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Hepcidin and Iron Parameters Determine Disease Outcome

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To mom, Tawar, Shayan, Kosar, Tara and my uncle.

And to the loving memory of my father.

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Abbreviations

ACLF: Acute-on-chronic liver failure

AFP: α-fetoprotein

AKA: Also known as

ALC: Alcoholic liver cirrhosis

ALT: Alanine aminotransferase

AP: Alkaline phosphatase

APS: Ammonium persulphate

AST: Aspartate- aminotransferase

BMI: Body mass index

BMP: Bone morphogenetic protein

BSA: Bovine serum albumin

CRP: C-reactive protein

DMSO: Dimethylsulphoxide

DNA: Deoxyribonucleic acid

dNTP: (Deoxynucleotides) nucleoside triphosphate

ECL: Enhanced chemiluminescence

EDTA: Ethylenediaminetetraacetic acid

ELISA: Enzyme-linked immunosorbent assay

H&E: Haematoxylin and Eosin

H_2SO_4: Sulfuric acid

HAMP: Hepcidin antimicrobial peptide

Hb: Haemoglobin

HCC: Hepatocellular carcinoma

HCL: Hydrogen chloride

HCV: Hepatitis C virus

HDAC2: Histone deacetylase 2

Hfe: Human hemochromatosis protein

HH: Hemochromatosis

HIV: Human immunodeficiency virus

Hjv: Hemojuvelin

HR: Hazard ratio

HRP: Horse radish peroxidase

ICU: Intensive Care Unit

IHC: Immunohistochemistry

IL: Interleukin-6

IRE: Iron response element

IRP: Iron response proteins

KCl: Potassium cholirde

KO: Knockout

LDH: Lactate dehydrogenase

MDA: Malondialdehyde

$MgCl_2$: Magnesium chloride

Na_2CO_3: Sodium carbonate

NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cell

NTBI: Non-transferrin-bound iron

PAGE: Polyacrylamide gel electrophoresis

PBS: Phosphate Buffered Saline

PCR: Polymerase chain reaction

PCT: Procalcitonin

PEG-IFN-α: Pegylated interferon alpha

PMSF: Phenylmethanesulfonyl fluoride

RBV: Ribavirin

RIPA: Radioimmunoprecipitation assay

RNA: Ribonucleic acid

RT: Room temperature

SD: Standard deviation

SDS: Sodium dodecyl Sulphate

Smad4: Mothers against decapentaplegic homolog 4

SPSS: Statistical Package for the Social Sciences

STAT3: Signal transducer and activator of transcription 3

suPAR: Soluble form of the urokinase plasminogen activator receptor

TAE: Tris acetate-EDTA

TBARS: Thiobarbituric Acid Reactive Substances

TBS: Tris-buffered saline

TBST: Tris-Buffered Saline and Tween

TEMED: Tetramethyl ethylene diamine

TfR1: Transferrin receptor protein 1

TNBS: Trinitrobenzenesulfonic acid

TSAT: Transferrin Saturation

WBC: White blood cells

WT: Wild type

1. Chapter One: Introduction

1.1. Biological role of iron

Iron is an essential trace element in almost every living organism and the fourth most abundant element on earth (T Ganz, 2013). It is a vital element that participates in many biological oxidations and in transport of molecules. Iron is responsible for transporting and storing oxygen and is involved in oxidation and reduction of substrates during energy production, intermediate metabolism, and the generation of reactive oxygen or nitrogen species for host defense (Le & Richardson, 2002; Nemeth & Ganz, 2009). Furthermore, many biological processes are iron dependent, including the production of proteins as well as the synthesis of DNA and RNA (Le & Richardson, 2002). It plays a role in transportation of electrons, cellular respiration, proliferation and differentiation as well as the regulation of gene expression (T Ganz, 2013). Many enzymes and other proteins vital to life contain iron such as catalase, lipoxygenases, and IRE-BP.

Iron is available in two oxidation states; the +2 and +3. The iron(II) compounds are called ferrous, while the iron(III) compounds are referred to as ferric. The complex of iron and molecular oxygen is part of hemoglobin and myoglobin, which are the two compounds responsible for oxygen transportation and storage in vertebrates (Hillman & Finch, 1971).

1.2. Iron distribution and homeostasis

Average human adult contains 3-4g of iron that are mainly stored as haemoglobin in erythrocytes, or in macrophages and hepatocytes in a specialized cytoplasmic iron storage protein called ferritin (Andrews, 1999). Muscles store iron mainly in the form of myoglobin. Iron is distributed to the tissue through blood plasma by binding to the iron-transport protein transferrin.

In Human body, erythrocytes contain the highest concentration of iron. In the recycling process, the main plasma iron is derived from the senescent erythrocytes that are recycled by macrophages in the spleen. Macrophages recover smaller amounts of iron from other cell types as well. The average lifespan of a normal human erythrocyte is around 120 days (Tomas Ganz & Nemeth, 2011). Accordingly, around 0.8% (or 15–25mg) of all erythrocyte iron is recycled everyday. Plasma iron is turning over every few hours so that around 25mg of iron passes through this dynamic pool every day. Regardless of this rapid turnover, the concentration of iron in plasma is relatively stable which is due to sophisticated regulation mechanisms. The process of hemoglobin synthesis through erythrocyte precursors is regulated by erythropoietin in response to tissue oxygenation (Hillman & Finch, 1971).

Studies from the early 1960s using iron tracers showed that iron losses from the body lie around 1–2mg/day, mostly as a consequence of

desquamation of epithelial surfaces (Saito et al., 1964).This loss is balanced by dietary iron absorption, mainly in the proximal part of the duodenum (Knutson, 2010). The homeostasis of iron in human is delicately balanced to keep the total body iron within normal range. Given that iron cannot be actively excreted from the body, a sufficient dietary iron absorption, that is tailored to suit the body requirements, represents the key regulating mechanism. This is important, since iron constitutes a toxic metal that leads to oxidative stress when being in excess (Knutson, 2010). In particular, free iron has the potential to readily accept or donate electrons, and therefore causes oxidative damage.

Once the iron is delivered into the circulation, it is chaperoned in the plasma by transferrin. This binding permits a controlled delivery of iron. The transferrin-bound iron is taken up by the cells via the transferrin receptor 1 (TFR1) (Tomas Ganz & Nemeth, 2011) and because of that, TFR1 expression is an important tool to regulate intracellular iron delivery. The majority of transferrin bound iron are processed into the erythroid precursors to be used in the production of haemoglobin (Breuer et al., 2000).

In normal status, only 30% of transferrin is saturated with iron, with still some 70% left to compensate in cases of increased iron release in plasma. Once the iron concentration exceeds the saturation capacity of transferrin (pathological conditions), non-transferrin-bound iron (NTBI) emerges in the circulation (Breuer et al., 2000). Compared to transferrin-bound iron, NTBIs are more reactive and the mechanisms of their cellular uptake are only

partially understood. Consequently, NTBIs lead to generation of oxidative stress species.

1.3. Ferritin - the cytoplasmic chaperone for iron

To prevent the oxidative damage from the intracellular iron and to provide a sufficient storage capacity, the iron is coupled intracellularly with ferritin. Ferritin is an acute-phase protein that under non-inflammatory conditions reflect the total body iron (T Ganz, 2013).

Ferritin is a spherical heteropolymeric 450kDa protein composed of 24 subunits of heavy (H) or light (L) type. Iron is released from ferritin through gated pores or once the ferritin undergoes autophagy and lysosomal degradation. The serum concentration of ferritin is regarded as a good marker for iron stores in the majority of physiological and pathological conditions. The two most prominent states where the ferritin doesn't reliably predict the iron load is when the macrophages are either extremely iron-depleted or extremely overloaded with iron compared to parenchymal tissue, or when ferritin production is induced by inflammation.

1.4. Hepcidin: the master iron regulator

There has been a major advancement in understating iron metabolism in the body since the start of the twenty-first century mainly due to the discovery of the cysteine-rich antimicrobial peptide hormone hepcidin. Hepcidin is the master iron regulator in the body (Hentze et al., 2010;

Pietrangelo, 2010, 2015). In year 2000, hepcidin was discovered in three independent laboratories in human urine and serum (Krause et al., 2000; Park et al., 2001), Tomas Ganz and his co-workers decided to call the peptide hepcidin, since they found out that it is mainly secreted by the liver and that it possess an antimicrobial (bactericidal) role (Park et al., 2001).

Hepcidin is encoded by the HAMP gene and is synthesised as an 84-amino acid prepropeptide. After signal peptide cleavage, the resulting product, prohepcidin, is processed by the prohormone convertase furin to obtain the biologically active 25-amino acid hepcidin (hepcidin-25) (T Ganz, 2013). Once released into the plasma, hepcidin inhibits the entry of iron into the plasma by interacting with ferroportin on the cell surfaces of enterocytes and macrophages. Ferroportin is the transporter that delivers iron into blood plasma. Hepcidin controls posttranslationally the membrane concentration of its receptor. The hepcidin-ferroportin interaction effectively controls the flux of iron into plasma and ensures a good iron supply to the tissues requiring it. Ferroportin is localized at the basolateral membrane of the corresponding cells. Once they react hepcidin causes the internalisation, ubiquitination, phosphorylation and degradation of ferroportin, thus preventing the absorption of iron from enterocytes and its release from the macrophages (Nemeth & Ganz, 2009).

Hepcidin release is triggered via iron. Iron overload causes an increase in the production of hepcidin, with the later trying to counteract the influx of iron. Anaemia causes a down-regulation of hepcidin, allowing more iron to

enter the plasma (Figure 1). Hepcidin is also down regulated in cases of hypoxia, to allow more oxygen supply to the tissue by increasing the iron. On the other hand, injection of 1–2μg/g synthetic hepcidin into mice showed a remarkable decrease in serum iron concentrations within the first hour, the hypoferremic state usually persists for many hours (Collins et al., 2008; T Ganz, 2013; Nemeth & Ganz, 2009). Erythropoesis homeostatically regulates iron as well, once an active erythropoiesis occurs, hepcidin production is suppressed in order to provide more iron for haemoglobin synthesis.

Hepatocytes are the major source of hepcidin production, but they are not the only cells producing it. Other cells such as macrophages and adipocytes are also expressing hepcidin mRNA but at a much lower level (Tomas Ganz & Nemeth, 2011). In addition to its central role in iron homeostasis, hepcidin is also known to be an acute-phase protein, and its inflammation-induced elevation plays a role in development of anemia of chronic diseases (Tomas Ganz & Nemeth, 2011).

Vertebrates also possess a hepcidin-independent iron regulation. For example, the expressioon of ferroportin, the sole known cellular iron exporter, was shown to be regulated translationally by intracellular iron via the IRE/IRP system (Iron response element/Iron response proteins) and transcriptionally via heme. The transcriptional and translational changes allow macrophages to match their iron export capacity to the fluctuating iron and heme load during episodic erythrophagocytosis (Tomas Ganz & Nemeth, 2011).

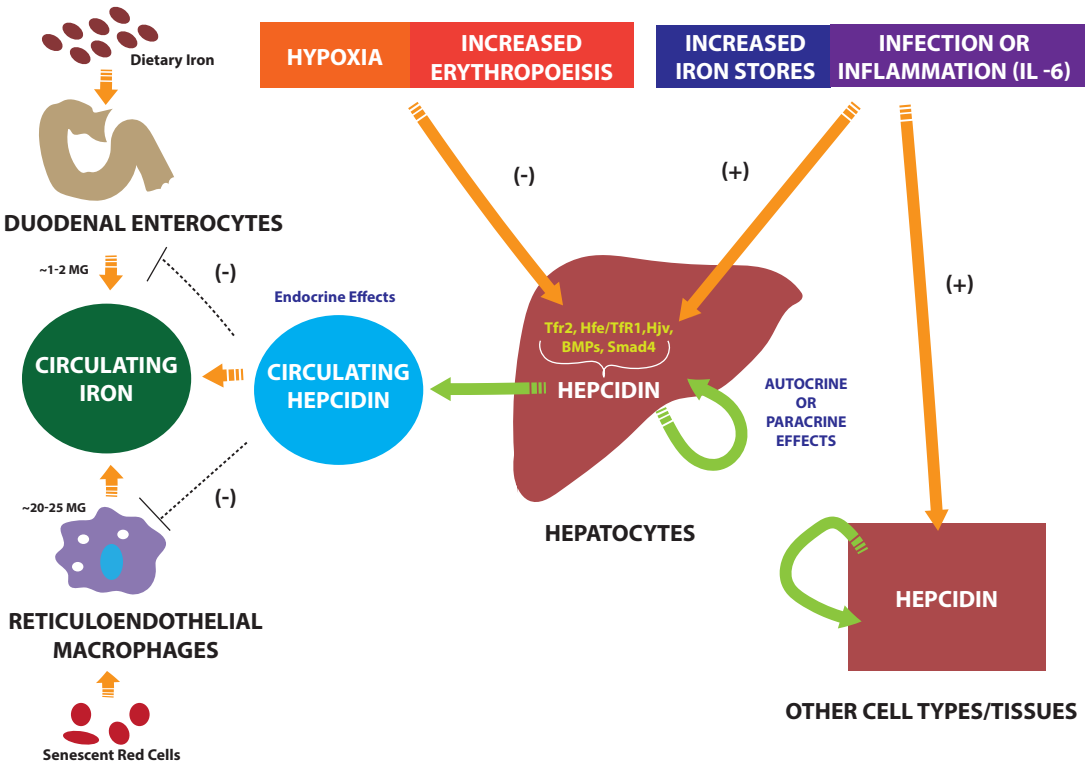


Figure 1 - Iron regulation via hepcidin. Hepcidin secretion is enhances by infection, inflammation or iron overload, and inhibited by erythropoiesis or hypoxia (Adapted from (Collins et al., 2008)). (IL=Interleukin-6, Hfe=Human hemochromatosis protein, TfR1=Transferrin receptor protein 1, Hjv= Hemojuvelin, BMPs= Bone morphogenetic proteins, Smad4=Mothers against decapentaplegic homolog 4)

1.5. Iron between host and microorganisms

The uncoupled iron does not only enhance tissue damage by generating free toxic radicals, but it also enhances the growth of the microorganisms in the body. The iron chaperones are there to prevent these toxic effects of iron and to limit the growth of intruding micro-organisms (Drakesmith & Prentice, 2012). As detailed above, the body has a

sophisticated process that chaperones iron by chelators and keeps it within macrophages during infections (Drakesmith & Prentice, 2012; Nemeth et al., 2004). Most of the proteins involved in iron coupling are acute-phase proteins that are upregulated during acute insults. Transferrin represents a rare exception, given that it decreases in various stresses and during infections. The synthesis of hepcidin by liver cells is transcriptionally regulated by IL-6 via the induction of STAT-3 (signal transducer and activator of transcription 3) signaling pathway. The induction of hepcidin through this pathway is responsible for hypoferremia in the acute state of infection; the hypoferrimic-state is a mechanism that inhibits the overgrowth of the pathogens. Bone morphogenetic protein (BMP) is another signaling pathway for the induction of hepcidin induction (Mayeur et al., 2014; Mayeur et al., 2014; Michels et al., 2015). During chronic inflammation, chronically elevated expression of hepcidin results in hypoferremia and later on, due to reduced availability of iron for erythropoiesis, in anemia of chronic disease that has been renamed to anemia of inflammation (Tomas Ganz & Nemeth, 2011; Kemna et al., 2005; A. Kim et al., 2014; Michels et al., 2015; Nemeth, et al., 2004).

There are many studies and major evidences that the state of the iron in the body can affect the infection outcomes (Drakesmith & Prentice, 2012). One trial that provided the supplementation of iron to patients in the Island of Zanzibar had to be ended prematurely due to major malaria-related and non-related sides effects (Sazawal et al., 2006). Another study showed that iron supplementation in bolus dosing, compared to a more evenly distributed administration, resulted in a higher rate of infection-related hospitalization and

mortality among hemodialysis patients (Brookhart et al., 2013). Furthermore, in experimental research studies, iron overload had a negative impact on infections with salmonella or malaria (Drakesmith & Prentice, 2012; D.-K. Kim et al., 2014; Portugal et al., 2011). There is a case report of lethal septicemic plague caused by an attenuated, nonpigmented Yersinia pestis isolates in a researcher that was later on diagnosed with hemochromatosis during an autopsy. This report was particularly striking since the causative Yersinia pestis KIM D27 strain represents aroutinely used, attenuated strain of Yersina that has been employed in many laboratories and until this finding it was not known to cause any human infection (Frank et al., 2011).

In contrast to these data that were obtained primarily for infections common in less developed countries, there isn't that much evidence regarding the role of iron metabolism in non-infectious, critically ill patients. One study reported that high transferrin saturation predicts a poor outcome in patients with acute-on-chronic liver failure (ACLF) (Maras et al., 2015).

