin has the ability to interact with proteins, which are involved in mitochondrial Fe-S biogenesis. This implicates that frataxin plays a role in Fe-S cluster assemble but the cellular mechanism that combine frataxin deficiency to mitochondrial iron overload is not clear. However, resveratrol, an antioxidant, was shown to modify frataxin levels in animal studies (Li et al. 2013). In a clinical study where Friedreich`s ataxia patients were treated with resveratrol for 12 weeks, no significant changes in frataxin protein expression could be observed. In this study frataxin levels in patient lymphocytes were assessed by an improved method using a combination of frataxin-ECLIA and a new infrared (IR)-based spectrometry system for more accurate frataxin quantification which is time saving in sample preparation and test execution.

Neurodegeneration with brain iron accumulation (NBIA)

belongs to a group of degenerative extrapyramidal monogenic disorders with radiological evidence of iron accumulation in the brain.

Using proteomics biomarkers of systemic iron trafficking, inflammation and oxidative stress in a patient with PLA2G6 associated neurodegeneration (PLAN) treated with the iron chelator Deferiprone showed reduced systemic transferrin expression at baseline, which is also present in other diseases of iron overload such as hemochromatosis. Inflammation parameters like IL-6 and NGAL were increased but declined during Deferiprone treatment. Ceruloplamsin was increased at the baseline in the patient’s plasma, which can also be induced by IL-6, but is decreased during Deferiprone

treatment. Iron trafficking proteins (haptoglobin, transferrin and hemopexin) may be elevated due in response to the iron chelator Deferiprone. MRM based proteomics was successful in measuring preliminary panel of markers in patient plasma at baseline and during deferiprone treatment.

6.2 Conclusion and future prospects

The present work aimed to

(1) investigate possible differences in biostability and efficacy of different intravenous iron preparations in a non-clinical study. Intravenous iron preparations are used when oral iron is insufficient to meet the body`s iron demand, like in patients with renal anemia.
(2) focus on the influence of resveratrol, an antioxidant, to improve frataxin expression in Friedreich`s ataxia patients. Frataxin is known to play a role in iron sulphur cluster assembly and heme biosynthesis.
(3) concentrate on a fingerprint analysis of plasma of a NBIA patient with brain iron accumulation, who was treated with the iron chelator Deferiprone. This approach should lead to the detection of biochemical markers of disease progression.

Taken together this work gave several new biochemical insights on iron related disorders by investigation, description and evaluation of the current state of medical knowledge on diagnostic tests and therapeutic techniques.

In case of anemia of chronic kidney disease we focused on treatment, safety and efficacy of intravenous iron preparations as well as aspects to decrease costs of the medical health care system. We found that generic iron sucrose AZAD and the iron sucrose originator Venofer have no differences regarding safety, biostability and metabolism in a non clinical study with liver parenchyma cells and macrophages.
Iron sucrose and ferric carboxymaltose, which represents a new a high dose intravenous iron formulation, were investigated regarding iron metabolism and biostability. We found that FCM has low bioavailability for liver parenchyma cells, therefore liver iron

deposition is unlikely. Ascorbic acid reduced transferrin-chelatable iron from ferric carboxymaltose, thus effects on hepcidin expression should be investigated in clinical studies.

Friedreich´s ataxia is an inherited disease with increased iron accumulation in mitochondria and in the brain and increased oxidative stress. It is known that the number of GAA repeats inversely correlates with frataxin expression, severity and age of onset of the disease. Resveratrol, a natural antioxidant has been shown to increase frataxin levels in a mouse model and in patient fibroblasts and lymphocytes. In a clinical pilot study with resveratrol in Friedreich`s ataxia patients for 12 weeks, no beneficial effect in terms of increasing Frataxin levels were observed. Further clinical studies with longer treatment periods and a different dose regime are warranted.

Neurodegeneration with brain iron accumulation (NBIA) with mutation in the PG2G6 gene displays a deficiency of the enzyme phospholipase A2G6. The underlying mechanism leading to the development of brain iron accumulation and oxidative stress generation is still unknown.
From clinical studies it is known that the iron chelator deferiprone is able to lower brain iron. In a clinical pilot study with Deferiprone in a PG2G6 patient, proteins involved in iron metabolism were screened during Deferiprone treatment to shed light on possible disturbances in iron metabolism. Analysis of the full panel of proteins involved in iron metabolism, oxidative stress and inflammation as proposed in this thesis should lead to the detection of biochemical markers of disease progression in the future.

Taken together, iron is a crucial component in the body and has to be tightly controlled and any misbalance has tremendous effects on human health. Knowledge of iron metabolism in health and disease is therefore a key component for identifying new active ingredients for innovative therapeutic approaches in the future.

7 Appendix: Materials and Methods

Chemicals and reagents were acquired from Sigma-Aldrich, Merck or Roth, unless otherwise indicated.

7.1 Cell culture

All materials were sterilized at 180°C. In addition sterilisation of the laminar flow hood by UV-radiation for 15 minutes was used.

7.1.1 Cell lines

7.1.1.1 THP-1 cells

THP-1 is a human monocytic cell line, which was created in 1980 from the blood of an one year-old boy, diagnosed with acute monocytic leukemia (Tsuchiya et al. 1982). The peculiarity of this cell line is the ability to differentiate into macrophages under certain conditions.

7.1.1.2 HepG2 cells

HepG2 (hepatocellular carcinoma, human) cells are an adherent cell line, growing as monolayer and in small aggregates. This cell line was derived from the liver tissue of a 15 year-old male with differentiated hepatocellular carcinoma.

7.1.2 Preparation of cell culture media

7.1.2.1 Cell culture medium for THP-1 cells

The powder medium (RPMI 1640 Instamed 10.34g/l, PAA Laboratories GmbH) was dissolved in 9L water and 10g sodium bicarbonate ($NaHCO_3$). A pH-value of 7.25 was adjusted using HCl and filled up to a volume of 10 liters. In the Laminar Air Flow (clanLAF), the medium was pumped into 0.5 L glass bottles which was previously sterilized through a filter with a pore size of 0.2 µm (Satorius). The bottles was stored at 4°C.

By addition of 10% fetal bovine serum (FBS, Bichrom), 2mL L-glutamine (PAA Laboratories GmbH) and 0.1mg/mL gentamycin (PAA Laboratories GmbH) the complete growth medium was obtained.

7.1.2.2 Cell culture medium for HepG2 cells

For the cultivation of HepG2 cells, Dulbecco's Modified Eagle's Medium (DMEM high glucose 4.5g/L, PAA Laboratories GmbH) was used. The production process was carried out as described before in 4.1.2.1.

7.1.3 Cell culturing

7.1.3.1 THP-1 cells

Sterile culture flasks (TPP, 75cm^2) with 20mL cell suspension were cultured in an incubator (Binder) at 37°C and 5% CO_2 atmosphere. Every second or third day fresh 37°C nutrient medium was added under sterile conditions (Laminar Air Flow) to the cells to obtain a cell density between 0.4 to 0.9 million cells/ml.

7.1.3.2 HepG2 cells

These cells are adherent cells and were cultured in steril culture flasks (TPP, 75cm^2) with 20 mL medium and grown in an incubator (Binder) at 37°C and 5% CO_2 atmosphere. To passage cells, the medium was sucked of and the cell monolayer was briefly rinsed with pre-warmed (37°C) 0.05% trypsin- EDTA solution for 2-3 minutes. Afterwards the trypsin was removed and again pre-warmed (37°C) 0.05% trypsin-EDTA solution was added and incubated at 37°C and 5% CO_2 atmosphere until the cells were detached from the surface (5-10 minutes). To deactivate trypsin an equal volume of complete growth medium was added. The cells were split every 2 to 3 days and seeded at a density of 0.5 millions cells/ml in new flasks.

7.1.4 Cell Counting

The cell number was measured with the automatic cell counter Microcellcounter CC-108 (Sysmex®). Under sterile conditions 1 mL of the cells was transferred from the culture flask into an Eppendorf tube. Afterwards under non-sterile conditions, 200µL of the cell suspension were moved into a special counter vessel and mixed with 9.8mL of a counter liquid (Cellpack PK-30, Sysmex®) and measured twice with the cell counter. In addition, this technique did not discriminate between vital and dead cells.

7.1.5 Cryopreservation of cells

To store cells for a longer period of time, liquid phase nitrogen storage is required. Therefore, the cells were counted and centrifuged at 1800rpm for 5 minutes at 4°C. The supernatant was removed and the cell pellet was resuspended in a cyroprotective medium (DMEM or RPMI full growth medium and 10% (v/v) DMSO) to obtain a cell count of 3 million cells per milliliter. In the laminar flow 1 or 1.5mL of the cell suspension was transferred into cryotubes (Cryovial) and placed into a special container (Nalgene Cryo 1°C Freezing Container). This container was filled with isopropanol to ensure a slow freezing rate of -1°C per hour at -80°C. After 72 hours the cryotubes were ready to be stored in the liquid phase nitrogen.

7.1.6 Thawing of THP-1 and HepG2 cells

Rapid thawing was very important to ensure high cell viability. Cryotubes were taken from the nitrogen storage and placed into ice. Afterwards the cryotubes were placed into a 37°C water bath before the cell suspension was diluted in 10mL RPMI or DMEM culture medium and transferred into culture vessels (THP-1 cells – in flasks for non-adherent cells, HepG2 cells – in flasks for adherent cells).

7.1.7 Differentiation and incubation of cells

7.1.7.1 Differentiation of THP-1 cells to macrophages

Differentiation of THP-1 cells to macrophages was induced by addition of PMA (phorbol 12-myristate 13-acetate; stock solution 810µM in DMSO, stored at -20 °C) (Tsuchiya et al. 1982).

2mL cell suspension per well (containing 0.5 million cells/mL) were seeded in 6 well-plates (Greiner-Bio-one GmbH) in the presence of PMA (160µM). After approximately 72 hours in the incubator differentiation was completed.

7.1.7.2 Incubation of differentiated THP-1 cells

Four days following initiation of differentiation by PMA, the cells in 6-well-plates were ready for the experiments. Just before the experiment the culture medium was removed

and the cells were covered with 1mL of different dilutions of the iron formulations in supplemented RPMI medium. Incubation periods varied between 3 and 24 hours at 37°C and 5% CO_2 atmosphere.

7.1.7.3 Incubation of HepG2 cells

HepG2 cells were seeded in 6-well-plates (0.5 Mio cells/mL per well) and stored in an incubator. After 3 days, the cells were used for the experiments. Again, just before the incubation with the reagent of interest the culture medium was removed and then the cells were covered with 1mL of different dilutions of iron formulations in supplemented DMEM medium. Incubation periods varied between 3 and 24 hours at 37°C and 5% CO_2 atmosphere.

7.1.8 Incubation with different intravenous iron preparations

All iron formulas in the experiments were used at maximal clinically relevant concentrations and were always corresponding to those expected in the plasma of patients after receiving the maximal recommended single dose. From pharmacokinetic studies in healthy volunteers it is known that the mean volume of distribution of the central compartment is 3L, hence close to the volume of plasma (Danielson et al. 1996). The expected plasma concentration of intravenous iron after infusion of 100mg IVI is therefore close to 600µmol/L serum, and with 300mg one can expect serum concentrations close to 1800µmol/L.

7.1.8.1 Preparation of cell lysates

Cell lysis was performed using the following solutions:
NP-40 cell-lysis buffer:
150mM NaCl
1 % IPEGAL CA630 (NP-40)
50mM Tris (hydroxymethyl)-aminomethan, pH 8.0
Storage at 4°C.
Before use 1mM phenylmethylsulfonylfluorid (PMSF), a protease inhibitor was added to the NP-40 cell lysis buffer.

The next steps were performed on ice.

The supernatant was removed and the cells were incubated with medium containing 50µM diethylene triamine pentaacetic acid (DTPA) for 5 minutes on the belly dancer. Following removal of the washing solution the cells were washed twice with cold FCS free medium to remove surface bound iron. The 6-well plates were stored overnight at -20°C.

Then, the cells were lysed with ice-cold NP-40 lysis buffer (350µL per well) containing 1mM of the protease inhibitor PMSF on the belly dancer for 10 minutes.

Then the cells were detached from the plate with a cell scraper (Greiner-Bio-One GmbH) and the lysate was transferred to the next well. This ensured that all cells from the 6-well plate were collected in a small volume of 1000µL lysis reagent and transferred to one single microcentrifuge tube. The lysates were shortly sonicated (Inula) and stored at -20°C.

7.1.8.2 Quantification of protein in the cell lysates

The protein content of the lysates was measured by the Bio-Rad Protein Assay. This method is based on a complex formation between Coomassie-Briliant Blue G-250 and free amino groups of the proteins which ensures a colour change form brown to blue and a measurable shift in the absorption spectrum from 465nm to 595nm (Bradford 1976).

Concentration (µg/µL)	µL BSA solution	µL H_2O dest.
0	0	800
2	20	780
4	40	760
6	60	740
8	80	720
10	100	700

Table 3: Pipetting scheme of protein standard solutions

Next, dilutions of bovine serum albumin (BSA) were prepared in duplicates from a 0.1mg/mL bovine serum albumin (BSA) solution according to the pipetting scheme shown in table 3. The samples (0.5 to 2µL) were diluted to a final volume of 800µL with distilled water. 200µL of Biorad reagens (Bio-Rad Laboratories GmbH) were

added to all standards and samples and mixed. After an incubation of at least 20 minutes at room temperature, 200µL of each sample were transferred into a 96-well microtiter plate (Greiner-Bio-One GmbH) and measured in a photometer at 595nm (Anthos Zenyth 3100).