1.6. Hepcidin and HCV replication

Chronic hepatitis C virus (HCV) infection is one of the main causes of liver cirrhosis worldwide. The state of iron in patients with HCV is still controversial. There are some discrepancy in the data whether iron is having an inhibitory effect on HCV replication or whether iron overload is enhancing the viral replication (Georgopoulou et al., 2014). HCV patients have lower hepcidin levels for given serum ferritin level (Fujita et al.; Girelli et al., 2009) and unlike other infections no hepcidin induction is usually noticed during

acute phases of HCV infection (Armitage et al., 2014). Hepatic iron accumulation is common in HCV infection and may promote both the development of liver fibrosis and of hepatocellular carcinoma (HCC) (Lambrecht et al., 2011). Furthermore, patients with HCV who also carry Hfe mutations are predisposed to a faster development of liver fibrosis (Tung et al., 2003). Many studies show that increased iron level in general is associated with worse prognosis and acts as a co-morbidity factor for the progression of the HCV disease (Martinelli et al., 2000). This is thought to be related to the fact that iron enhances oxidative stress and triggers chronic inflammation. The direct inhibitory effect of HCV on hepcidin synthesis is regarded as an alternative mechanism.

Before the emergence of direct acting antivirals, the main therapy option in chronic HCV combined pegylated interferon alpha (PEG-IFN-α) with ribavirin treatment (RBV) ("EASL Clinical Practice Guidelines: management of hepatitis C virus infection.," 2011; González-Grande et al., 2016; Zopf et al., 2016), the latter one constituting a nucleoside analog. Treatment of chronic HCV with interferon was not well tolerated with many patients experiencing significant side effects ("EASL Clinical Practice Guidelines: management of hepatitis C virus infection.," 2011; González-Grande et al., 2016; Zopf et al., 2016). Interestingly, a number of studies have reported the modulation of hepcidin by IFN in cell culture (Ryan et al., 2012; Zhang & Rovin, 2010). Furthermore, increased hepcidin expression by IFN-α has also been described (Bartolomei et al., 2011; Ryan et al., 2012; Zhang & Rovin, 2010).

Collectively, these data suggested the possibility that iron metabolism may influence the efficacy of IFN-based hepatitis C therapy.

1.7. Hepcidin in liver diseases

As mentioned earlier hepcidin is a hormone produced mainly by the liver, hence patients with chronic liver diseases often produce a reduced amount of hepcidin, the examples being chronic viral hepatitis, alcoholic liver disease, fibrosis or cirrhosis (Kessler et al., 2015; Meynard et al., 2014).

A lower hepcidin seen in patients with chronic liver disease may spur the development of an iron overload, which has been shown to promote the development of liver fibrosis in experimental models (Lunova et al., 2015). It has been also shown that iron overload associates with a higher risk of hepatocellular carcinoma (HCC). In particular, Nahon et al. showed in a large cohort that liver iron overload and HFE mutations are associated with a higher risk of HCC in patients with alcoholic but not HCV-related cirrhosis (Nahon et al., 2008).

HCC is one of the major causes of death in cirrhotic patients and is the third most common cause of death due to malignancy in the world (Mazzanti et al., 2016). Liver ultrasound is widely used as a screening method for early detection of HCC among high-risk population (Llovet & Bruix, 2000). Since this method is laborious and is of limited efficacy (Pavlov et al., 2016), an identification of predictive factors is of great importance.

The role of hepcidin in cirrhotic patients and HCC remains to be elucidated. To do that, we analyzed the prognostic relevance of baseline serum hepcidin in a large cohort of patients with alcoholic cirrhosis and a prospective follow-up for up to 5 years.

1.8. Extra-hepatic Iron overload in pancreas

Iron is a potentially toxic micronutrient. Its overload contributes to development of multiple human disorders such as liver cirrhosis, diabetes, cardiomyopathy, arthritis, testicular atrophy and bronze discoloration of the skin. Hepcidin deficiency as a result of malfunctions of hepcidin-ferroportin axis is a characteristic hallmark of diseases termed as hereditary hemochromatosis (HH). HH results in iron accumulation in multiple parenchymal tissues, especially heart, liver and endocrine glands such as pancreas (Brissot & Loréal, 2016; Fleming & Ponka, 2012; Pietrangelo, 2010, 2015), however, the exact mechanisms leading to accumulation in the particular tissues as well as the exact toxic effects of this overload remain to be systematically studied

Other causes of iron overload are defects in red blood cell production as seen in thalassemia or Diamond-Blackfan anemia (Fleming & Ponka, 2012; Pietrangelo, 2015). Apart from hepcidin deficiency, the need for repetitive blood transfusions is another major cause of iron accumulation, such as in patients with thalassemia (Brittenham, 2011; Fleming & Ponka, 2012).

Pancreas has been shown to be commonly affected in iron-overload syndromes; this has been particularly well documented in animal studies. The iron accumulation can involve both the exocrine and endocrine parts. A remarkable iron accumulation in the exocrine pancreas has been found in all mice with severe genetically determined iron overload, including BMP6-, hemojuvelin-, hepcidin- or Tfr2-deficient lines (Andriopoulos et al., 2009; Huang et al., 2005; Latour et al., 2016; Meynard et al., 2009; Ramey et al., 2007; Subramaniam et al., 2012). Furthermore, mice with hepcidin-resistant ferroportin mutation have been shown to develop both exocrine pancreatic iron-overload and dysfunction (Altamura et al., 2014). On the other hand, patients with HFE-associated HH may display iron overload both in the endocrine and exocrine pancreas (Ramey et al., 2007).

Given the consistent presence of marked iron overload in exocrine pancreas of different transgenic mouse lines, we decided to further explore the phenomena by characterizing its pathogenic consequences as well as the underlying mechanisms. For that we analyzed pancreata of hepcidin knockouts (KOs) and wild type animals (WTs) subjected to standard or iron-rich diet.

Aims of the Study

Multiple lines of evidence, both from human and animal studies, suggest that the host's iron status affects the development and outcome of different human diseases (Drakesmith & Prentice, 2012; T Ganz, 2013). Hepcidin, the master regulator of iron metabolism is mainly produced in the liver and accordingly, low hepcidin levels are noted in different liver diseases that may consequently lead to development of iron overload. While iron overload is seen in multiple human diseases, the clinical manifestation of iron-overload syndromes is colorful and far from being understood. While iron overload in exocrine pancreas was not systematically addressed in humans, it has been seen in multiple transgenic mouse lines with severe systemic iron overload.

To better understand the biological relevance of iron metabolism in human diseases as well as potential consequences of severe iron overload, I focused on the following topics:

1. Analysis of the iron status in a large cohort of well-characterized critically ill patients admitted to a medical ICU in Germany.

2. Evaluation of the prognostic relevance of baseline serum hepcidin levels in a large cohort of patients with alcoholic cirrhosis.

3. Exploration of the predictive value of hepcidin during the treatment of chronic hepatitis C.

4. Characterization of pancreata of aging hepcidin KOs and wild type animals.

2. Chapter Two: Materials and Methods

2.1. Materials

2.1.1. Chemicals and reagents

Product Name	**Company**
Acetic Acid (100%)	Merck
Agarose	Biozym
Albumin bovine Fraction V	Serva
Ammonium acetate	Sigma
Ammonium chloride	Sigma
Ammonium persulfat	Sigma
Bradford reagent	BioRad
Bromphenol Blue sodium salt	Sigma
Calcium Chloride	Sigma
Citric acid monohydrate	Fluka
Coomassie brilliant blue (R250)	Sigma
DEPC water	Invitrogen
Deoxynucleotides set (dNTP)	Invitrogen
Dimethylsulphoxide (DMSO)	Sigma
Direct Red 80	Sigma

DL-Dithiothretol-DTT	Sigma
DNA ladder (Gene ruler (50bp and 1kb)	Fermentase
ECL Plus Western Blotting Detection Reagents	GE HeaLhcare
ECL Western Blotting Detection Reagents	GE HeaLhcare
Eosin G (0.5%)	Merck
Ethanol	Sigma
Ethidium Bromide	Sigma
Ethylenediaminetetraacetic acid-disodium salt	Roth
Ethylene glycol tetraacetic acid (EGTA)	Sigma
Formaldehyd (37%)	Roth
Glycerine	Roth
Glycerol	Serva
Hematoxylin	Merck
Hydrochloric Acid (37%)	Sigma
Hydrogen peroxide (H_2O_2) 30%	Fischer
Isoflourane (Forane)	Baxter
Isopropanol	Sigma
Methanol	Sigma
Milk powder	Roth
Nonidet P40 (NP40)	Roche
Orange G	Merck
Page ruler plus	ThermoScientific
pHMeter-calibration solutions (pH 4, 7, 9)	Merck

Phenylmethanesulfonyl fluoride (PMSF)	Sigma
Phosphate Buffered Saline (PBS) (10x)	Gibco
Ponceau S	Merck
Potassium cholirde (KCl)	Merck
Potassium phosphate monobasic	Sigma
Protease Inhibitor Cocktail	Sigma
RNAlater® RNA Stabilization Buffer	Qiagen
SDS (Sodium dodeczl Sulphate)	Roth
Sodium carbonate (Na_2CO_3)	Merck
Sodium chloride VWR	Prolabo
Sodium hydrogen carbonates	Merck
Sodium hydroxide	Merck
Sulfuric acid (H_2SO_4)	Merck
TEMED (Tetramethyl ethylene diamine)	Sigma
Tris (tris-hydroxymethyl-aminomethane)	Sigma
Tween 20	Roth
Xylene	J.Y. Baker
Mercaptoethanol	AppliChem
Rotiphorese® Gel 30	Roth
Potassium hexacianoferrate (II) trihydrate	Sigma
Prussian Blue	Sigma
L-Ascorbic Acid	Sigma
Bathophenanthrolinedisulfonic acid disodium salt	Sigma

2.1.2. Instruments and Equipment

Device Name	**Company**
Agarose gel chamber	Bio-Rad
Blotting Trans-BlotTM Cell	BioRad
Cooling centrifuge 5417R	Eppendorf
Cryostat HM 550	Microm
Hypercassette (18x24)	Röntgen Bender
Image processor (550)	Leica microsystem
Incubator shaker Certomat R	Braun
Microscope (DM550B)	Leica microsystem
NanoDrop 2000c	PEQLAB
pH-Meter (Seven Easy pH S20-K)	Mettler
Pipettes	Eppendorf
Power supplies	BioRad
Protein minigel apparatus	BioRad
Protein transfer apparatus	Bio-Rad
Refrigerated centrifuge (5417R)	Eppendorf
Refrigerated centrifuge MuLifuge S-R	Heraeus
Refrigerated centrifuge Sorvall RC-5B	DuPont
Rotating incubator	Bachofer

SDS PAGE electrophoresis system	Bio-Rad
Shaking incubator Unimax 1010	Heidolph
Thermoblock	Eppendorf
Thermocycler	Bio-Rad
Thermomixer 5436	Eppendorf
UV Illuminator Modell 2011	LKB
Water bath Haake W13	Fisons
Balances	Sartorius

2.1.3. Consumables

Product Name	**Company**
Chamber cover glass, Lab-Tek	Nunc
Coverslips	Roth
Cryo tubes	Nunc
Disposable Scalpels sterile	Feather
Falcon 2059 polypropylene tube	BD Falcon
Filter papers	Whatman
Forceps	B2B
Injection needles	Braun
Tissue Culture Plate 96-Well	Nunc

Hyperfilm ECL (18x24cm)	Amersham
Nitrocellulose membrane Protran ®	Whatman
PCR-tubes	B2B
Polystyrene tubes	BD Falcon
PVDF membrane	Millipore
Safe –Lock Eppendorf tubes	Eppendorf
Sample-bottle (2 ml braun)	Roth
Slides	Roth
Super Frost slides	Roth
Syringe Discardit II (2, 5, 10, 20 ml)	Beckton Dickinson
Syringe Omnican®	Braun
Tweezers	B2B

2.1.4. Reaction kits and enzymes

Product Name	**Company**
Bradford Protein assay	Bio-Rad
DNeasy Blood & Tissue Kit	Qiagen
DNase Set (RNase-Free)	Qiagen
Proteinase K	AppliChem
RNeasy Mini Kit	Qiagen

Rneasy Fibrous Tissue Mini Kit	Qiagen
Rnase A	Qiagen
SYBR® Green qPCR SuperMix	Applied Biosystem
Stripping Buffer (Restore WB Stripping Buffer)	Thermo

2.1.5. Antibodies used for Western Blot and Immunofluorescence

2.1.5.1. Primary Antibodies

Table 1 – List of primary antibodies.

Product Name	Origin	Company or Laboratory	Cat. Number
Histone diacetylase 2 (HDAC2)	rabbit	Cell signaling	2540
NFKB/p65 (p65)	rabbit	Neomarkers	RB1638
β-Actin (monoclonal)	mouse	Sigma	A-1978
pH2AX	rabbit	Cell Signaling	RB-9718
F4/80	rat	Abcam	Ab-6641
K8	mouse	Progen	65138
K8 (Ks8.7)	mouse	Progen	65138

2.1.5.2. Secondary Antibodies

Table 2 – List of secondary antibodies.

Product Name	Company	Catalogue Number
Goat anti-mouse IgG (H+L)	Invitrogen	G21040
Goat anti-rabbit IgG (H+L)	Invitrogen	G21234
Goat anti-rat IgG (H+L)	Invitrogen	A10549
Goat anti-Mouse IgG (H+L) Biotinylated	Vector Lab	BA-9200
Goat anti-rabbit IgG (H&L) Biotinylated	Vector Lab	BA-1000
Rabbit anti Rat IgG (H+L) Biotinylated	Vector Lab	BA-4000

2.1.6. Oligonucleotides (Primers)

Table 3 – List of primers.

Hepcidin	HepcScr_hinWT-F	GGGGACACAGGGCAGTAGG
	HepcScr_hinKO-F	GCCTGAAGAACGAGATCAGC
	HepcScr_general-R	AACAGATACCACACTGGGAA

2.1.7. Buffers

APS (Ammonium persulphate) 10%:

1ml: 0.1g-ammonium persulphate

1000µl: distilled water

Coomassie Brilliant Blue Staining solution (per L)

Coomassie brilliant blue	1g
Methanol	500ml
Acetic acid	100ml
Distilled water	400ml

Coomassie de-staining solution (per L)

810mL dH_2O

Homogenization buffer

0.187 M Tris-HCl (pH 6.8)

3% SDS

5mM EDTA

Laemmli Buffer (4X Reducing and non-reducing)

125 mM Tris-HCl (pH 6.8)

4% SDS

20% Glycerol

4% β-Mercaptoethanol

0.01% Bromophenol blue

Resolving Gel Buffer

H_2O	2.05ml
Resolving gel buffer	1.3ml
30% Acrylamide	1.65ml
10% SDS	50µl
10% APS	25µl
TEMED	7.5µl

RIPA Buffer Concentrate

250mM Tris-HCl, pH 7.6,

750mM sodium chloride,

5% Tergitol (NP-40)

2.5% sodium deoxycholate

0.5% SDS

Running buffer (10x) (L)

SDS	50g
Tris Base	150g
Glycine	720g
Distillate water	4L

SDS (Sodium dodecyl sulphate) 10%

SDS 10g

100ml-distilled water

Stacking Gel buffer

H_2O	1.5ml
Stacking gel buffer	0.65ml
30% Acrylamide	375µl
10% SDS	25µl
10% APS	12.5µl
TEMED	2.5µl

TAE (Tris acetate-EDTA) buffer (10x)

48.5g Tris

11.4ml glacial acetic acid

48.720 ml 0.5M EDTA (pH 8.0)

TAE (Tris acetate-EDTA) buffer (1x)

100ml 10X TAE buffer

900ml-distilled water

TBS (10x) (L)

10xTBS, pH=7.6

24.2g Tris

80g NaCl

1L-distilled water

TBST (1x) (L)

100ml 10X TBS

900ml distilled water

0.2% Tween 20

Transfer buffer (10x) (L)

Tris base 3.16g

Glycine 7.21g

Methanol 200ml

1L-distilled water

Transfer buffer (1x)

100ml 10X Transfer buffer

900ml-distilled water

2.2. Methods

2.2.1. Human subjects

2.2.1.1. Selection of patients

To examine the hepcidin levels in systemic inflammatory conditions as well as it's role in the development and progression of different liver diseases, three patient cohorts have been studied (Fischer et al., 2012; Guldiken et al., 2014; Koch et al., 2009; Strnad et al., 2014; Usachov et al., 2012). For all studies, the protocols obtained approval from Human Subjects Committees of the participating centres (CPP, Aulnay-sous-Bois, France; Leipzig, Germany and Aachen, Germany; approved under reference number EK 150/06). Written informed consents to participate in the study were taken from the patients, their spouses or appointed legal guardians. Each study conducted on the participants was in compliance with the Declaration of Helsinki in 1975, as reflected in a priori approval by the institution's human research committee (Shephard, 1976).

2.2.1.2. Patients with alcoholic liver cirrhosis

In this cohort, all alcoholic liver cirrhosis (ALC) patients with first referral to the liver department of Hôpital Jean Verdier (Bondy, France) between January, 1st 1995 and December, 31st 2005 were included (Guldiken et al., 2014; Usachov et al., 2012).

Other causes of chronic liver disease were excluded by a thorough medical history as well as by appropriate clinical and diagnostic workups. To be included in the study, the following criteria had to be fulfilled: 1) a daily alcohol intake not less than 80g per day 2) serological exclusion of human immunodeficiency, hepatitis B and hepatitis C virus infections; 3) diagnostic exclusion of hereditary hemochromatosis, autoimmune liver disease and chronic cholestatic liver disorders 4) exclusion of hepatocellular carcinoma (HCC) at the time of inclusion through the conduction of an abdominal ultrasound and by a serum α-fetoprotein (AFP) level of <50ng/mL; 5) Caucasian origin; 6) residence in France; 7) declaration of compliance and acceptance of a regular follow-up and periodical HCC screening (Guldiken et al., 2014).

A total of 256 patients were recruited for the study. The study started with the detection of liver cirrhosis on the first liver biopsy, and all patients were prospectively evaluated at least twice a year by a physical examination, liver ultrasound and measurement of serum AFP levels. The diagnosis of HCC was based on either the histological evidence or the demonstration of a focal lesion of >2cm in size with an arterial hypervascularisation confirmed by two different imaging techniques or a combination of one imaging technique with a serum AFP level of ≥400ng/mL.

Genders, age, BMI, past medical history of diabetes mellitus, presence of ascites and/or hepatic encephalopathy, serum bilirubin and albumin, prothrombin time, serum alanine-aminotransferase (ALT) and aspartate-

aminotransferase (AST) activities were recorded at the time of inclusion. Daily alcohol intake was obtained by personal interview. To semi-quantitatively evaluate hepatic iron deposits, the Deugnier's histological hepatic iron score was used, adapted to cirrhotic samples as described previously (ranging from 0 to 33) (Nahon 2008).

All patients have been followed-up till their last visit prior to August 31st, 2011, unless the HCC, liver transplantation or death occurred prior to the aforementioned date. This deadline was used to upgrade the patients' files using a computerized database. The database also included contact information of the patients, their relatives or their family physician.

2.2.1.3. Patients with chronic hepatitis C infection

The collective of patients consisted of 74 patients with chronic hepatitis C infection, who received a dual therapy with PegIFN and RBV (PegIntron and Rebetol, respectively; Merck) at the University of Leipzig. The presence of hepatitis C has been determined by a positive hepatitis C virus (HCV) RNA test (Cobas Amplicor; Roche, Basel, Switzerland), the viral genotype has been examined by Inno-LipQA HCV II test (Innogenetics, Ghent, Belgium) (Fischer et al., 2012). All patients enrolled were genotype 1; subtype 1a and 1b (Strnad et al., 2014). To be eligible for the study patients should have been previously untreated, 18 years of age or older, weighing between 40-125kg, positive for HCV and genotype 1, with a plasma RNA level of ≥10,000IU/ml. Patients with other causes of liver disease, decompensated liver cirrhosis, renal insufficiency, human immunodeficiency virus (HIV) or hepatitis B

infection, active malignancies as well as pregnant or breastfeeding women, were excluded from the study. The administration of PegIFN followed subcutaneously at a weekly dose of 1.5µg/kg, RBV has been administered twice daily according to the body weight at 600-1,400mg/day.

Blood samples were collected after first referral, before starting the therapy, after one, four, 12 and 24 week(s) of therapy as well as 6 months after the end of the therapy. Age, sex, basal metabolic index (BMI), ALT, AST, haemoglobin (Hb), white blood cells (WBC), platelet count, serum bilirubin, alkaline phosphatase (AP), serum protein, albumin, blood urea, serum creatinine, glucose, triglycerides, cholesterol, ferritin and AFP have been measured by the local clinical biochemistry department. Histologically HCV patients were subdivided into individuals with mild (fibrosis stage + inflammation grade ≤2.5) and active disease (fibrosis stage + inflammation grade ≥3.0). The inflammation, fibrosis, steatosis and cirrhosis stagings were documented prospectively. In case of anaemia with a haemoglobin level of <10g/dl, RBV has been reduced with increments of 200mg. RBV was completely stopped with a haemoglobin level of <8.8g/dl. Once anemia subsided, the treatment was preceded either with full dose or in gradual steps.

2.2.1.4. Characteristics of sepsis and non-septic critically ill patients

The study included 155 patients (92 male, 63 female with a median age of 64 years; range 18–90 years). Patients were included on admission to the Intensive Care Unit (ICU) of the University Hospital Aachen due to critical illness. Patients that were expected to have a short-term (<72 hours) intensive

care treatment, for example due to a postinterventional observation or an acute intoxication, were not included in this study. Peripheral venous blood was withdrawn at ICU admission before any medical procedures have been performed. Patient's data and clinical information were recorded prospectively.

Among the 155 critically ill patients enrolled in this study, 112 patients conformed to the criteria of bacterial sepsis, according to the criteria of the American College of Chest Physicians & the society of Critical Care Medicine Consensus Conference Committee for severe sepsis and septic shock (Bone et al., 2009; Dellinger et al., 2008).

Each therapy that took-place in ICU, such as volume therapy, vasopressors, ventilation, including the duration, antimicrobial therapy, renal replacement therapy, and parenteral/enteral nutrition, were recorded, alongside a large number of laboratory parameters that were routinely assessed during intensive care treatment (blood gas analysis, potassium, sodium, chloride …etc). All patients were prospectively followed-up for a period of up to three years via telephone interview. The data were obtained from the patients themselves, relatives or the responsible primary care physicians.

2.2.1.5. Characterization of control group

The control group consisted of 156 healthy non-diabetic blood donors (91 males and 65 females, with a median age of 34.5 years; ranging from 20–

71 years). The study protocol has been approved by the institutional review board (EK 150/06). Written informed consent was obtained from the patients themselves. Venous blood was withdrawn before donation. Serum was obtained via centrifugation at 2000g for 10 minutes and the specimens were immediately placed at -80°C where they were kept until further use.

2.2.1.6. Quantification of Hepcidin in Serum

Human hepcidin serum concentrations were determined by using a commercially available ELISA (bioactive) kit (EIA-5258, DRG Instruments, Marburg, Germany) according to manufacturer's protocol. In this competitive ELISA, the hepcidin available in the sample competes for binding with biotinylated hepcidin molecules that are subsequently detected after binding with horse radish peroxidase (HRP)-coupled streptavidin. Briefly, 100µl of Assay Buffer, 20µl of the sample and 50µl Enzyme Conjugate (Hepcidin-25 conjugated to biotin) were added into each well followed by a thorough mixing for 10 seconds. Afterwards, the plate has been incubated for 60 minutes at room temperature under agitation (300-700rpm). After 60 minutes, the contents of the wells were briefly shaken and then rinsed with 300 µl diluted Wash Solution four times. 100µl of Enzyme Complex (Streptavidin conjugated to HRP) has been added into the wells and incubated at room temperature for 30 minutes. Subsequently, the plates were shaken and washed with 300µl of diluted Wash Solution three times. 100µl of HRP Substrate Solution (Tetramethylbenzidine) has been added to each well and incubated for 20 minutes at room temperature. To stop the reaction, 100µl of Stop Solution

(containing 0.5M H_2SO_4) has been submerged into each well. The absorbency has been determined with a micrometer plate reader at 450nm.

2.2.1.7. Serum enzymes concentrations and serum iron parameters

The presented parameters were measured by the local clinical chemistry department using routine methods. Levels of soluble form of the urokinase plasminogen activator receptor (suPAR, ViroGates, Birkeroed, Denmark) were determined with commercially available ELISA kits. Interleukin 6 (Siemens Healthcare, Erlangen, Germany) and procalcitonin (Kryptor, B.R.A.H.M.S. Diagnostica, Henningsdorf, Germany) serum concentrations were quantified by commercial chemiluminescence assays. All data are shown as means ± SD.

2.2.2. Animal experiments

To study the consequences of deficient hepcidin signaling, we used previously described hepcidin knockout (KO) mice on C57BL/6N background(Lesbordes-Brion et al., 2006; Lunova et al., 2014). To induce iron overload, hepcidin KO mice and their non-transgenic littermates have been fed with 3% carbonyl iron - containing diet starting at the age of 28 days (Ssniff, D12450B including 3% Iron carbonate, Sigma) (Lunova et al., 2014). The used diet has been pre-mixed and ordered as pellets from the vendor (Sniff). Mice fed standard diet (Ssniff, D12450B) were used as controls. We kept the animals in plastic cages (26.5cm x 20cm x 14cm) at a constant

temperature of 22°C in the animal facility of the University of Ulm (Ulm, Germany). Both water and chow were given ad libitum. The animals were kept at a 12-hour day-night cycle with a light period from 7.00-19.00 o'clock. Mice were sacrificed at the age of 6 months by CO2-inhalation, blood was obtained by cardiac puncture, for the measurement of serum parameters, and pancreas was very rapidly removed and cut into pieces that were divided into a part that was immediately fixed in 10% formalin for histological/immunohistochemical (IHC) staining, a snap- frozen section in liquid nitrogen for protein analysis, and the rest was submerged into RNAlater stabilization reagent (Ambion) for mRNA analyses. All animal experiments have been approved by the state of Baden-Württemberg in Germany and the University of Ulm animal care committee and were conducted in compliance with the German Law for Welfare of Laboratory Animals. All treatments have been made in accordance with the German guidelines for animal research and animal welfare.

2.2.2.1. Deoxyribonucleic acid (DNA) purification

For DNA isolation we used DNeasy Blood and Tissue kit (Qiagen). Tail-tips from the mice were cut with a clean scissor and then digested in a mixture of proteinase K and buffer ATL on 56°C- floating bath over night. To ensure an optimal binding of DNA to the column, 200µl buffer AL and then 200µl 96 – 100% ethanol (Sigma) were added, samples were thoroughly mixed after each step and placed onto DNeasy Mini spin columns. This step was followed by subsequent centrifugation. During that, DNA was selectively bound to the column and the flow-through was discarded. To wash the column, 500µl of

AW1 buffer and then 500µl of AW2 were added, with a centrifugation following each step. To remove the washing buffer AW2 completely, the collection tubes was replaced with new ones and columns were centrifuged once more at 20000g for 1 minute. Finally, a low - salt elution buffer AE (provided in the kit) with basic pH was used to elute the DNA from the column.

2.2.2.2. Polymerase Chain Reaction (PCR)

The mice were genotyped using polymerase chain reaction (PCR). For that we used previously described primers (see section 2.1.5 for details). The PCR was used to amplify DNA segments and includes cyclic temperature changes of denaturation, annealing and extension. For the PCR, an automated thermal cycler (PTC-200 DNA Engine Cycler, BioRad Laboratories GmbH, Munich, Germany) has been used. The reaction mixture consisted of 3µl 10x buffer (without $MgCl_2$), 2,4µl $MgCl_2$ (25mM), 0,6µl dNTP-Mix (10mM/each), 1µl upstream and downstream oligonucleotide (F and R), 0,2µl Fermentase Taq polymerase (5U/µl) and 20,8µl H2O that was complemented with 1µl of DNA isolated from mouse-tails. The reaction mix was placed into the PCR tubes. The first step of the reaction was started with an initial denaturation of DNA at 94°C for 4 minutes. After that, 40 PCR cycles were performed. These cycles consisted of a denaturation step at 94°C for 30 seconds, an annealing step at 54°C (optimal primer melting temperature) for 30 seconds, and an extension step performed at 72°C (optimal working temperature for Taq DNA Polymerase) for 30 seconds (extension time is dependent on DNA length and Polymerase. Under normal conditions this time is about 10 second per 100bp of DNA). To ensure that all single strands of the

DNA become fully extended, the final extension was performed at 72°C for 10 minutes and the DNA fragments were stored at 4°C.

To detect any possible contamination with foreign DNA, a reaction mixture without DNA template has been run as a negative control. To ensure a successful amplification, a positive control has been loaded with each reaction that contained a previously successfully amplified DNA.

2.2.2.3. Agarose gel electrophoresis of DNA

To estimate the size of DNA products, agarose gel electrophoresis was performed (Grabar, 1957). The agarose gel separated molecules based on their sizes. By applying an electric field the negatively charged DNA molecules move through the agarose matrix. The short molecules migrate faster through the gel. After PCR amplification, 9 µl of the sample was mixed with 1µl of 6x DNA Loading Dye (Fermentas) and loaded on 1.5% agarose gel (Biozym; this gel concentration was used to allow detection of this relatively small DNA fragments). To detect DNA fragments, 0.0005 % ethidium bromide (Sigma) was added to the mixture. This dye intercalates between two DNA strands and omits fluorescence under UV light. Electrophoresis was performed in TAE buffer at 130V for 45 minutes. Gene Ruler 50bp (Fermentas) was used to identify the DNA fragment size.

2.2.2.4. Bradford Protein Assay and Protein Immunoblot (Western Blot)

All procedures concerning protein isolation and latter application were performed on ice. To isolate protein, tissues (approximately 25mg) were homogenised on ice in 600µl homogenisation buffer (Toivola DM, 2000) using the Potter-Elvehjem grinder with Teflon pestle that is suited for disruption of soft tissues, such as pancreas. The employed buffer contained 3% sodium dodecyl sulphate (SDS), which efficiently denatured the proteins, preventing them from degradation. The homogenised liver tissues were heated for 5 minutes at 95°C for denaturation. Homogenates were subsequently sheared with a 1ml syringe and a 21-22 G needle for approximately 20 times to disrupt DNA then centrifuged for 2 minutes at 14.000rpm to remove non-dissolved debris. After extraction, samples were mixed with 4x Reducing Laemmli buffer. All samples were then stored until use at -20°C. Prior to use, protein extracts were briefly denaturated at 95°C and applied to a gel. The protein bands were visualised by Coomassie staining (Brilliant Blue R, Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Coomassie staining was done for at least 30min at room temperature (RT) and then gels were destained in the destaining solution for at least 1h at RT.

Equal amounts of protein were separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE). Briefly, denatured proteins bind to SDS to ensure relatively constant negative charges and are therefore separated based on their molecular weights. The gel consisted of a stacking gel, which has large pores and enable concentration of proteins in thin zones, and a resolving gel (10% gel was used in most gels, for that exact composition of the gels please

refer to section 2.1.5.), which separates proteins according to their size. The electrophoresis was performed at 70V for 30 minutes followed by 110V for 1 hour using Bio-Rad system. After the electrophoretic separation, the negatively charged proteins were transferred onto a PVDF membrane (0.45µm pore size, Millipore), prior to that the PVDF was activated for 5 minutes in Methanol. For the transfer we used a constant current of 0.2A for 70 minutes and a wet-blot device (Bio-Rad) by using the trasfer buffer (for the composition please reffer to section 2.1.5.). Afterwards the PVDF membranes were blocked for 1 hour at room temperature with 5% milk powder dissolved in Tris Buffered Saline containing 0.1% Tween (TBST) buffer (Roth) to decrease nonspecific bindings to the membrane. To detect the proteins of interest, the membranes were incubated with the primary antibody (diluted in blocking buffer) at +4°C overnight. Afterwards, the membrane has been washed at least 4 times (7 minutes each) with TBST buffer to remove the unbound antibody before the membranes were incubated with the corresponding secondary antibody (diluted in 5% blocking buffer) for 1 hour at room temperature. After washing with TBST for 4 times (with 7 minutes interval), these membranes were incubated with enhanced chemiluminescence reagent (GE Healthcare/Amersham Biosciences). This reagent contains substrate for horseradish peroxidase (HRP), i.e. the enzyme coupled to secondary antibodies. The reaction between HRP and this substrate leads to formation of hydrogen peroxide that oxidizes a compound termed luminal and therefore leads to an emission of light. For the antibodies used see section 2.1.4.

2.2.2.5. Tissue staining

The pancreas tissue were embedded in paraffin then cut into 3µm sections and fixed overnight in 10% buffered formalin at room temperature. Thereafter the sections were stained with Haematoxylin and Eosin (H&E), iron staining or were used for immunohistochemistry (IHC).

2.2.2.5.1. Haematoxylin and Eosin staining (H&E)

For the overall histological assessment, H&E staining was used. The tissue was deparaffinised in xylol and hydrated with serial dilutions of ethanol (100%; 96%; 90%; 80% and 70%). After that, probes were incubated in deionised water for 1 minute at RT. To visualise the nuclei, slides were incubated with haematoxylin solution (Dako) for 5 minutes. The stain was removed by washing with tap water. The samples were then placed into 0.5% Eosin G-solution for 5 minutes (cytoplasmic staining) and finally being washed in deionized water. The slides were then dehydrated with graded ethanol series (70%; 80%; 90%; 96% and 100%, each step for 1 minute) and finally incubated with xylol for 10 minutes. The slides were covered with a glass coverslip using Entellan medium (Merk). The slides were analysed with a light microscope equipped with digital camera (model DM 5500, Leica Microsystems Imaging Solutions GmbH, Wetzlar, Germany) and Leica Application Suite 3.8 has been used for acquisition of digital images.