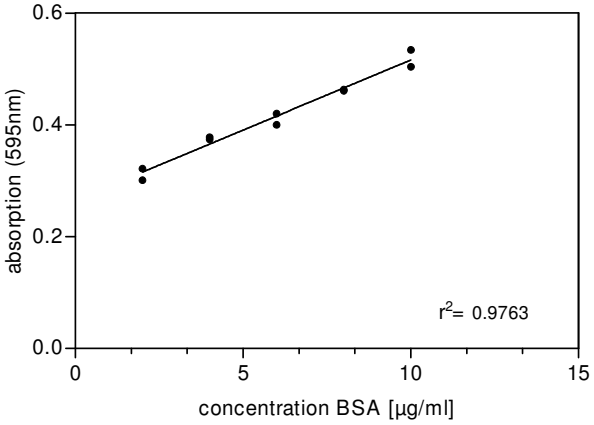

Figure 14: Example of a typical standard curve for protein quantification with Bradford.

The protein content of the samples was achieved by comparison with the standard curve. Evaluation was performed by the program GraphPadPrism, Version 5.

7.2 Quantification of iron in biological samples

7.2.1 Measurement of cellular iron content by the Ferrozine method

Ferrozine is an iron chelator for ferrous iron and forms a purple ferrous-ferrozine-complex which can be measured spectrophotometrically at 540nm (Fish 1988).

For the ferrozine method the following solutions were prepared:

Reagent A:

10mL 1.2M HCl

10mL 4.5% (w/v) $KMnO_4$

First 0.45g of $KMnO_4$ was diluted in 10mL distilled water and mixed with 1.2M HCl (10mL) protected from-light and used immediately.

Reagent B (for 20mL reagent)
64mg 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p′-disulfonic acid monosodium salt hydrate (ferrozine)
64mg neocuproin
7.04g ascorbic acid
7.76g ammonium acetate

Ascorbic acid and ammonium acetate were dissolved in a small volume of distilled water. In two separate microcentrifuge tubes neocuproin and ferrozine were dissolved in 1mL distilled water and added to the other solution. Distilled water was added to this solution to a final volume of 20mL. Reagent B was stored at 4°C, protected from light for up to 4 weeks.

iron concentration (µg/mL)	µL iron solution (µl)	µL H_2O dest.
0,2	5	495
0,4	10	490
0,6	15	485
0,8	20	480
1,5	40	460
2,4	60	440
3,2	80	420
4,0	100	400
0	0	500

Table 4: Pipetting scheme to generate standard solutions of different iron concentrations for the ferrozine assay

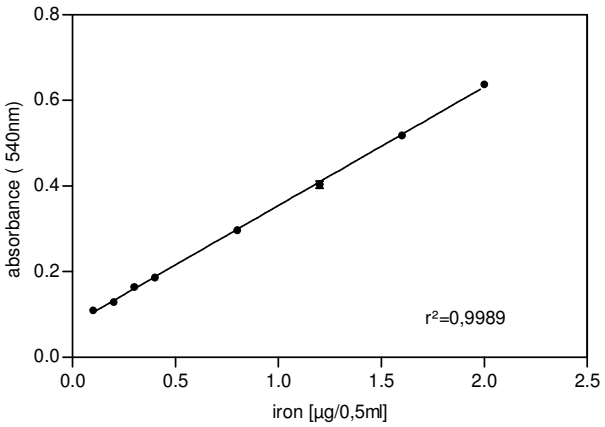

Figure 15: Representative ferrozine standard curve

All standards, samples and blanks were prepared in duplicates. Standard solutions of different iron concentrations were prepared in 2mL microcentrifuge tubes according to table 4. All samples (cell lysates) were diluted with deionized water to a final volume of 500µL. The blank consisted of 500µL deionized water.

Then 250µL of reagent A were added to each standard, sample and blank. For complete digestion all tubes were incubated in a water bath at 60°C for two hours. After cooling to room temperature 50µL reagent B were added to each tube and mixed. To clear the samples from possible precipitates they were centrifuged at 650g for 5 minutes.

Then from each tube 200µL were transferred into the wells of a 96-well microtiter plate (Greiner-Bio-One GmbH). After a total incubation time of 30 minutes with reagent B, absorbance was measured with a plate reader (Multilabel Counter, Wallac Victor 1420) at 540nm. The amount of iron measured in each sample was then normalized to the amount of protein in the sample using the BioRad protein assay.

7.2.2 Release of iron from iron loaded cells

The protein transferrin is responsible for iron circulation in our body. Under physiologic conditions the plasma concentration of transferrin is in the range from 2.2 to 3.7mg/mL (26-42µmol/L). It is known, that transferrin bound iron is the only source of iron for erythroblasts and therefore it is very important for an adequate heme synthesis.

Therefore, apotransferrin, at a physiologically relevant concentration of 2.5mg/mL, was used to support iron release from cells, previously loaded with different iron preparations.

HepG2-cells and THP-1 macrophages were cultivated in 6-well plates and were incubated with 1200µM intravenous iron (Venofer, ISA, Ferinject) in DMEM or RPMI containing 10% fetal calf serum, 2mM glutamine, gentamycin (50µg/mL) for 3h at 37°C. After removal of the incubation medium, the cells were washed once with ice cold medium containing 50µM DTPA, a strong impermeant iron chelator and twice with pure medium to remove surface bound iron. Finally the cells were incubated with medium, supplemented with 20mM Hepes (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid, pH-7.4) and 2.5mg/mL apotransferrin to promote cellular iron release. Control values represent the basal iron release obtained by cells not loaded with iron. The release of iron was measured in a time dependent manner in a Hitachi 8000 atomic absorption spectrometer with heated graphite tubes and longitudinal Zeeman effect background correction, in aliquots of the release medium. All measurements were carried out at 248.3nm using a hollow cathode lamp (HCL), the slit width was 0.2nm.

The sample volume used was 15µL and no matrix modifier was applied. The amount of iron in the release medium was correlated to the amount of protein in the well using the BioRad protein assay.

7.2.3 Intracellular labile iron pool (LIP)

The principle of the measurement of the labile iron pool is shown in a cartoon in figure 16 (Kakhlon and Cabantchik 2002b).