2.2.2.5.2. Perls' Prussian Blue staining

For the visualisation of non-heme iron in the tissue, Perl's Prussian Blue staining has been used. The formaldehyde-fixed, paraffin-embedded pancreatic tissue sections were washed twice with xylol, 10 minutes each time to remove the paraffin. Afterwards the sections were incubated in a stepwise descending ethanol series (100%; 96%; 90%; 80% and 70% - 2 minutes each) to be hydrated. The incubations were followed by washing with deionised water 3 times, 5 minutes each. To produce blue iron complexes, slides were incubated for 30 minutes in a mixture of 2g of potassium hexacyanoferrate (II) trihydrate (Sigma) dissolved in distilled water and then mixed with 2.7% HCL to denature the binding proteins of the haemosiderin molecule, and thereby release ferric (3+) ions. The sections were then washed in a deionised water and stained with 0.5 % Eosin G-solution (Merk) for 5 minutes at RT, which helps in visualizing the cytoplasm. Finally, the sections were washed with deionised water, dehydrated in a graded ethanol series (70%; 80%; 90%; 96% and 100%, 1 min each) and incubated in xylol for 10 minutes. The slides were covered with glass coverslip using the Entellan medium (Merk).

2.2.2.5.3. Immunohistochemical staining

For immunohistochemical staining, a mouse ABC Staining System (Vectastain ABC Kit, Vector Laboratories) has been used. Briefly, deparaffinized, hydrated sections were boiled in Antigen Retrieval Solution (Vector Laboratories) and then incubated with a 3% hydrogen peroxide solution (H_2O_2) (Fischer) for 10 minutes to remove the endogenous

peroxidase activities. The slides were then incubated with a blocking buffer containing bovine serum albumin (BSA, fraction V) (Serva) for 20 minutes at room temperature, to remove the unspecific background. Afterwards, the slides were incubated with a primary antibody for 1 hour, then washed at least 3 times with TBST, and incubated with biotinylated secondary antibody for 30 minutes at RT. To visualize the specific signal, a chromogenic reporter - AB enzyme reagent (Vector Laboratories) was used. To that end, the slides were incubated with AB enzyme reagent for 30 minutes and washed with TBST. The substrate Vector Nova Red (Vector Laboratories, INC) reacts with the enzyme to yield a product with an intense colour that can be easily analysed with a microscope. The slides were then dehydrated by submerging into a stepwise ethanol series (70%; 80%; 90%; 96% and 100%, each step for 1 minute) and finally incubated with xylol for 10 minutes. The slides were covered with a glass coverslip using Entellan medium (Merk).

2.2.2.6. Terminal deoxynucleotidyl transferase dUTP nick end labeling Assay (TUNEL Assay)

To detect the DNA fragments in apoptotic cells we used the TUNEL Universal Apoptosis Detection Kit. The assay depends on the presence of nicks in the DNA which can be identified by terminal deoxynucleotidyl transferase (TdT), an enzyme that will catalyze the addition of dUTPs that are secondarily labeled with a marker or cells that have suffered severe DNA damage (Gavrieli, Sherman, & Ben-Sasson, 1992). After incubating the sections for 30 minutes at 60°C, the samples were washed twice in xylene for five minutes each and then rehydrated at gradient concentrations of ethanol

(100%, 95%, 90%, 80%, 70%) for five minutes each time. The slides were then washed by rinsing them with PBS for five minutes. Afterwards the slides were digested for 15 min at 21-37°C with Proteinase K solution (the later contains 2µl 50X Proteinase K in 98µl PBS buffer). The slides were rinsed again twice with PBS for 5 minutes. The sections were then incubated in the 3% H_2O_2 in methanol containing Blocking solution. The slides were then rinsed with PBS twice (5 minutes each). A 50µl TUNEL Reaction Mixture were added to the slides and were covered with a coverslip to be incubated in the dark under wet conditions for 60 minutes at 37°C. The slides were rinsed in PBS 3 times (5 minutes each). The sections were assayed with a fluorescence microscope with an excitation wavelength of 450-500nm and an emission wavelength of 515-565nm.

2.2.2.7. Quantifying iron in pancreas

Pancreas iron content was measured as described previously (Ramey et al., 2007). Firstly, the tissue has been weighted, then dried at 120°C for 24 hours. Subsequently, the specimens were hydrolysed in 1ml of 100mM citric acid (Calbiochem) at 60°C for 4 hours. For the detection of the iron, 500µl of the samples were mixed with 500µl of 100mM citric acid and 50µl of L-ascorbic acid (Sigma). Ascorbic acid reduces the iron (III) to iron (II), and then the iron (II) reacts with 100µl of 5mM Bathophenanthrolinedisulfonic acid disodium salt (BPS, Sigma) to form a red colour complex. The samples were mixed well and incubated for 30 minutes in the dark to develop the described red-complex. The absorbance was measured with a spectrophotometer at wavelength 535nm. The data were presented as average ± SD.

2.2.2.8. Thiobarbituric Acid Reactive Substances (TBARS) Assay

TBARS assay was used for assessing lipid peroxidation, which is a well-established mechanism of cellular injury in animals and is used as an indicator of oxidative stress in cells and tissues (Yagi, 1998). Malondialdehyde (MDA) is a naturally occurring product of lipid peroxidation. Lipid peroxides, derived from polyunsaturated fatty acids, are unstable and decompose to form a complex series of compounds, which include reactive carbonyl compounds, such as MDA (Janero, 1990).

After cutting and placing approximately 25mg of pancreatic tissue into a 1.5ml centrifuge tube, we homogenised it with 250µl of Radioimmunoprecipitation assay buffer concentrate (RIPA). The homogenised samples were then sonicated for 15 seconds at 40V on ice. The tubes were then centrifuged at 1,600xg for 10 minutes at 4°C. The supernatants were then collected and kept on ice. All samples and standards have been assayed in duplicates. 100µl of sample/standard were added to MDA 5ml vial. Afterwards 100µl of sodium dodecyl sulfate (SDS) Solution was added to the vial and swirled for mixing. 4ml of the Color Reagent was added to the downside of the vials. The vials were boiled for 1 hour in an upright position with each being closed by a cap. To stop the reaction the vials were kept in an ice bath for 10 minutes. Afterwards another centrifugation followed for 10 minutes at 1,600xg at 4°C. After adding 150µl from the vial to each well, the absorbance was read at 530-540nm.

2.2.3. Analysis and software

2.2.3.1. Statistical Analysis

Given that most samples were not normally distributed, the data are given as median and range. Mann-Whitney- U-test assessed the differences between two groups. Multiple group comparisons were performed with Kruskal-Wallis- ANOVA and Mann-Whitney-U-test for post hoc analysis.

Differences were considered statistically significant at $p<0.05$. Correlations between variables have been analysed using the Spearman's Rank correlation coefficient tests. Conducting univariate and multivariate analyses in the Cox regression model tested the prognostic value of the variables. The prognostic value of the variables was tested by univariate and multivariate analyses in the Cox regression model. The associations between the parameters and the risk of mortality, hazard ratios (HR) and 95% confidence intervals (CI) were estimated using Cox proportional hazards models with iron parameters and other biomarkers employed as continuous parameters.

The distribution of death and HCC was compared with the log-rank test. A significant level <0.10 was used to select variables for Cox proportional hazards models using a stepwise procedure with a threshold of α=0.05. Kaplan Meier curves were plotted to display the impact on survival and to

estimate the occurrence of HCC and death. The Youden-index was employed to find the optimum cut-off values. Values of $p<0.05$ were considered statistically significant. All statistical analyses were performed with SPSS version 12.0 (SPSS, Chicago, IL, USA).

2.2.3.2. Image analysis

The image processing and analysis were conducted on Adobe Photoshop CS5 and Image J (http://rsbweb.nih.gov/ij/) program.

2.2.3.3. Software

Microsoft Office Excel, Word, Powerpoint (University of Ulm, Germany or RWTH-Aachen, Germany)

Adobe Photoshop CS5 and Adobe Illustrator

The gene bank data (http://www.ncbi.nlm.nih.gov)

SPSS V12.0 software (SPSS, Chicago, IL, USA)

2.2.4. Ethics statement

The mice received the utmost humane care and all the experiments were approved by the appropriate local authorities and the Institutional Animal Care Committee state of Baden-Württemberg, Germany (Regierungspraesidium Tübingen, registration number: 35/9185.81-3).

2.2.5. Safety measures

All operations with genetically modified organisms and plasmid DNA were performed in accordance to the “Gentechnikgesetz” from the year 1990 and to the rules described in the ‘‘Gentechnik-Sicherheitsverordnung’’ from the year 1990. Ethidium-bromide, formaldehyde, DEPC and other chemicals that are dangerous for the environment has been carefully handled and properly disposed in accordance with guidance by the institution.

3. Chapter Three: Results

3.1. Role of Iron parameters in ICU

3.1.1 ICU cohort characterization

The analyzed ICU cohort comprised of 155 patients including 112 septic patients and 43 non-septic ones, with approximately 3:2 male to female ratio (Table 4). 23.9% of the patients died within the ICU. The ICU death rates amounted to 26.8% among the septic patients and 16.3% among the non-septic patients. All patients were followed-up for three years. During this interval, 52.7% of the septic subjects passed away compared to 37.2% of the patients that didn't fulfill the sepsis criteria. As expected, the septic subjects showed significantly higher levels of procalcitonin (PCT) and C-reactive protein (CRP) ($p<0.001$) at admission compared to the non-septic patients. The former also had a worse renal function as evidenced by higher creatinine levels ($p<0.001$).

Table 4 – Characterization of the patients admitted to the ICU at the University Hospital Aachen. For quantitative variables, median and range (in parenthesis) are given. Abbreviations: ICU, intensive care unit; n, number; BMI, body mass index; CRP, C-reactive protein, GFR, glomerular infiltration rate, WBC; white blood cells.

Characteristics	All Patients	Non-Sepsis	Sepsis
Number of patients	155	43	112
Sex (male/female)	92 / 63	25 / 18	67 / 45
Age median (range) [years]	64 (18-90)	62 (18-85)	66 (20-90)
APACHE-II score median (range)	17 (3-40)	14.5 (3-28)	18 (3-40)
ICU days median (range)	9 (1-83)	6 (2-42)	12 (1-83)
Death during ICU n (%)	37 (23.9%)	7 (16.3%)	30 (26.8%)
Death during follow-up n (%)	75 (48.4%)	16 (37.2%)	59 (52.7%)
Mechanical ventilation n(%)	112 (72.3%)	29 (67.4%)	83 (74.1%)
Ventilation time median (range) [h]	129 (0-1584)	46 (0-986)	203.5 (0-1584)
BMI median (range) [m^2/kg]	25.0 (15.3-61.0)	26.5 (15.9-40.5)	24.9 (15.3-61.0)
WBC median (range) [x10^3/µl]	12 (<0.1-208)	9.8 (2.5-26)	12.8 (<0.1-208)
CRP median (range) [mg/dl]	116.5 (5-230)	13 (5-230)	165 (5-230)
Procalcitonin median (range) [µg/l]	0.9 (0.1-207.5)	0.2 (0.1-36.5)	2.3 (0.1-207.5)
Creatinine median (range) [mg/dL]	1.15 (0.1-11.5)	0.9 (0.2-11.5)	1.4 (0.1-10.7)
GFR Cystatin median (range) [ml/min]	45 (4-379)	55 (7-379)	30.5 (4-379)

3.1.2. Iron parameters undergo dramatic changes in ICU patients

Compared to the blood donors, the iron profile of the patients admitted to the ICU showed marked changes, septic patients exhibited hypoferremia (median: 4.4µmol/l vs. controls 17.7µmol/l; $p<0.001$) and decreased transferrin levels (median: 1.1g/l vs. controls 2.7g/l; $p<0.001$). TSAT values were similar in controls and ICU subjects but significantly lower in septic vs. non-septic patients (medians: septic 15.8%, non-septic 26.4%; $p=0.04$). Serum ferritin levels were higher in ICU patients (median 365.1ng/ml, vs. controls 32.9ng/ml; $p<0.001$), the same did also apply to hepcidin (50.3ng/ml vs. controls 8.0ng/ml, $p<0.001$). Moreover, both parameters were significantly

higher in septic vs. non-septic individuals (medians 445.7ng/ml vs. 135.3ng/ml; p=0.001 and 63.2ng/ml vs. 19.8ng/ml; p<0.001, respectively) as described previously (Nemeth et al. 2003; Table 5). The ICU subjects also frequently displayed anemia that was not seen in the control subjects (median Hemoglobin: controls 15.2g/dl; ICU 10.3g/dl; (Table 5)).

Table 5 – Iron parameters in patients admitted to the ICU and in healthy blood donors (controls). Blood for measurement of the highlighted parameters was drawn shortly after the admission to the ICU or immediately prior to blood donation.

Iron metabolism	All	Non-Sepsis	Sepsis	Controls
Hepcidin median (range) [ng/ml]	50.3 (4.1-544.4)	19.8 (4.1-120.1)	63.2 (4.2-544.4)	8.0 (0-62.9)
Serum iron median (range) [µmol/l]	5.7 (2.0-42.2)	9.7 (2.0-26.2)	4.4 (2.0-42.2)	17.7(5.7-54.3)
Serum transferrin median (range) [g/l]	1.3 (0-171.6)	1.5 (0.9-3.1)	1.1 (0-171.6)	2.7(1.8-3.9)
Transferrin saturation median (range) [%]	17.7 (4.2-100)	26.4 (6.3-100)	15.8 (4.2-100)	24.65 (3.7-82.6)
Serum ferritin median (range) [ng/ml]	365.1 (15.6-53977)	135.3 (15.6-7187.4)	445.7 (23.3-53977)	32.9 (7.5-283.8)

Furthermore, hepcidin levels of the septic patients admitted to the ICU differed according to the etiology. As mentioned above the septic subject in general showed a marked increase in hepcidin levels compared to non-septic subjects, and sepses originating from a urinary tract infection were the ones with the highest levels (Figure 2), while patients with liver cirrhosis showed generally a decrease in the level of hepcidin.

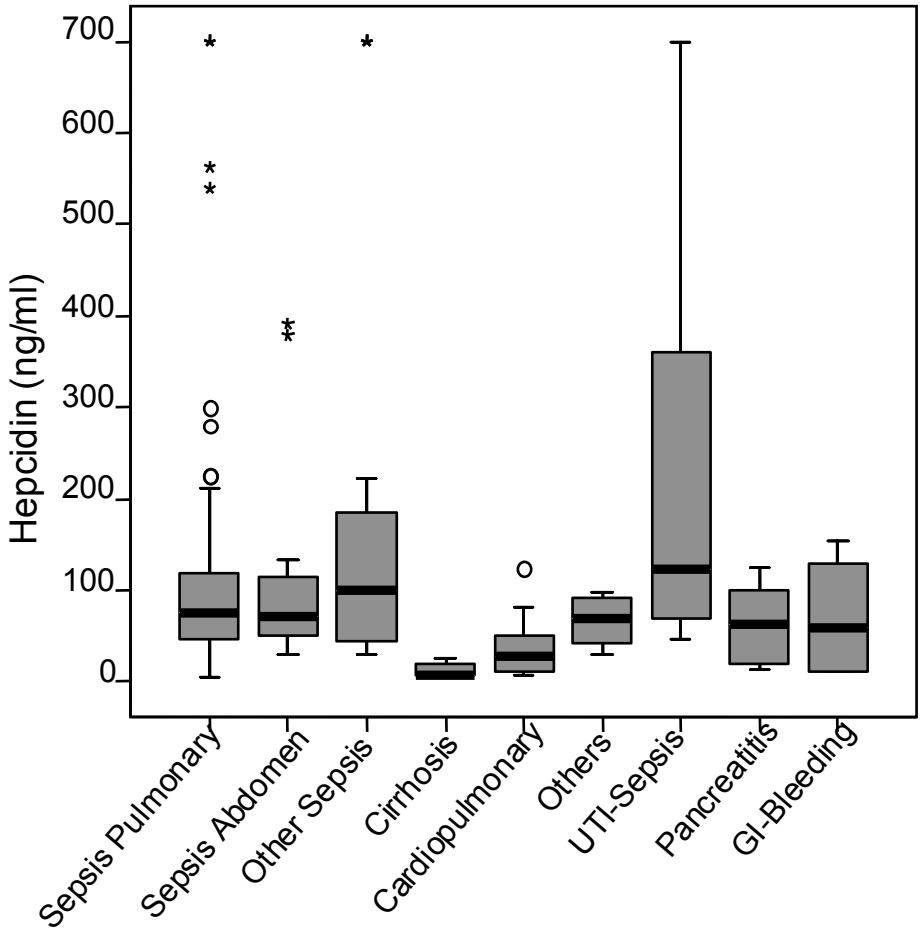


Figure 2 – In patients admitted to intensive care unit (ICU), serum hepcidin levels differ according to the disease etiology. Admission levels of serum hepcidin were determined in all patients and subcategorized according to the main admission diagnosis. Hepcidin levels are higher in the septic subjects, while the cirrhotic subjects show low hepcidin levels. Boxplots display median with first and third quartile, while whiskers indicate smallest and largest non-outlier observations. Abbreviations: UTI, urinary tract infection; GI, gastrointestinal.

Using Spearman rank's correlation coefficient test, serum hepcidin was moderately correlated with serum ferritin (=0.583, p<0.001), while it showed a weak negative correlation with serum iron (r=0.295, p<0.001), and transferrin (r=—0.282, p<0.001). Surprisingly, TSAT, which represents a ratio of iron and transferrin, showed no significant correlation with hepcidin in the studied

population (Figure 3). A worse renal function, as determined by creatinine and cystatin C levels, was associated with increased hepcidin levels (r=0.226 and r=457: respectively) (Figure 3e and 3f). Our analysis further demonstrated moderate and week positive correlations of hepcidin with C-reactive protein (CRP) and (suPAR), respectively (Figure 3g and 3h).