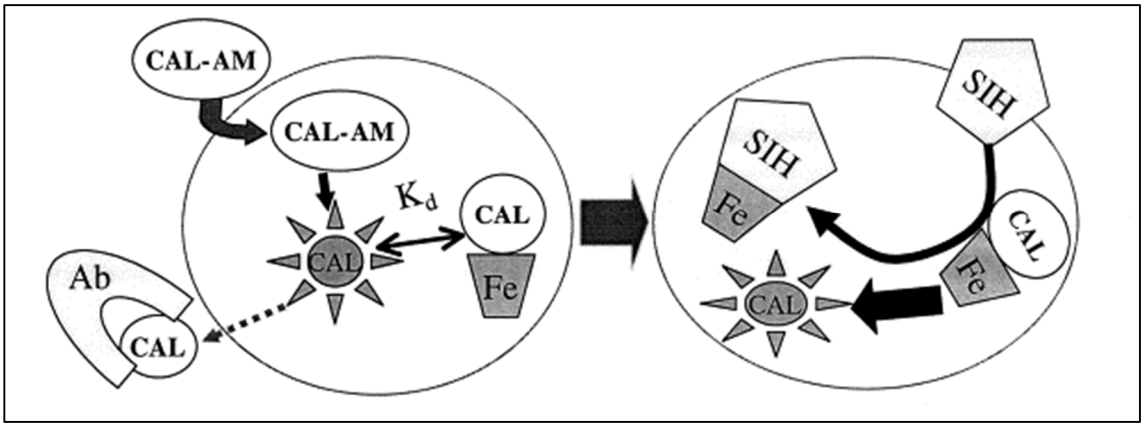

Figure 16: Measuring the labile iron pool (LIP) (Kakhlon and Cabantchik 2002b)

„Cells are loaded with the acetomethoxy derivative of CAL (CAL-AM) which is nonfluorescent and membrane permeant. Upon entry to cells, CAL-AM is hydrolyzed to give the fluorescent CAL, which is quenched upon binding of iron. Anti-CAL antibodies are added to ensure that the measured fluorescence is intracellular. SIH, a strong permeant iron chelator evokes fluorescence dequenching by binding CAL-bound iron in a manner proportional to CAL-bound iron [CAL-Fe]“ (Kakhlon and Cabantchik 2002b).

Quantification of cellular labile iron was performed using the following solutions:

Hepes:

400mM 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, pH 7.3 (Hepes) was dissolved in distilled water and the pH was adjusted with HCl to 7.3. Next, sterile filtration was conducted following storage at 4°C.

DTPA:

5mM N,N-Bis(2-[bis(carboxymethyl)amino]ethyl)glycine (DTPA)was also dissolved in distilled water and pH 7.3 was set with HCL following sterile filtration and storage at 4°C.

SIH:

2,5mM Salicylaldehyde isonicotinoyl hydrazone (SIH) was dissolved in DMSO.
21mg of SIH was dissolved in 18mL DMSO and 22mL of distilled water and divided into aliquots and stored at -20°C. 10µL of the solution were added to each well for the experiment.

Calcein –AM:

0.25µM Calcein-AM (acetoxymethyl ester) were used for the experiments therefore
50µg of Calcein-AM were dissolved in 100µL DMSO (anhydrous) and protected from light, divided into aliquots of 10µL and stored in the dark at -20°C.
10µL Calcein-AM were added per mL of Hepes buffered medium to quench extracellular iron.

HepG2-cells were cultivated in 96 well-plates and incubated with 1200µM, 600µM and 300µM intravenous iron (Venofer, ISA, Ferinject) for 3 hours diluted in DMEM, 10% fetal calf serum, 2mM glutamine, gentamycin (50µg/mL) for 3h at 37°C. At the end of the incubation time the medium was removed and the cells were incubated for 5 minutes with medium containing 50µM DTPA and additionally washed twice with pure medium to remove surface bound iron. Further, the cells were loaded with 100µL of 0.25µM Calcein-AM in 20mM Hepes-buffered medium for 15 minutes at 37°C. Fluorescence was measured at Ex 485nm/Em535nm (measurement A) in a fluorescence plate reader (Microplate multimode reader, Anthos Zenyth 3100). Three minutes after the addition of 10µL SIH (100µM) per well, the plate was measured again (measurement B). The difference between measurement B and measurement A represents the labile iron pool. The labile iron pool of control cells (without treatment with iron preparations) was set as 100%.
Due to technical reasons the LIP could not be measured in THP-1 macrophages.

7.2.4 Cellular ferritin content

To quantify cellular ferritin, the cells had to be first lysed with NP-40 cell lysis buffer followed by quantification of ferritin in the lysate by a commercially available ferritin-ELISA.
The NP-40 cell-lysis buffer consisted of 150mM NaCl, 1% PEGAL CA630 (NP-40) and 50mM Tris (hydroxymethyl)-aminomethan, pH 8,0 and was prepared by mixing.

1.5mL of NaCl solution (5M Stock) and 2.5mL of Tris (hydroxymethyl)-aminomethan in a 50mL tube and filled up with dest. water. Then 0.1mL of 1% PEGAL CA630 (NP-40) were added to the tube, mixed and stored at 4°C.

Directly before use a protease inhibitor 1mM phenylmethylsulfonylfluorid (PMSF) dissolved in DMSO was added to the NP-40 cell lysis buffer.

HepG2-cells and THP-1 macrophages cultivated in 6 well plates were washed first with 1mL of pure medium (37°C), then the cells were incubated with 1mL of 1200µM intravenous iron (Venofer, ISA, Ferinject) in DMEM or RPMI containing 10% of fetal calf serum, 2mM glutamine and 10% gentamycin for 3h, 6h and 24h. At the indicated time points the incubation medium was removed and the cells were washed once with medium containing 50µM DTPA and once with pure medium. After complete removal of the washing solution, the plate was stored at -20°C for 24h. Next day each well was lysed with 100µL NP-40 buffer containing 1mM PMSF. The amount of ferritin in the samples was measured using a human ferritin ELISA (BioCheck Inc., California, USA). The assay system used one rabbit anti-ferritin antibody for solid phase immobilization and a mouse monoclonal anti-ferritin antibody in the antibody-enzyme (horseradish peroxidase) conjugate solution. Protein concentrations were determined by the Bradford assay (BioRad Protein assay, BioRad, Vienna, Austria) and the ferritin content was normalized to the protein content in the cell lysates.

7.2.5 Transferrin-chelatable iron

Transferrin-depleted human serum was incubated with 75µM intravenous iron (Venofer, ISA, Ferinject) alone or in the presence of 0.56mM ascorbic acid (Asc) for 1

hour at 37°C. The samples were then mixed with reagent A (HBS containing 0.6µM fluorescein-labeled apotransferrin, Fl-aTf) or reagent B (same as reagent A, but containing 5mM EDTA) and incubated at 37°C in the dark. After 1h, 2h and 3h the fluorescence was measured at Ex 485nm/ Em 535nm in a fluorescence plate reader (Anthos Zenyth 3100) from Perkin Elmer. The ratio between the incubation with and without EDTA (reading B/A) was calculated and correlated to a standard curve generated with ferrous ammonium sulfate at concentrations ranging from 0-20µM.

7.2.5.1 Fluorescein-labeled apotransferrin

To obtain Fluorescein-labeled apotransferrin (Fl-aTF), 8mg/mL apotransferrin (100µM/L, based on MW 80.000Da) was diluted in 100mM $NaHCO_3$, pH 8.4, and 100µM of 5-(4,6-dichlorotriazinyl) aminofluorescein (DCTAF) from a freshly prepared 10mM solution in dimethylsulfoxid where added.

After incubation in the dark for 30min at 37°C, 5mM L-lysine, pH 8, was added to stop the reaction. Next, the Fl-aTf was dialyzed against HBS, divided into aliquots and stored at -20°C.

To test the final preparation of 100µM Fl-aTf a polyacrylamide gel electrophoresis was performed. This gave a single fluorescent band at approximately 80.000Da. The concentration of fluorescein in FL-aTf was measured by DCTAF as standard solution (a-Tf: fluorescein of 1:0.84) at 496nm.

7.2.5.2 Redox-active iron in iron sucrose preparations

The measurement of redox-active iron was performed according to the method of Esposito (Esposito et al. 2003), with slight modifications as reported by Schaller (Schaller et al. 2005). This method is based on the principle that dichlorofluorescein (DCF) is converted from its non-fluorescent to its fluorescent form by several oxidants.

2´,7`-Dichlorofluorescein diacetate (DCFH-DA) was hydrolysed to receive DCF. For hydrolysis 0.5mL 1mM DCFH-DA were mixed with 2mL 0.01M NaOH and incubated at 25°C for 30min in the dark followed by neutralisation with 10ml 25mM Na-phosphate buffer (pH 7.2).

Redox-active iron was assessed in plasma like medium or human serum (20µl) which was supplemented with various concentrations of i.v. iron preparations and transferred in quadruplicates to black, clear bottom 96 well plates (Greiner, Bio-one, Kremsmünster, Austria). Plasma like medium was composed of 20mM Hepes, pH 7.4, 150mM NaCl, 120µM sodium citrate, 40µM ascorbic acid, 1.2mM Na_2HPO_4, 10mM $NaHCO_3$ and 40mg/mL bovine serum albumin and was rendered iron free before use by treatment with 1g/100mL Chelex-100 (Sigma). Two wells were incubated with iron free HBS (20mM Hepes, 150mM NaCl, pH 7.4) containing 150µM ascorbate and 5µM DCF at 37°C in the dark. The other two wells were incubated with 180µl of the same solution containing 50µM of the iron chelator Deferiprone (L1). HBS was rendered iron free by treatment with 1g/100mL Chelex-100 (Sigma). The kinetics of fluorescence increase was measured in a fluorescence plate reader (Anthos Zenyth 3100) from Perkin Elmer with excitation/emission filters of 485nm/530 nm. Measurements between 120 and 375 minutes were used to calculate slopes of DCF fluorescence intensity over time. The fluorescence increase measured in the presence of L1 represents oxidation of DCF by several other oxidants, e.g. peroxidases or hypochlorous acid generated by myeloperoxidases. Therefore, the difference in the rate of oxidation of DCF with and without addition of the chelator L1 represents the redox-active component of NTBI. The duplicate values of the slopes with and without addition of L1 were averaged, and redox-active iron (in µM) was determined from calibration curves correlating the difference in slopes with and without L1 against the iron concentration (Schaller et al. 2005).

7.2.6 Cytotoxicity

7.2.6.1 MTT- assay

The MTT assay measures the reduction of yellow MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) into an insoluble purple formazan product in metabolically active cells. This reaction relies in the presence of active reductase enzymes, conversion is directly attributable to the number of viable cells (Mosmann 1983). This mechanism is shown in figure 9.

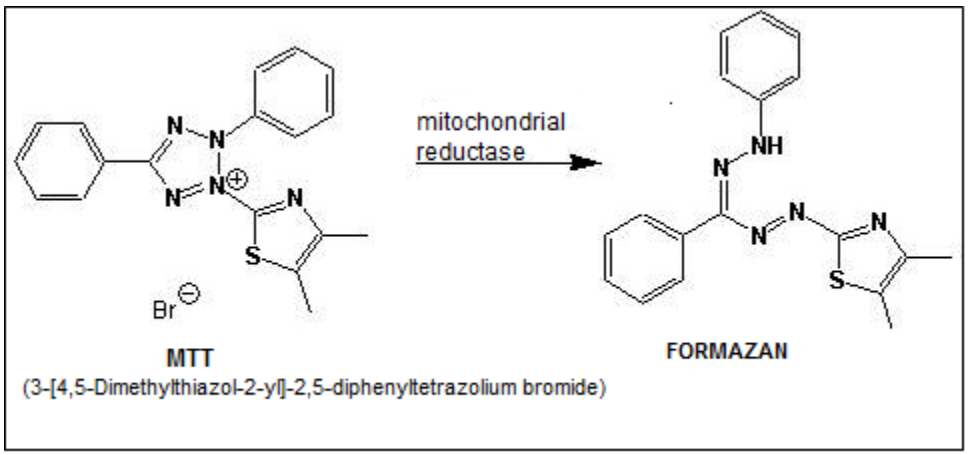

Figure 17: MTT-assay

(http://en.wikipedia.org/wiki/File:NewMTTscheme1.pdf; retrieved in June 2013)

Quantification of cell viability by using the MTT assay was performed using the following solutions:

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was dissolved in PBS (1x) reaching an concentration of 5mg/mL, filtered through a 45µm membrane filter and stored at -20°C in the dark.

MTT solvent consists of 200µL 2-propanol, 200µL Nonident P40 (Fluka) and 160µL of 5M HCl which were mixed and stored at 4°C.

HepG2-cells or THP1-macrophages, cultivated in 96-well plates were incubated with various concentrations of intravenous iron preparations in DMEM or RPMI-medium, supplemented with 10% fetal calf serum, 2mM L-glutamine, and gentamycin (50µg/mL) at 37°C and 5% CO_2 for 24 hours. After removal of the incubation medium and washing of the cells, they were incubated with 100µL/well of fresh medium and 20µL/well MTT-solution for 30min at 37°C. The MTT solution was prepared by dissolving 5mg MTT per milliliter phosphate buffered saline, at pH 7.4. Subsequently, the wells were carefully emptied and filled with 150µL MTT-solvent (4mM HCl, 0.1% Nonident P-40 in isopropanol) to lyse the cells and dissolve the coloured crystals under constant agitation for 15min. Absorption was measured at 595nm (measuring wavelength) as well as 620nm (reference wavelength) in a fluorescence plate reader (Anthos Zenyth 3100) from Perkin Elmer.