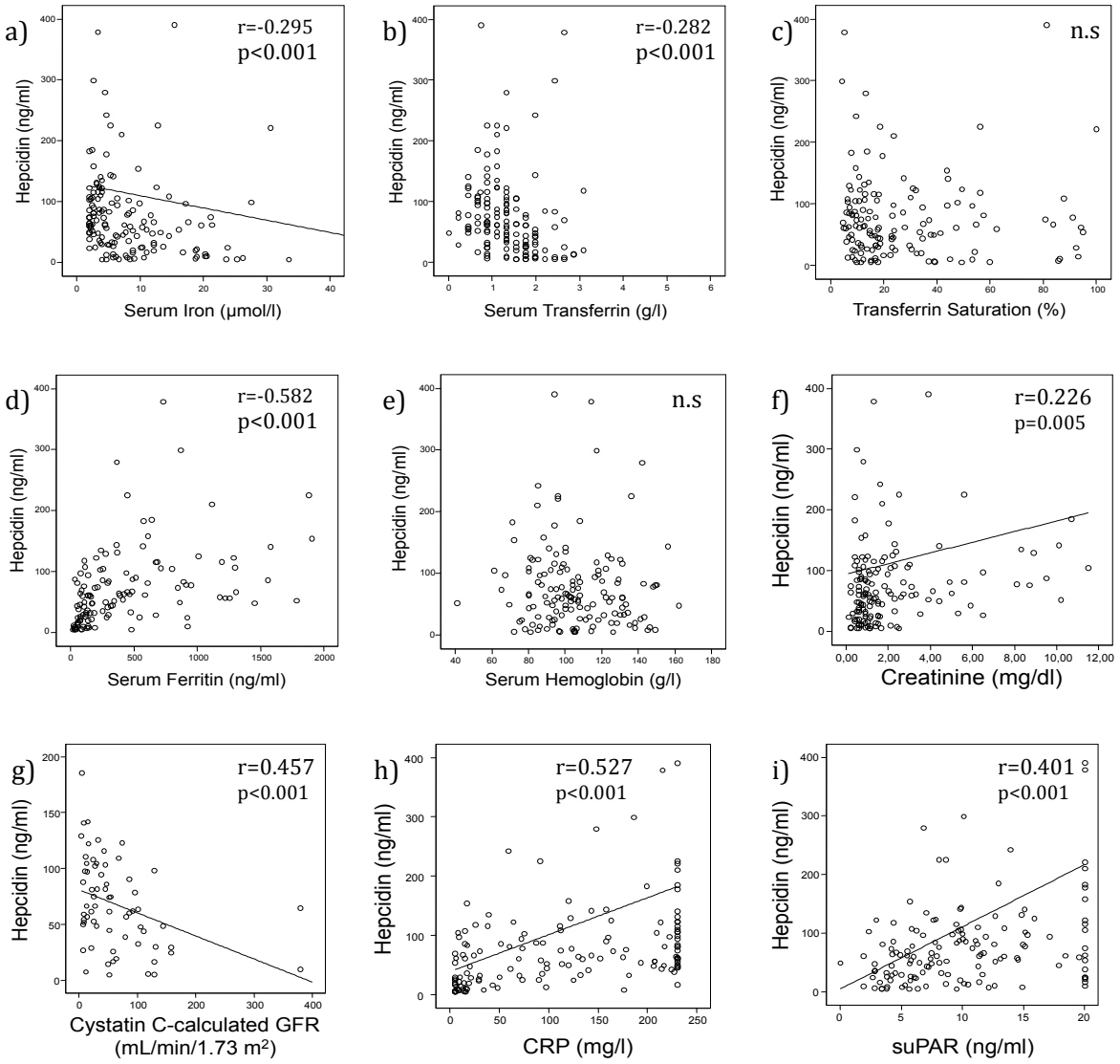


Figure 3 – Hepcidin correlation with other iron, inflammatory and renal parameters. All correlations refer to the first day of admission to the intensive care unit and were calculated using Spearman rank's correlation coefficient test. As expected hepcidin moderately correlates with ferritin (d) and a mild negative correlation is seen with both iron and transferrin (a and b). As an acute phase protein, hepcidin positively associates with the examined inflammatory parameters (g,h). Hepcidin also positively correlates with serum creatinine and negatively with GFR calculated based on serum cystatin C values (e,f). Abbreviations: CRP: C-reactive protein; SuPAR, soluble urokinase-type plasminogen activator receptor.

3.1.3. Iron parameters but not hepcidin predict mortality of the ICU subjects

Next, we assessed the prognostic value of iron parameters in the ICU cohort. In our cohort, 37 patients (24%) passed-away during the ICU care, while the total mortality during the whole follow-up period reached 75 (48%; Table 4). We conducted univariate Cox regression analyses, which illustrated a prognostic importance of iron (p=0.005), TSAT (p=<0.001) and ferritin (p=0.02) for ICU-mortality, while showing a prognostic value of iron (p=0.007) transferrin (p=0.008), TSAT (p=<0.001) and ferritin (p=0.02) for the overall mortality (Table 6). In the multivariate analyses TSAT showed to be the only iron parameter that can independently predict both short and long term mortalities (Table 6).

Table 6 – The prognostic value of iron parameters of the analyzed ICU patients according to Cox's proportional hazards model.

	Short-term survival		Long-term survival	
Parameters	**Cox univariate analysis**	**Cox multivariate analysis**	**Cox univariate analysis**	**Cox multivariate analysis**
Hepcidin	n.s.	n.s.	n.s.	n.s.
Iron	1.052 (1.015-1.089) p=0.005	n.s.	1.040 (1.011-1.071) p=0.007	n.s.
Transferrin	0.586 (0.335-1.027) p=0.06	n.s.	0.587 (0.396-0.870) p=0.008	n.s.
TSAT	1.022 (1.012-1.032) p<0.001	1.022 (1.012-1.032) p<0.001	1.023 (1.015-1.031) p<0.001	1.023 (1.015-1.031) p<0.001
Ferritin	1.000 (1.000-1.000) p=0.02	n.s.	1.000 (1.000-1.000) p=0.02	n.s.

To demonstrate a difference in survival, we performed Kaplan-Meier curve analyses (Figure 4), the cut-off values were determined by using Youden-Index. High serum iron levels were strongly associated with both ICU

and long-term mortality, when using a cut-off value of 10.5µmol/ml, while low transferrin (cut-off 1.6g/l) and high ferritin levels (cut-off 1500ng/ml and 800ng/ml for short and long-term mortality, respectively) were also associated with increased mortality. High transferrin saturation with a cut-off of 55% turned out to be the best discriminator among iron parameters for both short- and long-term survival (Log rank 28.5, $p<0.001$; log rank 27.4, $p<0.001$, respectively). Of note, no significant difference in survival was observed among subjects with high vs. low hepcidin values.

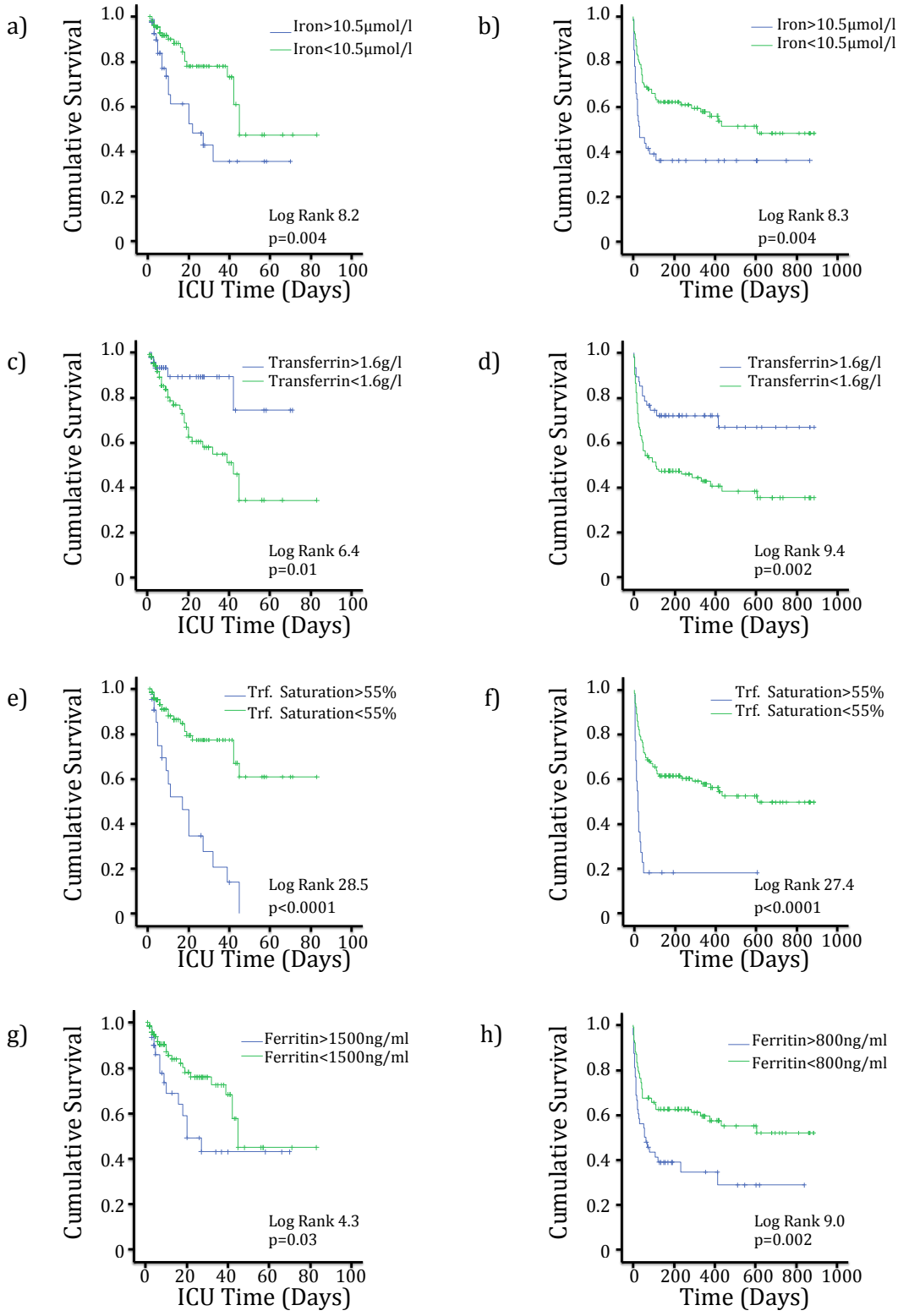


Figure 4 – Kaplan Meier survival curves based on iron parameters of the ICU patients. Short-term mortality was defined as death during the ICU stay (a, c, e and g), whereas long-term mortality took into consideration a follow-up period of up to 3 years (b, d, f and h). High transferrin saturation with a cut-off of 55% turned out to be the best discriminator for both long- and short-term survival (e,f).

3.2. Hepcidin and the treatment response in chronic HCV patients

3.2.1. Chronic HCV cohort characterization

Cumulatively seventy-four patients with chronic hepatitis C (all genotype 1) undergoing dual therapy with pegylated interferon and ribavirin were included in this study. The age of the population ranged from 18-68 years (Table 7). Only two patients were already diagnosed with cirrhosis at baseline. 55% of the population had genotype 1b, while 28% had genotype 1a, with the rest showing either a mixed genotype 1 (a+b) or an unknown subtype. Both genders were represented in the population in an evenly distributed manner. Most of the patients (76%) displayed a mild to moderate amount of inflammation in the histological observation (grade 1-2). Male subjects had median hemoglobin (Hb) of 15.7g/dl, while the female population displayed median Hb of 13.8g/dl.

Table 7 – Baseline characteristics of the analyzed patients with chronic hepatitis C infection. For quantitative variables, median and ranges (in parenthesis) are given. Abbreviations: BMI, body mass index; SVR, sustained viral response.

Characteristics		All Patients	Male	Female
Number of Patients		74	41	33
Age in years (range)		44 (18-68)	43 (24-67)	43(18-68)
BMI (range)		25.63 (18.1-35.2)	26 (18,1-35,2)	24.8 (18.3-33.5)
Viral genotype n (%)	**1a**	21 (28%)	8 (20%)	12 (36%)
	1b	41 (55%)	22 (54%)	19 (58%)
	Unspecified	13 (18%)	11 (27%)	2 (6%)
Fibrosis stage n	**No to mild/ Moderate/Severe**	37/21/11	21/12/5.0	16/9/6.0
Inflammation Grade n (%)	**0**	5 (7%)	2 (5%)	3 (9%)
	1	28 (38%)	15 (37%)	13 (39%)
	2	28 (38%)	17 (41%)	11 (33%)
	3	6 (8%)	2 (5%)	4 (12%)
Cirrhosis (no/yes/unknown)		67/2/5	37/1/3	30/1/2
Steatosis (no/yes/unknown)		44/10/20	24/5/12	20/5/8
SVR n (%)		50 (68%)	25 (61%)	25 (76%)
Hemoglobin g/dl (range)		14.7 (11.6-16.8)	15.7 (12.8-18.7)	13.8 (11.6-16.8)

3.2.2. Hepcidin doesn't predict the treatment response in chronic HCV on dual therapy

Serum hepcidin levels were evaluated at the baseline, at weeks 1, 4, 12, 24 of the therapy and 6 months after the end of the treatment. Patients with chronic hepatitis C infection showed a marked decrease in hepcidin levels compared to healthy controls (4.9 vs. 8.0ng/ml, $p<0.001$). Male patients displayed significantly higher hepcidin levels throughout the study compared to female counterparts (6.0 vs 2.5ng/ml at baseline; $p<0.001$). One week after starting the therapy, a significant increase (median: 12.68ng/ml, $p<0.001$) in

serum hepcidin has been detected compared to baseline. However, the levels started to decrease by week four, and almost returning to the baseline levels 6 months after the end of therapy (Figure 5).

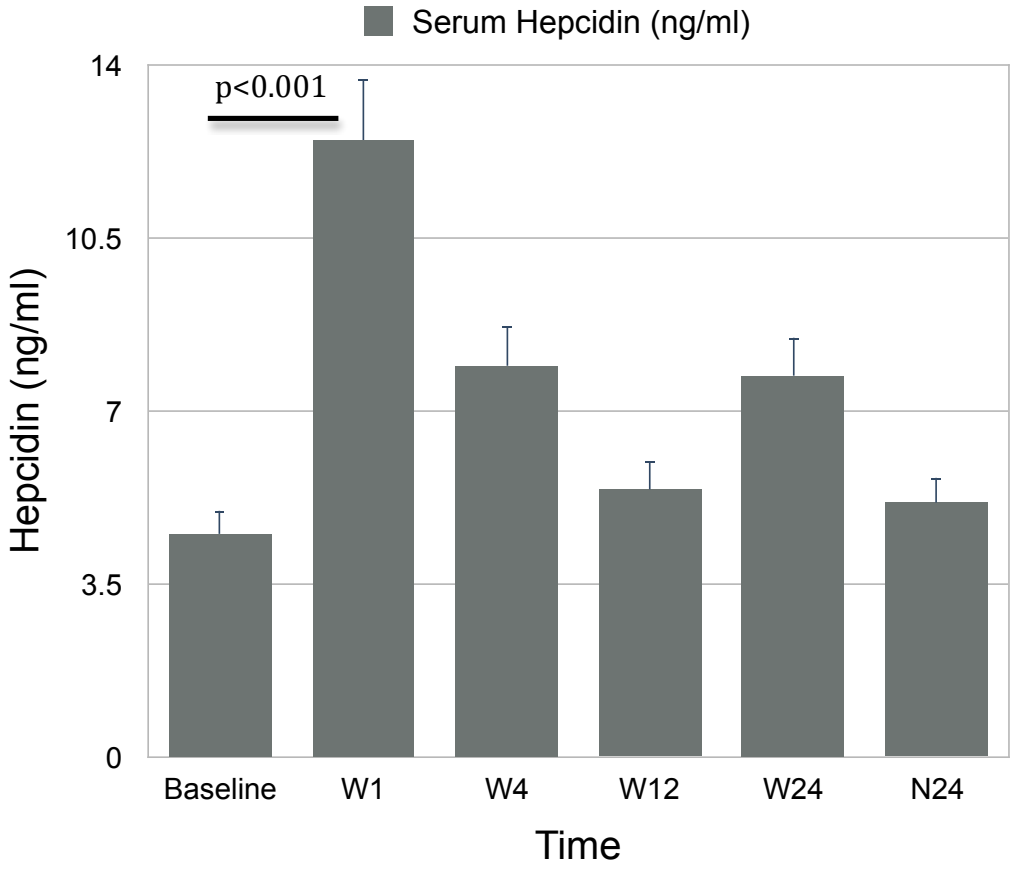


Figure 5 – Serum hepcidin values before, during and after a dual antiviral, IFN-containing therapy. Patients with chronic hepatitis C infection have low hepcidin levels at the baseline. After one week of pegylated-interferon therapy, they show markedly increased levels of hepcidin ($p<0.001$), which decrease gradually to almost baseline levels three months after the beginning of the therapy. (Abbreviations: W1, at week 1; W4, at week 4; W12 at week 12; W24, at week 24; N24: 6 months after the end of the therapy)

At baseline, weak correlations between hepcidin levels and serum ferritin ($r=0.27$, $p=0.04$), body mass index ($r=0.29$, $p=0.011$), and hemoglobin

levels (r=0.32, p=0.005) were observed. On the other hand, we detected a negative relationship between hepcidin and Aspartate transaminase (r=—0.25, p=0.029) as well as platelet levels (r=—0.25, p=0.029) (Figure 6).