7.2.6.2 Resazurin- assay

The resazurin-assay is a colorimetric test were Resazurin (7-hydroxy-3H-phenoxazin-3-one 10-oxide) is reduced to red fluorescent resorufin shown in figure 4. Viable cells retain the ability to convert resazurin into resorufin the fluorescent end product, non-viable cells do not. The metabolic capacity is too low to generate a fluorescent signal.

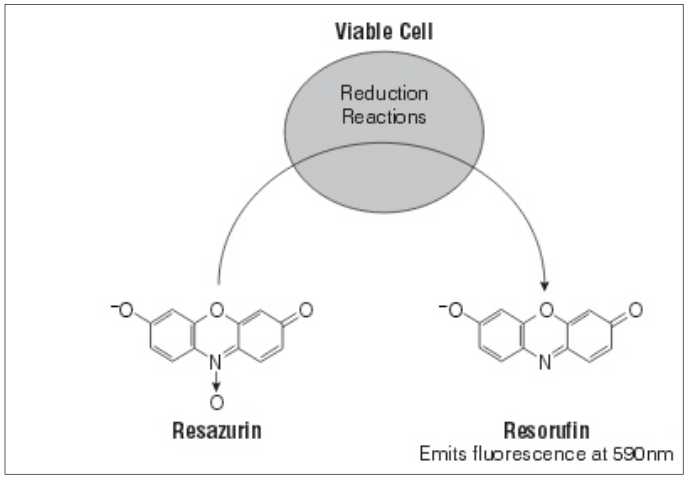

Figure 18: Mechanism of intracellular reduction of resazurin to resorufin

(http://www.promega.de/resources/protocols/technical-bulletins/101/celltiter-blue-cell-viability-assay-protocol/; retriever in June 2013)

Quantification of metabolic capacity by the resazurin-assay was performed by using the following solutions.

DFO:

50µM/L Deferioxamin (DFO) diluted in dest. water was used to mimic anemic conditions in the cell culture.

HEPES buffered phenol red-free medium:

400mM 2-(4-(2-Hydroxyethyl)- 1-piperazinyl)-ethansulfonsäure (HEPES) was dissolved in dest. water and added to phenol red-free medium to obtain a final concentration of 20mM HEPES.

Resazurin solution (4mM):

4mM 7-Hydroxy-3H-phenoxazin-3-one-10-oxide sodium salt was dissolved in distilled water, proportionalized in 1.5mL and stored light-protected -20°C.

Used Resazurin solution (3µM):

Previously prepared 4mM resazurin solution was diluted in HEPES buffered phenol red-free medium (20mM) to reach a concentration of 3µM resazurin.

HepG2 cells were cultured in 96-well plates (Greiner Bio-One) and preincubated with 50µM/L DFO for 6 hours to induce iron deficiency. Then the iron depleted cells were incubated with different concentrations of iron sucrose or iron carboxymaltose (225 – 3600µM iron) for 24 hours. The next day the supernatant was removed and washed once with phenol red-free DMEM. 4mM resazurin solution (see above) was diluted in HEPES buffered phenol red-free medium (20mM) to reach a concentration of 3µM resazurin. 200µL of this solution was added in each well. Next the absorbance at 595nm was measured at 37°C in the bottom read mode in a plate reader (Anthos Zenyth 3100) every 15 minutes for 3 hours.

The calculations were performed with the program GraphPad Prism 5 (GraphPad Software, Inc., San Diego, California, USA).

7.3 Measurement of Frataxin in Friedreich´s Ataxia lymphocytes by ECLIA

The Meso Scale Discovery platform was used to quantify the frataxin levels in FRDA lymphocytes. Electrochemiluminescent Immunoassay (ECLIA) involve 96 well multi-array high bind plates (Meso Scale Discovery) which were first coated with monoclonal mouse anti-frataxin antibody. Then the sample was applied, next the second antibody (polyclonal rabbit anti frataxin) was added and binds subsequently to frataxin. The last, third antibody (goat anti rabbit) is linked by a sulfo tag which acts as the detection antibody for quantification of frataxin (Figure 19B). This Principe is an "antibody-antigen –antibody" formation, which is used in a Sandwich- Enzyme-linked Immunosorbet Assay (ELISA). The only difference is that the detection antibody is a labelled non- radioactive and stable reagent called MSD SULFO-TAG™ (Figure 19).

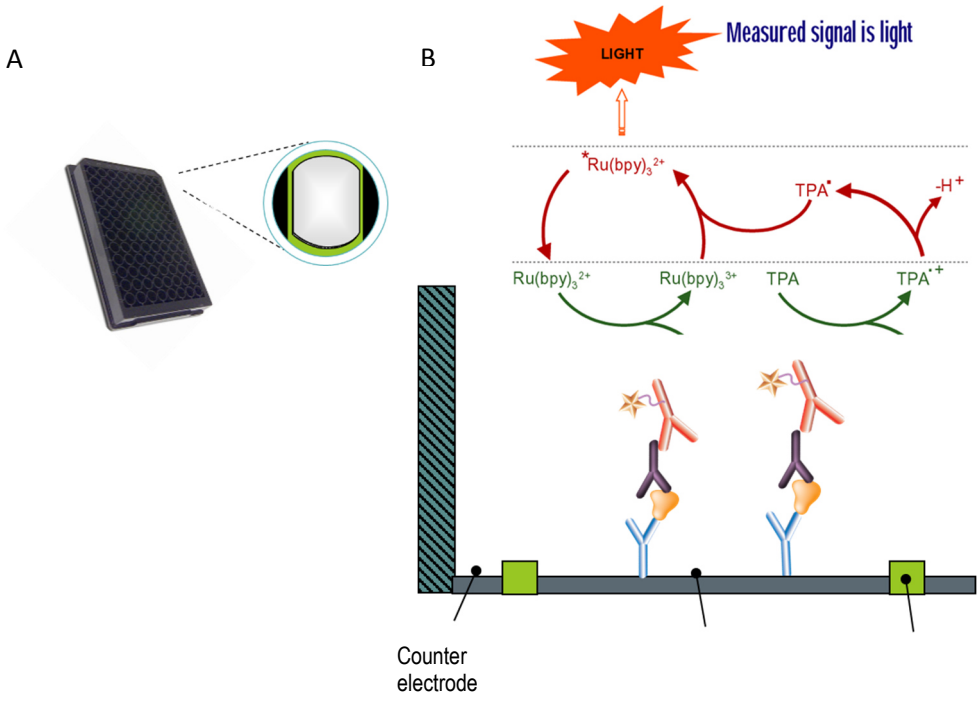

Figure 19: Measurement of frataxin with ECLIA (modified)

A: Each spot within the 96 well Multi-Array High Bind plates (Meso Scale Discovery) shown above posses a working electrode surface that binds capture reagent. B: The working electrode is coated with the capture antibody (blue), a monoclonal mouse anti frataxin antibody and after blocking, the wells are incubated with the samples. Frataxin in the sample (yellow) binds to the capture antibody in the well. Then a second Antibody (polyclonal rabbit anti frataxin) binds to Frataxin (purple) and will be detected by a sulfo tag goat anti rabbit detection antibody (orange). Following addition of a read buffer containing tripylamin (TPA)

and in the presence of an applied voltage the Sulfo-Tag undergoes a rapid redox- reaction and emits light. The electrochemical reaction occurs within the plate and light is measured through a CCD camera.

7.4 Measurement of FRDA patient lymphocytes

The following antibodies were used for the measurement of frataxin in FRDA patient lymphocytes:

first antibody ("capture antibody")
mouse anti-human frataxin monoclonal antibody (MAB2594), (Chemicon)

second antibody
frataxin (H-155) rabbit polyclonal antibody (Santa Cruz Biotechnology)

third antibody ("detecting antibody")
goat polyclonal anti-rabbit labelled with MDS-SULFO-TAG™ (Meso Scale Discovery)

The established method in our lab to measure frataxin in lymphocytes (Steinkellner et al. 2010) was improved in terms of efficiency, high throughput rates and protein quantification to handle clinical study samples.

Each well of the Multi-Array High Bind plates (Meso Scale Discovery) were coated with 5µL of monoclonal mouse anti-frataxin antibody for 1h at room temperature in the dark. Right after, the wells were emptied and subsequently blocked with 125µL blocking solution (MSD Blocker A) for 90 min at room temperature and finally washed three times with PBS–Tween (0.05%). Afterwards, samples (25µL per well diluted in 1% MSD Blocker A and 0.02% sodium dodecyl sulfate were added and the plates were incubated at room temperature for another 90 min. The polyclonal rabbit anti-frataxin antibody (5µg/mL in 1% MSD Blocker A) was added to each well for 1 h and after that 0.75µg/mL of MSD Sulfo-TAG™ goat anti-rabbit detection antibody in 1% MSD Blocker A was added. After 1h incubation at room temperature on the belly dancer, free detection antibody was removed by washing. Finally, 150µL Tris-based Read Buffer T (1x) with surfactant (Meso Scale Discovery) containing tripropylamine as a co-reactant

for light generation was added to the plate. Upon applied voltage, ruthenium label bound to the carbon electrode emitted luminescence light at 620nm. ECL signals were captured by a sensitive cooled CCD camera in a Sector Imager 2400 reader (Meso Scale Discovery) and recorded as signal counts.

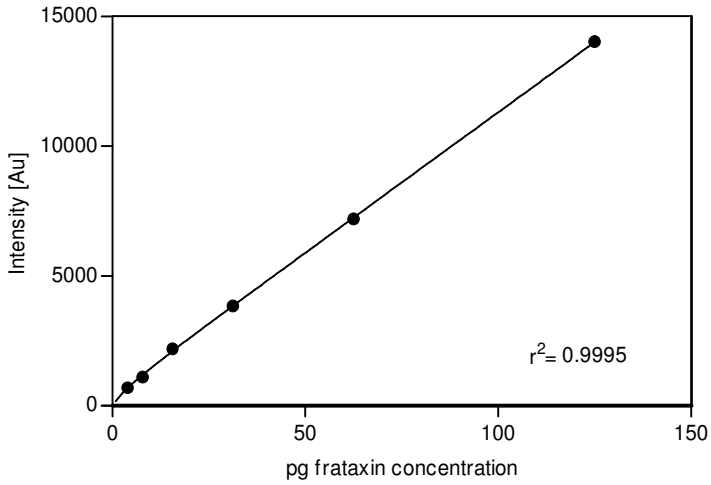

Figure 20: ECLIA measurement standard curve for frataxin

A representative standardcurve of frataxin measured by ECLIA

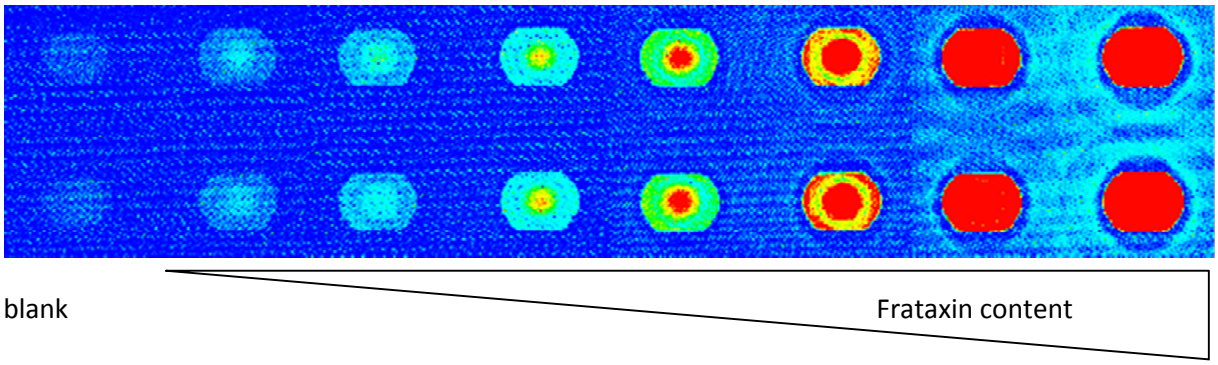

Figure 21: Signal of the frataxin standards detected by the MSD Sector Imager 2400

Shown is a representative ECL signal of the frataxin standards measured by a CCD camera in the MSD Sector Imager 2400 in the image view.

Since frataxin levels always have to be normalized to the protein content in the samples, it is of tremendous importance to quantify protein contents accurately and reproducible.

Therefore, we decided to combine frataxin-ECLIA and Infrared (IR)-based spectrometry protein quantification.

7.5 Infrared (IR)-based spectrometry method for protein quantitation (Direct Detect™, from Millipore)

In general, this IR-based method measures amide bonds in protein chains. A possible advantage is that a single time standard curve is used which is performed with a certified BSA in PBS calibration standard from the National Institute of Standards & Technology. Furthermore, it is independent of amino acid composition, dye binding properties or redox potential and in addition, no chemical reactions are necessary.

In contrast, conventional protein quantification methods like the Bradford protein assay (from Bio-Rad),the bicinchoninic acid (BCA),the Protein assay from Pierce have high day to day variations (necessity to generate each day a new standard curve). In addition the absorbance maximum of the dye-protein complex varies from 595nm to 620nm depending on dye source (Sedmak and Grossberg 1977).