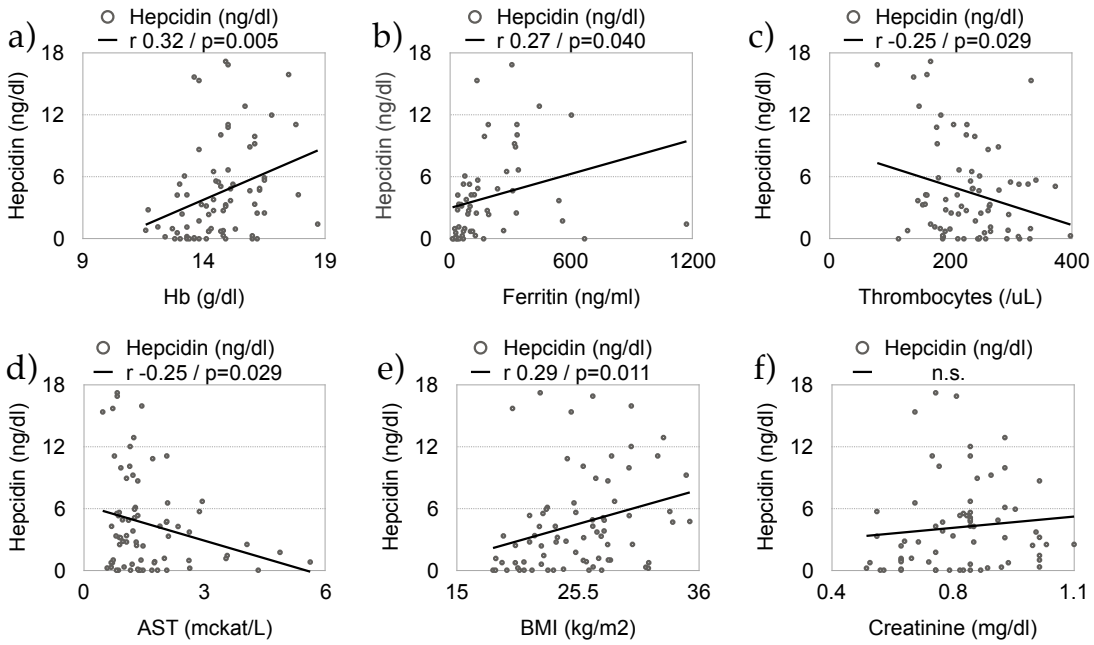


Figure 6 – Correlations between hepcidin and selected parameters in patients with chronic hepatitis C infection. The depicted parameters were compared at baseline before the start of the antiviral treatment. AST shows a weak negative correlation with hepcidin (p=0.02) (d), while ferritin shows a weak positive correlation (b). In this cohort, creatinine displayed no significant correlation with hepcidin. Abbreviations: Hb, Hemoglobin; AST, Aspartate transaminase; BMI, body mass Index; n.s., not significant.

Neither baseline hepcidin levels nor the increase in the hepcidin levels at week one or four predicted the end-of-treatment response or the achievement of a sustained virological response (data not shown). Of note, patients with steatosis showed a more prominent increase (p<0.001) in levels

of hepcidin one week after starting therapy versus non-steatotic subjects (Figure 7).

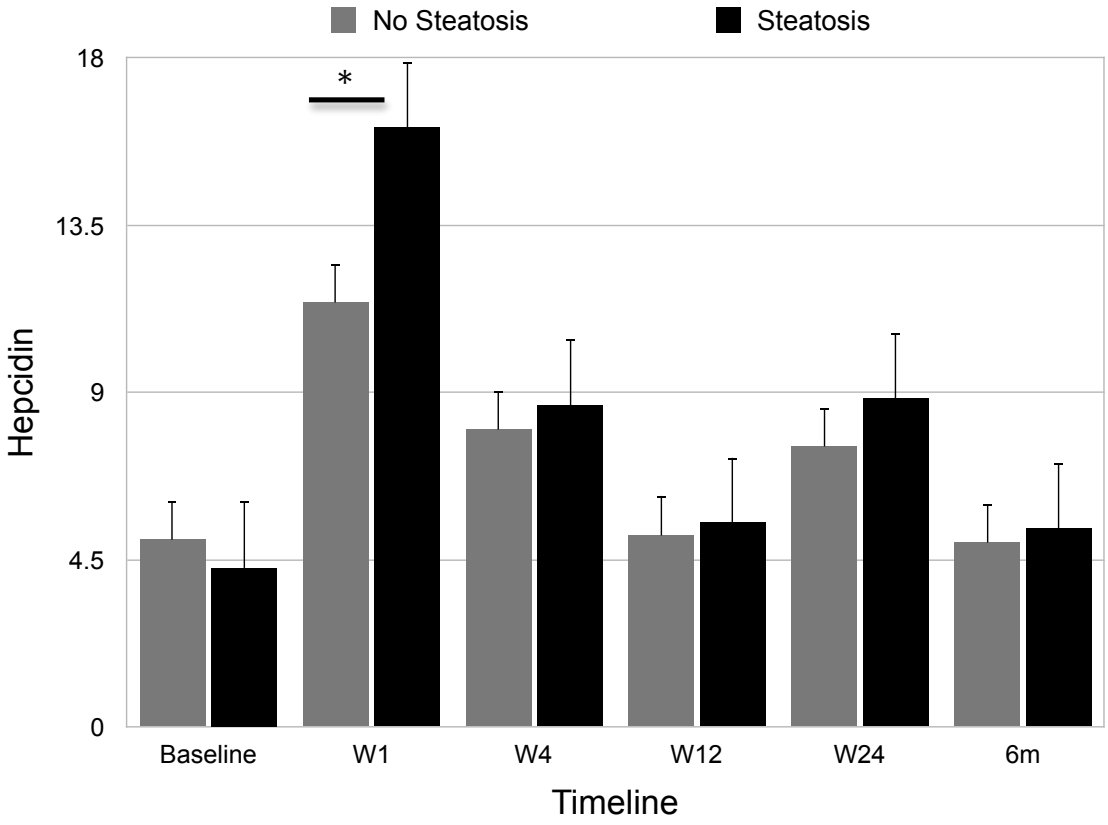


Figure 7 – Hepcidin levels in steatotic vs. non-steatotic subjects with chronic hepatitis C infection before and after receiving an antiviral dual therapy. Patients with steatosis showed a more prominent increase in levels of hepcidin 1 week after the beginning of the therapy than non-steatotic subjects. (Abbr.: W1, week 1; W4, weeks 4; W12 week 12; W24, week 24; 6 mo: 6 months after the end of the therapy; asterisk, $p<0.001$)

3.3. Hepcidin in alcoholic liver cirrhosis (ALC)

3.3.1. ALC cohort characterization

The baseline characteristics of the cohort and the outcome of alcoholic liver cirrhosis patients are described in Table 8. Overall 237 Patients were enrolled, with median age of 56.8 years and approximately 5:1 male to female ratio. The median follow-up time was around 65.8 months, with a maximum follow-up of a patient reaching 150 months. 133 patients passed away during the follow-up, 54 of them due to HCC, while 24 patients were lost to the follow-up. Only 17 patients have been transplanted in the cohort, and thus reaching the end of the follow-ups.

Table 8 – Baseline characteristics of the analyzed patients with alcoholic liver cirrhosis. For quantitative variables, medians are given if not mentioned otherwise. Abbreviations: ALC, alcoholic liver cirrhosis; BMI: body mass index; HCC, hepatocellular carcinoma.

Characteristics	ALC Cohort
Number of patients	237
Age in years median	56,8
Male n (%)	190 (81%)
Female n (%)	47 (19%)
BMI median (kg/m2)	27,7
Child-Pugh Score	7,7
Follow-up months (range)	65.8 (22-150)
Lost follow-ups n (%)	24 (10,1%)
Death n (%)	133 (56%)
HCC Related death n (%)	54 (23%)
Liver related death n (%)	74 (31%)
Extra-hepatic cause of death n (%)	5 (2%)
Transplantation n (%)	17 (7%)
Diabetes n (%)	23 (10%)

The median hepcidin level in the cohort was 8ng/ml [Q1: 3ng/ml - Q3: 13ng/ml], which has been used as the cut-off value for the analyses (Table 9). Baseline parameters regarding age, gender, Diabetes mellitus and liver function among others were comparable in both subgroups. Ferritin and body mass index (BMI) were the only exceptions. Expectedly, ferritin showed a positive correlation with hepcidin ($p<0.001$). On the other hand, BMI was higher in the subgroup of patients with lower hepcidin levels (Table 9).

Table 9 – Characteristics of subgroups of patients with low versus high hepcidin levels. A hepcidin cut-off of 8ng/dl has been used. Abbreviations: n.s., not significant; BMI: body mass index; AST aspartate transaminase; ALT: alanine transaminase.

Parameters	Hepcidin<8 N=117 (49.4%)	Hepcidin>8 N=120 (50.6%)	p
Age in years (median)	56.5	57.3	n.s.
Male n (%)	90 (76.9%)	100 (83.3%)	n.s.
BMI (kg/m2)	28.7	27.1	0.04
Diabetes n (%)	33 (28.4%)	39 (32.5%)	n.s.
Platelet count median (/nl)	127	141	0.09
Child-Pugh score	7,8	7,3	0.06
Serum iron (µmol/l)	19.5	21.2	n.s.
Ferritin median (ng/ml)	327	567	<0.001
Transferrin saturation (%)	50.8	43.1	n.s.
Liver iron score	2.1	2.9	n.s.
AST (x upper limit of normal)	2.1	2.1	n.s.
ALT (x upper limit of normal)	1.2	1.5	n.s.

3.3.2. Hepcidin predicts the occurrence of HCC and mortality in ALC

During the follow-up, patients with low serum hepcidin levels displayed a higher incidence in developing HCC (34 vs. 20; p=0.02) and overall death (61 vs. 39; p=0.002; Table 10). No significance difference has been associated in non-HCC liver related deaths between the two groups (p=0.442). We further confirmed our data by using Kaplan Meier survival curves to demonstrate a difference in survival, which showed an increase in rate of HCC (HR=1.75 [1.02-3.12]; p=0.045, Figure 8a) and overall death (HR=1.63 [1.09-2.43]; p=0.019, Figure 8b) among the ALC patients with lower hepcidin levels (hepcidin <8ng/ml).

Table 10 – Hepcidin as a prognostic marker for development of hepatocellular carcinoma (HCC) and death in patients with alcoholic liver cirrhosis. Patients with low hepcidin values (<8ng/dl) are more likely to develop hepatocellular carcinoma than those with high hepcidin levels (p=0.02). Low hepcidin is also associated with higher rates of the overall death in those patients (p=0.002). Abbreviation: HCC, hepatocellular carcinoma; n.s., not significant.

Parameters	Hepcidin<8 N=117 (49.4%)	Hepcidin>8 N=120 (50.6%)	P
HCC	34 (29.1%)	20 (16.6%)	0,02
Liver-related death without HCC	29 (24.8%)	23 (19.1%)	n.s.
Overall death	61 (52.1%)	39 (32.5%)	2
Alcohol withdrawal	65 (69.1%)	65 (69.2%)	n.s.

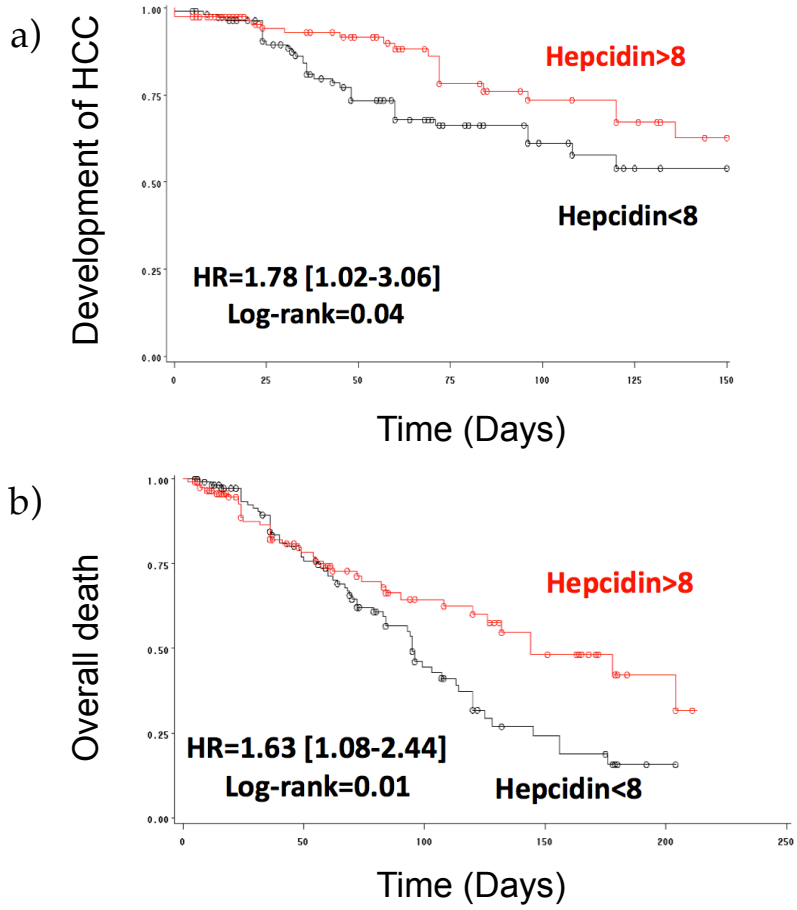


Figure 8 – Higher hepcidin increases the risk of HCC and overall death in patients with alcoholic liver cirrhosis. To assess the risk of developing of hepatocellular carcinoma (HCC) (a) and the overall mortality (b), patients were subdivided into groups with low vs. high hepcidin levels and Kaplan-Meier survival curves were plotted. Mortality was defined as death during the follow-up period of up to three years. The graphs indicate that lower hepcidin levels associate with lower survival rates and the development of HCC. Abbreviations: HR, hazard ratio.

To further evaluate the prognostic value of hepcidin we performed a univariate Cox regression analysis. A lower hepcidin level was associated with an increased risk of overall death (Hazard ratio, HR=1.63 [1.07-2.44]; p=0.019). Of note, higher liver iron score, higher transferring saturation, older age, higher Child-Pugh score and obesity were all associated with overall

death (Table 11). In the Cox proportional hazards model, lower hepcidin levels showed an independent association with overall mortality (HR=2.84 [1.29-6.25], p=0.009). The same applied to higher Child-Pugh score (HR=1.20 [1.04-1.38], p=0.01) and lower platelet count (HR=0.993 [0.988-0.999], p=0.02; Table 11). Of note, the association between hepcidin and HCC occurrence could not be confirmed in the multivariate analysis (p=0.119).

Table 11 – Parameters associated with mortality in patients with alcoholic liver cirrhosis according to Cox's proportional hazards model. Uni- and multivariate analyses have been conducted. In the univariate analysis low serum hepcidin level was associated with a worse prognosis. In the multivariate analysis, platelet count, Child-Pugh score and hepcidin were independently associated with the overall mortality. Abbreviations: BMI, body mass index; HR, hazard ratio.

Parameters	Univariate analysis	Multivariate analysis
Age	HR=1.03 [1.01-1.05] P=0.002	
BMI	HR=1.04 [1.006-1.07] P=0.02	
Platelet count	HR=0.997 [0.993-1.000] P=0.09	HR=0.993 [0.988-0.999] P=0.02
Child-Pugh score	HR=1.2 [1.1-1.3] P<0.0001	HR=1.20 [1.04-1.38] P=0.01
Alcohol withdrawal	HR=0.6 [0.4-1.0] P=0.05	
Iron saturation	HR=1.005 [1.000-1.01] P=0.05	
Liver iron	HR=1.06 [1.009-1.13] P=0.02	
Hepcidin<8	HR=1.63 [1.07-2.44] P=0.01	HR=2.84 [1.29-6.25] P=0.01

3.4. Pancreatic iron overload in hepcidin KO mice

3.4.1. Hepcidin KO mice exhibit a massive iron accumulation in pancreas

To examine the pancreatic iron accumulation in hepcidin knockout mice, hepcidin WT mice were fed standard or iron rich diet and sacrificed at the age of 6 or 12 months. Hepcidin KO mice were administered with the same standard diet and were sacrificed at the same age as their non-transgenic littermates. The 6 month hepcidin KO mice showed a marked increase in pancreatic iron content in the prussian blue staining (Figure 9f), while the 12 month old animals displayed an even more advanced accumulation of iron with signs of atrophic degeneration of the parenchyma (Figure 9 i and l), both displayed a deliberate accumulation in the exocrine parts of the pancreas. On the other hand, the WT animals on standard and iron rich diet showed no to fractional histological changes at both ages (Figure 9).