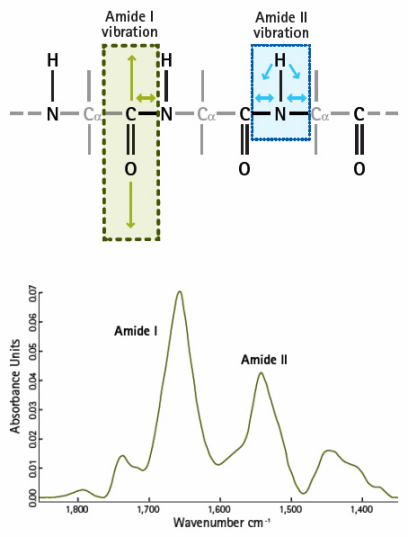

Figure 22: Infrared protein detection

The determination of the concentration of proteins as peptids measures the intensity (peak height) of the Amide I band, which is assigned to C=O stretching vibration of the peptide bond (about 80%) with a minor contribution from C-N stretching vibration (about 20%) (FTIR Technology, 2014).

7.6 Assessment of Iron Biomarkers in Plasma with multiplex Multiple Reaction Monitoring (MRM) proteomics

We used multiplex Multiple Reaction Monitoring (MRM) proteomics to investigate a patient with PLA2G6 associated neurodegeneration (PLAN) being treated with Deferiprone. The investigation focused on the patient's systemic state of iron trafficking proteins prior to and during Deferiprone treatment. Long-term goals include investigation of the systemic state in other diseases with neurodegeneration with brain iron accumulation (NBIA) in order to assess possible biomarkers of disease progression.

Possible iron trafficking proteins were selected and further assessed via UniProt Knowledgebase (UniProtKB). This database represents a collection of functional information on proteins, like the amino acid sequence, protein name or description, taxonomic data and citation information. This further includes widely accepted biological ontologies, classifications and cross-references, and clear indications of the quality of annotation in the form of evidence attribution of experimental and computational data (UniProtKB,2014)Next, external and an internal data alignment of UniProtKB and tracker of the proteomic centre was performed and results are shown in the results section.

7.6.1 Plasma sample collection for MRM analysis

Whole blood from the PLAN patient and a control subject was collected in sodium heparin tubes every 3 months over the course of the Deferiprone treatment. Until plasma isolation, the blood was kept on ice. Within 30min of collection, the blood samples were centrifuged at 2900x g for 15min at 4°C to separate plasma, which was stored at −80 C until further analysis.

7.6.2 Sample preparation for multiplex MRM analysis

For sample preparation the following reagents were used:

- ✓ 25mM ammonium bicarbonate, AmBic (99mg + 50mL LC-MS water)
- ✓ 10% sodium deoxycholate, NaDOC (200mg + 2.0mL of 25mM AmBic)
- ✓ 0.05 TCEP (30µL of 0.5M TCEP + 270µL of 25mM AmBic)
- ✓ 0.1M iodoacetamide (53.9mg + 2.915mL of 25 mM AmBic) always fresh and kept in the dark
- ✓ 0.1M dithiothreitol, DTT in 25mM Am. Bicarb (30.8mg + 2mL of 25mM AmBic) always fresh

The deep-frozen patient plasma specimen were defrosted 5 min at room temperature, vortexed, centrifuged briefly, and then kept on ice. The digestion was conducted as described by (Proc et al. 2010). Briefly, each plasma sample was diluted in 90µL of 25mM of AmBic, vortexed, centrifuged and kept on ice. The digestion procedure was started by using 30µL of the aliquot of the 1/10 diluted plasma sample and denatured by adding it to a tube containing 175µL of 25mM AmBic, and then adding 30µL of the NaDOC solution. Reduction was performed by adding 26µL TCEP and incubation for 30 min at 60°C. Alkylation was done by adding 29µL of the iodoacetamide solution, and incubating for 30 min at 37°C. Tryptic digestion was performed by adding 4.5µg of trypsin to each tube, to give a 1:50 trypsin: sample ratio, followed by incubation at 37°C for 16h. Digestion was stopped after 16h by placing the plate on ice.

Next day, a mixture of stable isotope standards (SIS peptide coded with [$^{13}C_6$]Lys, [$^{13}C_6$]Arg) were added to the samples post digestion at 50fmol per peptide per 1µg protein of sample digest.

The NaDOC was then removed by precipitation through addition of formic acid to 0.5% and subsequent removal by centrifugation for 10 min at 13.200×g. Desalting and concentration of the supernantant by solid phase extraction was performed using a Waters Oasis 10-mg HLB. The colum was rinsed and pre-equilibrated with 1mL of methanol, followed by 1mL of water. The sample was applied, and eluted with 100µL

of 50% acetonitrile/0.1% formic acid. The eluted samples were frozen (-80°C) and lyophilized to dryness. Before multiplex MRM, samples were rehydrated in 0.1% (v/v) formic acid (mobile phase A) to produce a 1µg/µL protein concentration.

In this study, 45 SIS peptides were used for 17 corresponding proteins known to be involved in iron metabolism. In our pilot study 5 target proteins were assessed: Lipocalin-2, Ceruloplasmin, Transferrin, Hemopexin and Haptoglobin;

All samples were run on an Agilent 6490 with IonFunnel and analyzed as described previously (Domanski et al. 2012).

7.7 Statistics

Statistical analysis were performed by using GraphPad Prism 5.0 and MS Excel 2010. MRM-Data analysis was performed by Mass-Hunter and calculated via MS Excel 2010.

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9 Curriculum Vitae

Mag. Monika Praschberger

Educational Profile

Ph.D. in Nutritional Sciences (Submitted Thesis); Advisor: Prof. Barbara Scheiber-Mojdehkar

Conducted at Medical University of Vienna, Department of Medical Chemistry, Austria
PhD Thesis:
"Biochemical aspects to improve treatment of anemia in endstage renal disease and advanced biomarker assessment in iron related disorders"

Degree: Magister in Nutritional Sciences (2004-2011);

Nutritional Sciences, University of Vienna, Austria

Diploma thesis: "Influence of H_2S on stabilization of HIF-1α protein in THP-1 macrophages"

Teaching Experience

Medical University of Vienna

- Teaching assistant, "Biochemistry of the cell" for medical students, 2011-current.
- Teaching assistant, "Digestion and Nutrition" for medical students, 2011- current.

Awards

- **"**Rafael Estrada in Memoriam 2013 Award" at Neurocuba 2013.

Peer-Reviewed Publications

1. **Praschberger** M., Hermann M., Wanner J., Jirovetz L., Exner M., Kapiotis S., Gmeiner BM., Laggner H.
   The uremic toxin indoxyl sulfate acts as a pro- or antioxidant on LDL oxidation
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2. **Praschberger** M., Cornelius C., Schitegg M., Goldenberg H., Scheiber-Mojdehkar B.and Sturm B.
   Bioavailability and -stability of intravenous iron sucrose originator versus generic iron sucrose AZAD.
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   Carbamoylation abrogates the antioxidant potential of hydrogen sulfide
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