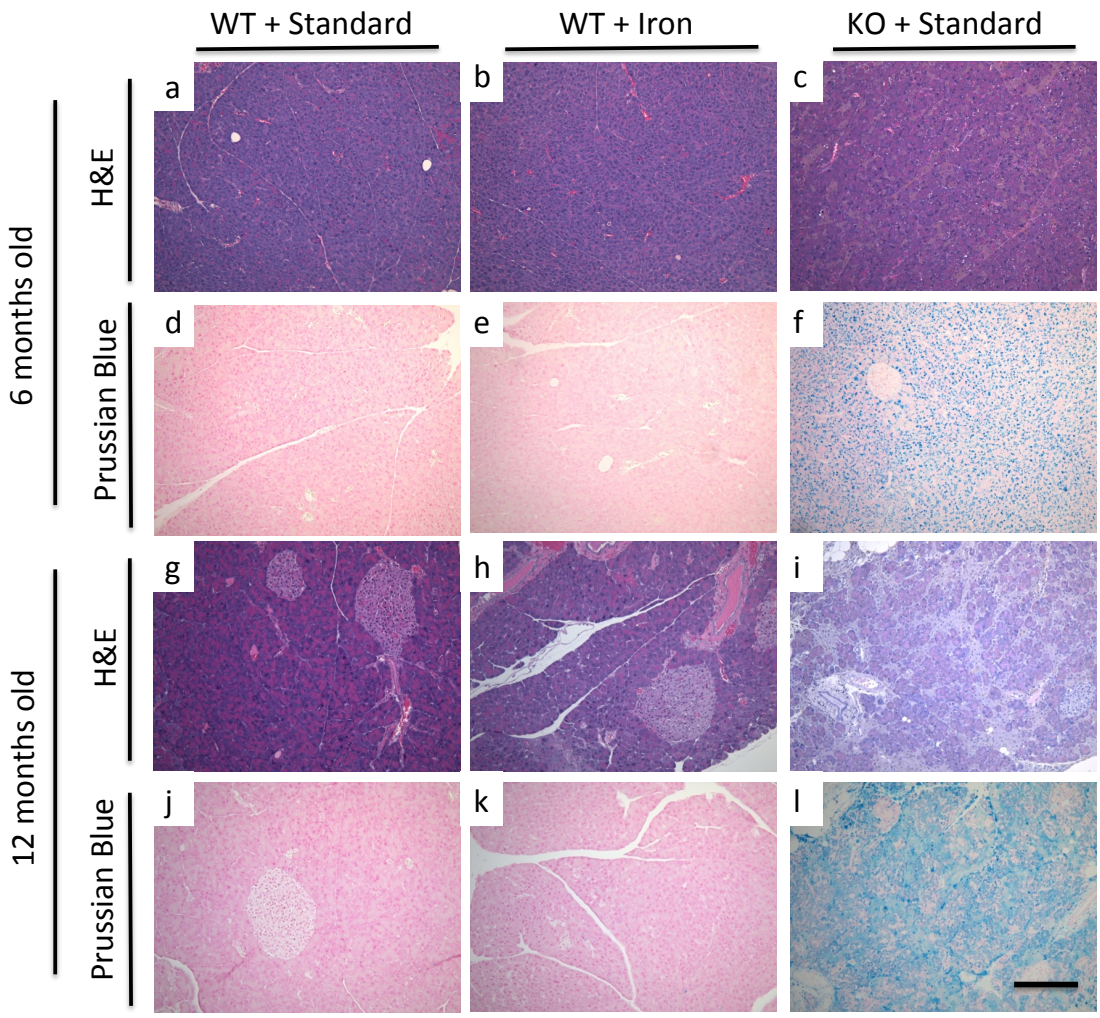
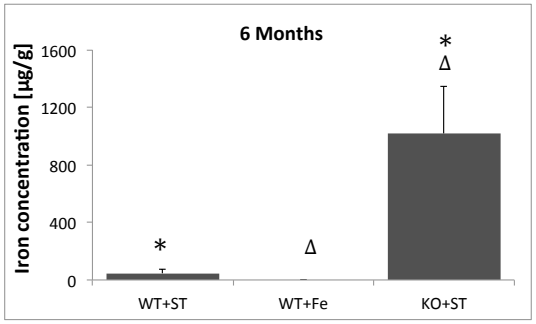


Figure 9 – Pancreatic iron accumulation and pancreatic atrophy in hepcidin knockout mice. Hepcidin WT mice were fed standard or iron rich diet (starting at one months of age) and sacrificed at the age of 6 or 12 months. Hepcidin KO mice were administered with the same standard diet and were sacrificed at the same age as their non-transgenic littermates. Prussian blue staining was conducted to determine the distribution of non-heme bound iron (d,e,f,j,k,l), whereas hematoxylin and eosin staining (H&E) was performed to evaluate the overall tissue morphology (a,b,c,g,h,i). 6 month old hepcidin KO mice showed a marked increase in pancreatic iron content (f), while the 12 month old animals displayed an even more pronounced accumulation of iron with signs of atrophic degeneration of the parenchyma (i,l). Scale bar 100µm.

We further confirmed our finding by conducting a biochemical quantification of pancreas iron; here we found a significant difference in the iron levels between the WT and KO mice (6 months: KO 1020µg/g vs WT 47µg/g, p=0.03; 12 months: KO 1498µg/g vs WT <1µg/g, p<0.001; Figure 10). The KO mice displayed moderate increases in serum lipase levels as a sign of mild pancreatitis (data not shown).

a)



b)

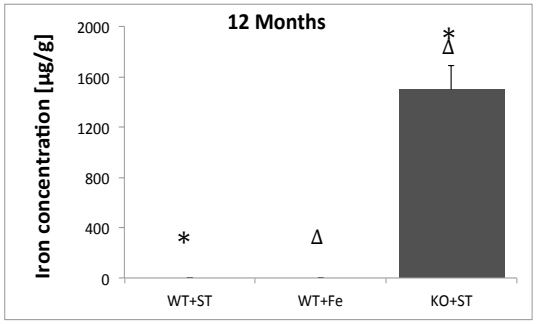


Figure 10 – Massive iron accumulation in pancreas of hepcidin KO mice. Biochemical measurement of non-heme bound iron was performed in pancreata of 6 (a) and 12 (b) months old hepcidin KOs as well as their age-matched non-transgenic littermates that were fed either standard (WT+ST) or iron-rich diet (WT+Fe). (a: *:p=0.03, Δ: p=0.01; b: */Δ: p<0.001)

3.4.2. Six month old hepcidin KO mice activate NFkB, undergo apoptosis and show signs of oxidative stress

To further understand the phenomena, we conducted further experiments to see whether the KO subjects show any sign of NF-κB activation, oxidative stress or apoptosis in the 6-month-old animals. As an injury sign, hepcidin KO showed an increase in NF-κB activation. p65 immunohistochemistry (Figure 11a) was performed on the pancreas tissues obtained from WT fed with standard and iron rich diet as well as hepcidin KO

mice, which showed nuclear translocation of p65. Furthermore, NF-κB Western blot was conducted by quantifying the nuclear translocation of p65. The hepcidin KO showed a marked over-expression of p65. HDAC2 was used as a loading control for the nuclear extract (Figure 11b).

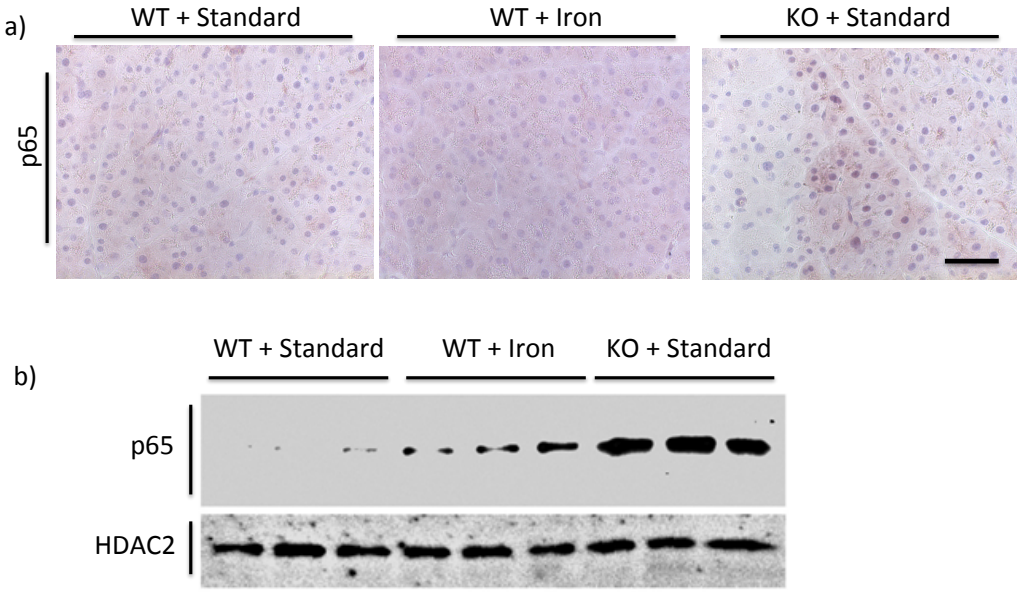


Figure 11 – 6 months old hepcidin KO mice show activation of NF-κB signaling. p65 immunohistochemistry (a) was performed on the pancreas tissues obtained from WT fed with standard and iron rich diet as well as hepcidin KO mice kept on standard diet. Scale bar 50μm. To evaluate the activation of NF-κB signalling, western blot (b) was assessed the amount of p65 in the nuclear fraction. Hepcidin KOs display a marked increase in nuclear p65. HDAC2 was used as loading control for the nuclear extract.

We conducted TUNEL staining to study apoptosis in 6 months WT and KOs, in the quantification we saw that hepcidin KO mice showed a marked elevation ($p<0.001$) in the level of apoptosis compared to the WT littermates

(Figure 12). Furthermore, Thiobarbituric acid reactive substances (TBARS) were assessed for the degree of oxidative stress as a lipid oxidation marker (Figure 13a). The levels of TBARS were significantly higher in KO mice vs. WT on standard diet ($p=0.03$) as well as WT on iron rich diet ($p=0.04$). To further confirm the DNA damage, Phospho-H2A.X Western blot was conducted by using total lysates. The hepcidin KO mice further displayed a marked increase in oxidative stress by over-expressing Phospho-H2A.X protein in their Pancreata ($p<0.05$; Figure 13b).

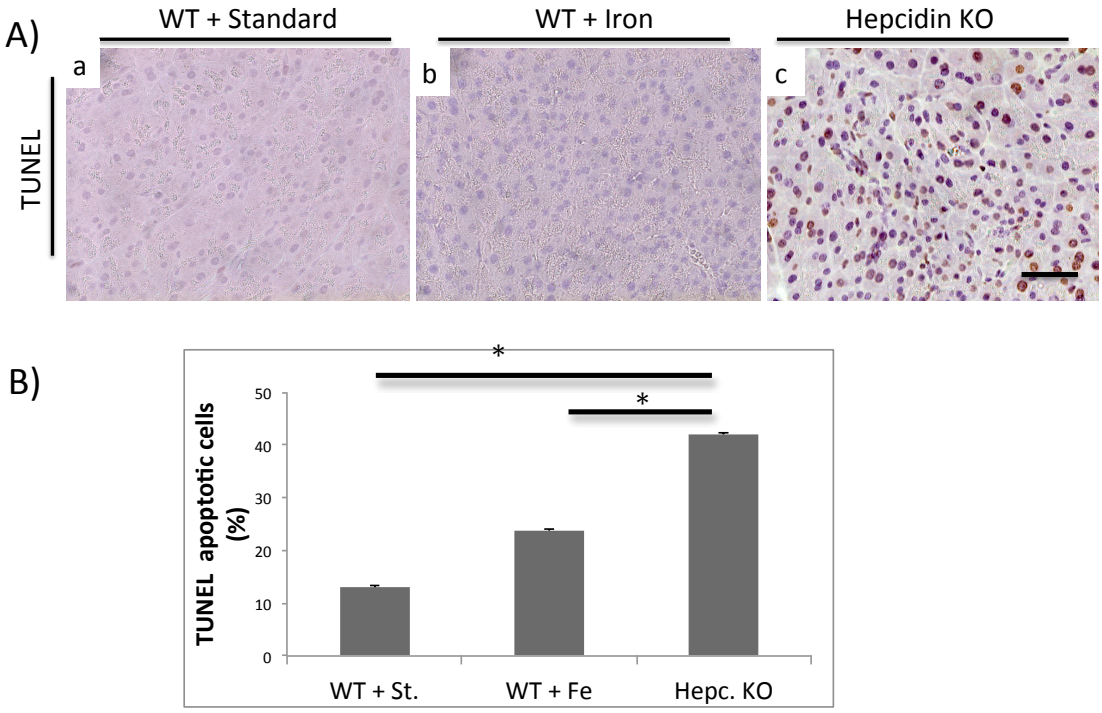


Figure 12 – 6-month old hepcidin KO mice display higher amounts of apoptotic cells. TUNEL assay was performed on pancreata from 6 months old WT mice fed with standard and iron rich diet as well as hepcidin KO mice of the same age. Hepcidin KO mice showed a marked elevation ($p<0.05$) in the level of apoptosis compared to WT littermates. (*=$p<0.05$) Scale bar 50µm.

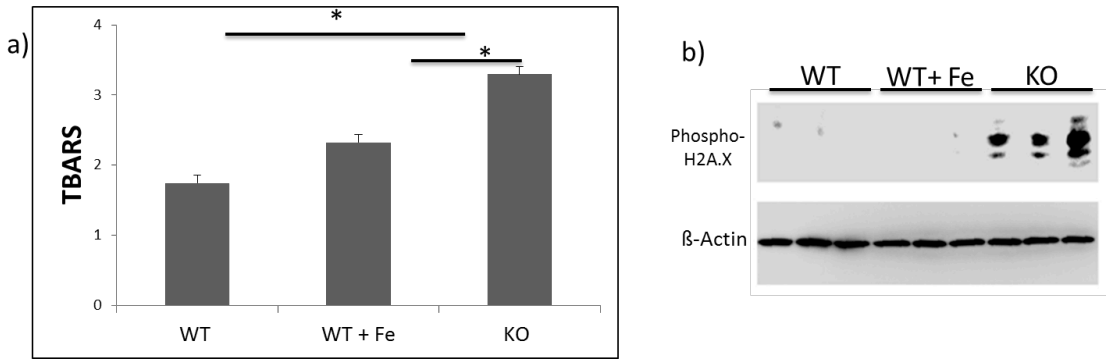


Figure 13 – 6-month old hepcidin KO mice show an increased level of oxidative stress in pancreas. Thiobarbituric acid reactive substances (TBARS) were assessed as a marker of oxidative stress (a). The KO mice are displaying higher levels of TBARS compared to the WT littermates treated with standard or iron-rich diet (p=0.03 and 0.04, respectively). Phospho-H2A.X Western blot (b) was conducted on total pancreas lysates. Beta-Actin has been used as a loading control. The hepcidin KO mice showed a marked increase in oxidative stress as detected via increased presence of Phospho-H2A.X protein. (*=p<0.05).

3.4.3. Mice treated with minihepcidin undergo iron redistribution

To further analyze the role of hepcidin on pancreas, 12-month old hepcidin knockout (KO) mice were injected with minihepcidin (aka PR73, a synthetic substance similar to hepcidin), which led to redistribution of iron from pancreatic acinar cells to macrophages and their accumulation in the spleen (Figure 14). The mice that were treated with minihepcidin generally displayed signs of health enhancement by boosting serum parameters. Minihepcidin treatment resulted in a significant decrease in serum iron, lipase, lactate dehydrogenase and aspartate aminotransferase levels, while serum ferritin,

which mirrors the overall iron storage in all tissue, remained unchanged (Figure 15).

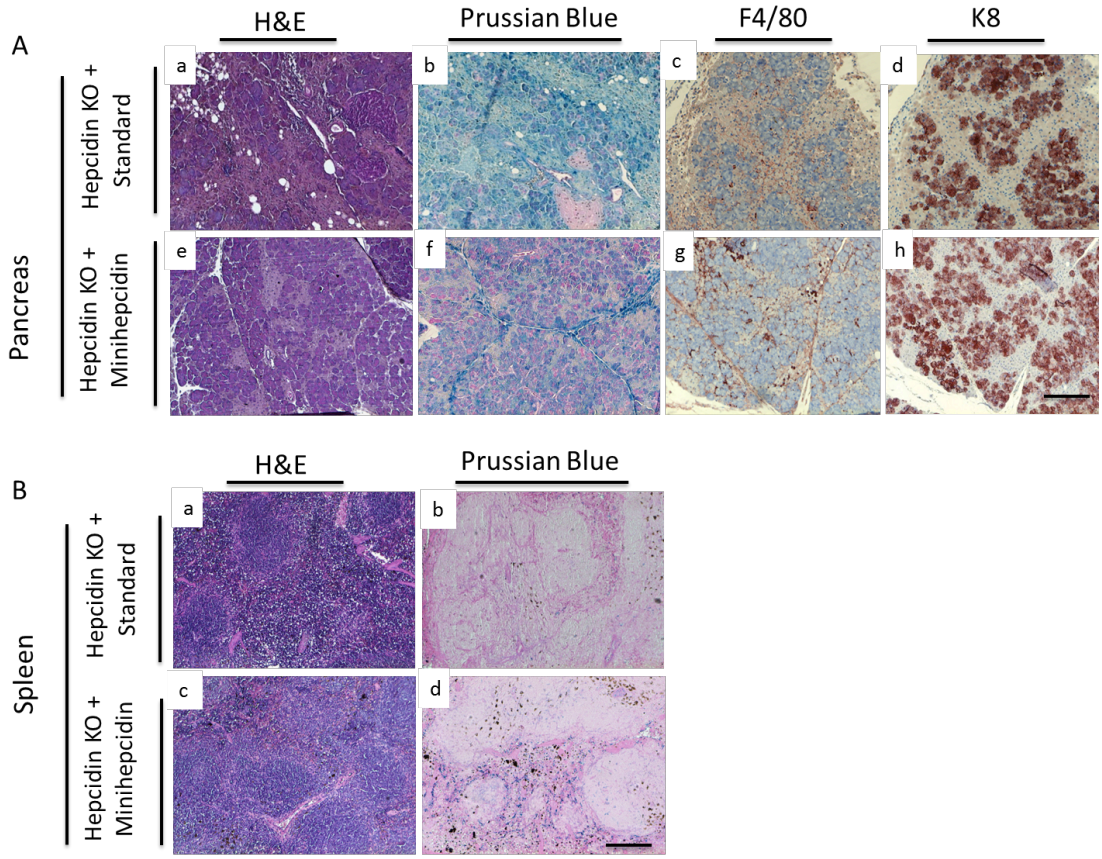


Figure 14 – Treatment of 12-month old hepcidin knockout (KO) mice with minihepcidin leads to redistribution of iron from pancreatic acinar cells to macrophages. Prussian Blue staining, F4/80, and K8 immunohistochemistry were performed on pancreatic (Aa-Ah) and splenic sections (Ba-Bd) from 12 months old hepcidin KOs that were either treated with carrier only (controls-Aa-Ad,Ba and Bb) or with minihepcidin (Ae-Ah, Bc and Bd) daily for a week. Scale bar 200μm.

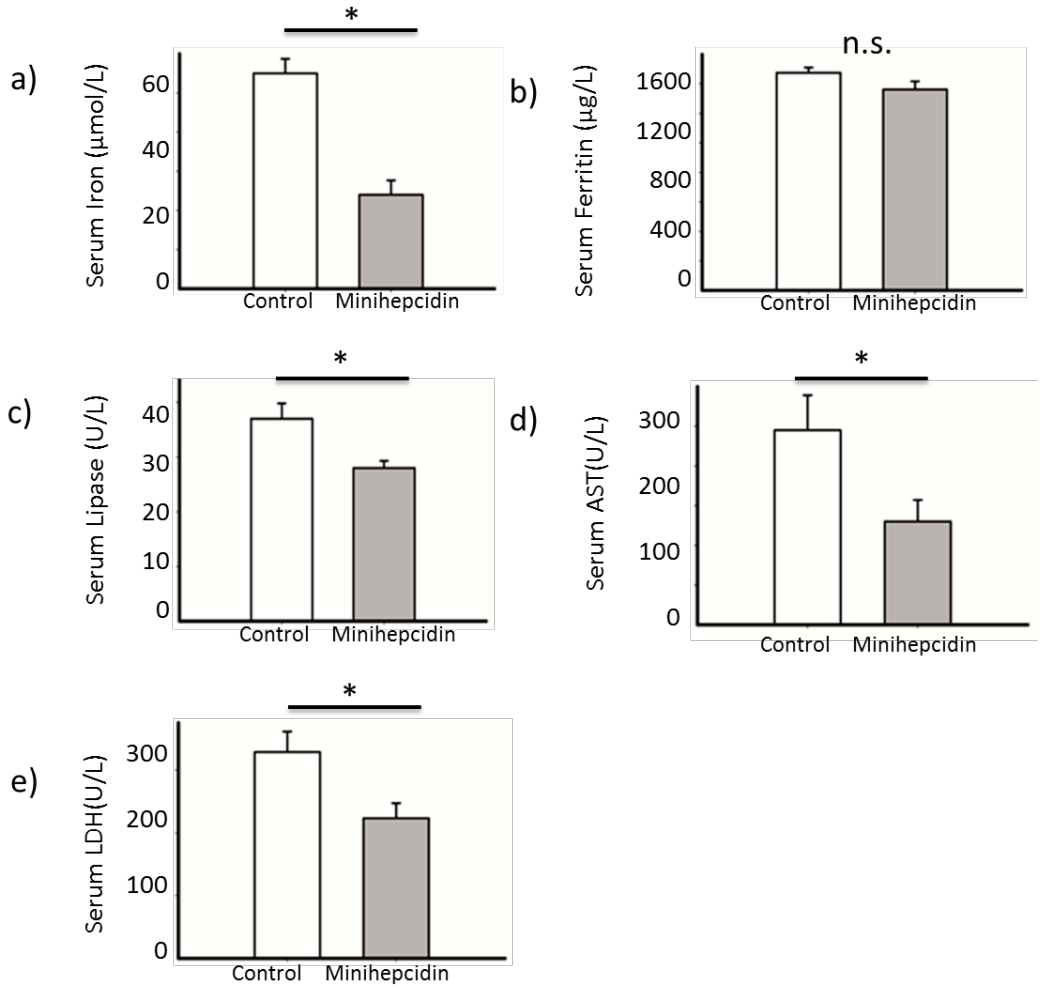


Figure 15 – Treatment of 12-month old hepcidin KO mice with minihepcidin leads to an improvement in several serum parameters. Serum iron (a), ferritin (b), lipase (c), AST (d) and LDH (e) levels were measured and results are presented as mean ± SEM. Minihepcidin treatment led to a significant decrease in most of the assessed parameters. (*$p<0.05$), AST: aspartate aminotransferase; LDH: lactate dehydrogenase; n.s.: not significant.

4. Chapter Four: Discussion

Given the crucial importance of iron in multiple different processes, the major aim of our study was to analyze the parameters of iron metabolism in various disease cohorts. To that end, I studied the changes and prognostic role of hepcidin and iron metabolism in critically ill patients, chronic hepatitis C infection as well as in alcoholic liver cirrhosis. To further understand the iron overload-related tissue injury, I investigated the pancreatic phenotype of hepcidin KO mice.

4.1. Iron parameter determine the prognosis of critically ill patients

In our ICU-cohort, we studied the changes and clinical significance of iron parameters in septic and non-septic ICU patients. ICU patients displayed decreased serum iron and transferrin levels, while a marked elevation in ferritin and hepcidin were noted in those patients, which is in accordance with previous studies (Darveau et al., 2004; Heming et al., 2011). These data are in line with the fact that both hepcidin and ferritin represent established acute phase proteins that become up-regulated during stress conditions (Darveau et al., 2004; Heming et al., 2011). Moreover, the elevated hepcidin levels lock the iron within macrophages and lead to hypoferremia, that was seen in the described cohort (Darveau et al., 2004; Tomas Ganz, 2013; Heming et al., 2011). On the other hand, serum transferrin showed a decrease in level in the ICU patients, this decrease was also expected since transferrin acts as an

anti-acute phase protein during stress conditions (Darveau et al., 2004; Finch & Huebers, 1982; Heming et al., 2011).

TSAT was the only studied iron parameter that didn't show any major changes among the studied population at the time of admission, which is largely because of the marked decrease in transferrin seen in those patients that counterbalanced the hypoferremia. This finding was surprising since TSAT is known to decrease in inflammatory conditions (Darveau et al., 2004; Finch & Huebers, 1982; Heming et al., 2011). The reason might be that parameters of iron start to change and develop further during the course of the disease. Alternatively, TSAT seem to correlate with severity of the underlying condition (see below for details) and because of that, the differences between the cohorts may be responsible. Future studies involving the course of the disease are needed to understand this issue.

Iron parameters showed good correlation with the individual prognosis of critically ill patients. Transferrin and TSAT levels represented attractive prognostic biomarkers in the ICU, while ferritin expressed only moderate prognostic significance for the short-term mortality. The negative prognostic value of low transferrin might be partially explained through its behavior as a negative acute phase reactant (Ritchie et al., 1999). Moreover, the negative prognostic value of high TSAT has been shown to apply to other acute stress situations such as patients with acute on chronic liver failure and those with acute lymphocytic leukemia (Maras et al., 2015; Potaznik et al., 1987).

The high TSAT might be contributed to the decreasing concentration of transferrin that is subjected to negative acute phase regulation and becomes lower during liver dysfunction (Darveau et al., 2004; T Ganz et al., 2008). The reasons why critically ill patients face derangement in iron metabolism are probably multiple. They undergo catabolism decreasing iron consumption and production and this situation is facilitated by increased destruction of erythrocytes and other cells. Last but not least, the state is further aggravated by increased need for blood transfusions, further enhancing the serum iron loading that is measured by TSAT (Darveau et al., 2004; T Ganz et al., 2008).

The association with mortality might be due to the observed decrease in transferrin, which leads to an insufficient supply of iron to the tissues (T Ganz, 2013; Ritchie et al., 1999). In addition, the increased TSAT levels associate with formation of the highly reactive NTBI, which represents the labile iron that can be easily utilized by the microorganisms and therefore increases the thread of infection (Drakesmith & Prentice, 2012; Ritchie et al., 1999; Ritter et al., 2004). Furthermore, it leads to generation of reactive oxygen species contributing to the development of multi-organ failure (Ritter et al., 2004).

Of note, patients with liver cirrhosis admitted to the ICU expressed lower levels of hepcidin compared to non-cirrhotic subjects, which is in line with previously published data (Kessler et al., 2015). Overall, serum hepcidin at ICU admission didn’t show any prognostic significance regarding survival. As a potential explanation, hepcidin regulation is a complex mechanism that

involves factors such as hypoxia, anemia, growth hormones, endoplasmic reticulum stress response and many others (Tomas Ganz 2013; Collins et al. 2008, Ganz et al. 2008; Galesloot et al. 2011). Furthermore, although hepcidin increases in response to many stress conditions occurring in ICU-patients, it is known to decrease during hepatic insults and may therefore diminish in patients with multi-organ failure (Tomas Ganz, 2013).

4.2. Hepcidin does not predict the treatment response in chronic HCV infection

In a patient´s cohort with chronic hepatitis C infection lower hepcidin levels than in healthy controls were noted which is in line with previous findings (Fujita et al.). A quick hepcidin increase after the beginning of the treatment with pegylated-interferon and ribavirin is also in aggrement with previous observations (Ryan et al., 2012).

In chronic HCV infection, hepcidin showed only a weak correlation with ferritin, unlike healthy controls, which is in agreement with the published studies (Galesloot et al., 2011; Girelli et al., 2009). The explanation might be related to the fact that in injured liver, hepcidin production is regulated by factors other than body iron store.

Furthermore, patients with chronic hepatitis C Infection with steatosis exhibited a significantly higher rise in hepcidin levels after the start of the therapy compared to baseline. Steatosis has already been described to be a negative predictive marker for viral clearance (Adinolfi et al., 2013). Further

studies are needed to determine whether the dual-therapy itself or the concurrent events are responsible for the observed hepcidin rise.

4.3. Hepcidin has a prognostic value in alcoholic liver cirrhosis

Just like patients with chronic hepatitis C, patients with injured liver in alcoholic liver cirrhosis showed reduced hepcidin levels. These findings support recent reports that detected lower hepcidin levels in cirrhotic patients with acute-on-chronic liver failure as well as those with HCC (Kijima et al., 2008; Maras et al., 2015). Although the reason behind the lower hepcidin is yet to be fully understood, oxidative stress and tissue hypoxia has been shown to suppress hepcidin synthesis in animal models and human cell lines (Chaston et al., 2011; Harrison-Findik et al., 2006). Another study showed that alcohol ingestion by mice affected several iron-related parameters, while preventing hepatic iron accumulation leading to the suppression of hepcidin (Varghese et al., 2016).

In our study we showed that a low hepcidin level is a negative prognostic marker in patients with alcoholic liver cirrhosis and that it might indicate an increased risk of HCC development and death. The reason behind the association with higher mortality might be the resulting iron overload but also the diminished antimicrobial effects that predispose to bacterial infection, which is one of the major causes of acute on chronic liver failure and eventually death (Arroyo et al., 2015; Drakesmith & Prentice, 2012; Park et al., 2001).

4.4. Hepcidin KO mice develop chronic pancreatitis

In our animal study, both 6 and 12-month hepcidin KO mice showed a marked increase in pancreatic iron content, mainly in the exocrine parts. On the other hand, the WT animals on standard and iron rich diet showed no significant iron accumulation at both ages. This is in line with other previously published data (Altamura et al., 2014; Andriopoulos et al., 2009; Huang et al., 2005; Latour et al., 2016; Meynard et al., 2009; Ramey et al., 2007; Subramaniam et al., 2012) supporting the idea that iron overload in exocrine pancreas develops as a consequence of a severe dysfunction in the hepcidin-ferroportin axis whereas less severe changes, such as dietary-induced iron overload in mice with an functioning hepcidin signalling, do induce neither pancreatic iron accumulation nor tissue damage.

As a mechanism leading to pancreatic atrophy, hepcidin KO displayed an increased NFkB activation with a nuclear translocation of p65. Moreover, hepcidin KO mice showed a marked elevation in the level of apoptosis and oxidative stress compared to the WT littermates. This supports observations from mice with mutant ferroportin that were resistant to hepcidin. These animals developed an injury exocrine pancreas, that was paralleled by oxidative damage, degeneration, severe body weight loss and death (Altamura et al., 2014).

To further analyze the role of hepcidin on pancreatic iron accumulation and tissue injury, we subjected 12 months-old hepdicin KOs to minihepcidin, which led to redistribution of iron from pancreatic acinar cells to macrophages

and to iron accumulation in the spleen (Figure 14). This finding is surprising since ferroportin expression is relatively low in the acinar cells (Ryan et al., 2012). Furthermore, we demonstrated that treated mice show signs of health improvement as evidenced by reduced levels of several important serum parameters. In a recent study minihepcidin has been shown to be disease modifiers in mice affected by β-thalassemia and polycythemia vera (Casu et al., 2016). Our results also suggest that iron overload in macrophages might be better tolerated than an accumulation in parenchymal tissues and because of that, hepcidin analogues may become useful as novel therapeutics for disorders of iron metabolism (Ruchala & Nemeth, 2014).

5. Chapter Five: Summary

Iron represents both an essential and toxic element and its metabolism is regulated via hepcidin, the master regulator of iron in human body. Inappropriately low hepcidin levels result in iron accumulation in parenchymal tissues, whereas an overproduction of hepcidin leads to anemia of chronic inflammation. Accordingly, hepcidin knockout (KO) mice represent a model of severe iron overload. Increased iron availability may predispose to development of bacterial infection whereas hepatic iron overload may accelerate the progression of liver disease and development of hepatocellular carcinoma. Ryan et al. suggested that the extent of pegylated interferon-induced hypoferremia correlates with the reduction of hepatitis C viral load.

We evaluated the role and prognostic significance of hepcidin and other iron parameters as outcome predictors in multiple patients' cohorts with different human conditions. To further understand the downstream consequences of iron overload we analyzed the changes that occur in pancreas of hepcidin KO mice.

In our intensive care unit cohort, parameters of iron metabolism at admission constituted strong outcome predictors. In Kaplan-Meier analyses, low iron levels (cutoff 10.5 µmol/mL), low transferrin saturation (cutoff 55%), and high serum transferrin concentrations (cutoff 1.6 g/L) were associated with short- and long-term survival. In patients with alcoholic liver cirrhosis, lower serum hepcidin was independently associated with death and this

association remained significant in multivariate analysis [HR = 2.84 (1.29–6.25), P = 0.009]. In a third patients cohort, dual therapy of hepatitis C infection with pegylated interferon and ribavirin led to a marked up-regulation of hepcidin level, however, no association between hepcidin levels and the virological response was noted.

Aged hepcidin KO mice exhibited massive cytoplasmic acinar iron overload, acinar atrophy, macrophage infiltration, fatty changes and fibrosis as sign of chronic pancreatitis. As potential underlying mechanisms, increased oxidative stress with elevated DNA damage, apoptosis and activated NF-kB signaling were observed. Treatment of the mice with daily minihepcidin injections led to iron redistribution from acinar cells to macrophages and to an overall improvement in health status.

In conclusion, parameters of iron metabolism constitute attractive predictors of disease course in several human disorders. Severe iron overload seen in hepcidin KOs leads to development of chronic pancreatitis that the iron overload-induced chances become rapidly reversed after minihepcidin supplementation.

6. Chapter Six: References

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