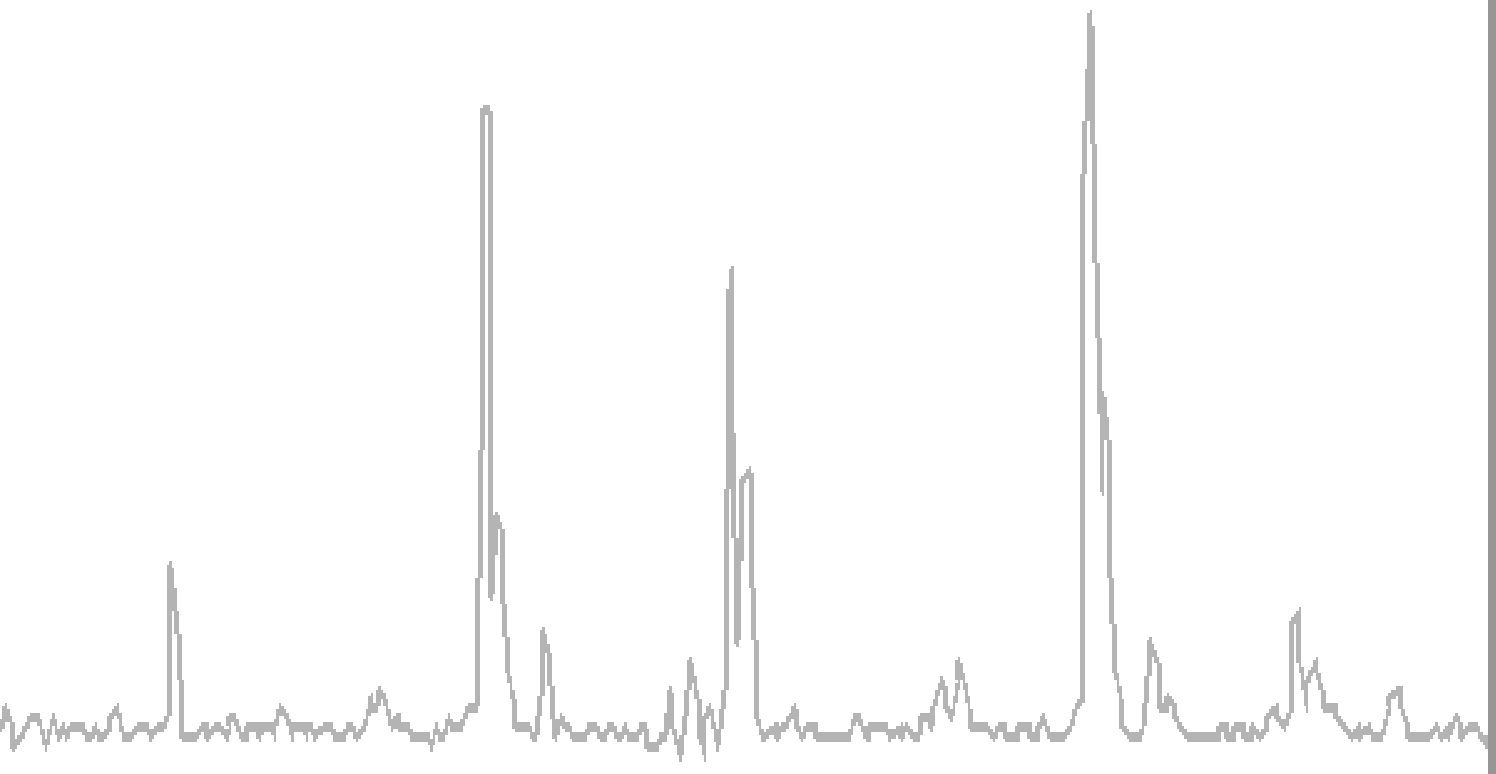
Hepcidin:
Analysis, Regulation and Clinical Perspectives



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Cover: A magnet in the middle of a surface covered with iron powder. The magnetic field gives direction to the scattered iron particles. This picture symbolizes the regulation of body iron by the peptide hormone hepcidin.

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Hepcidin: analysis, regulation and clinical perspectives

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Chapter 1

Introduction

Hepcidin: from discovery to differential diagnosis

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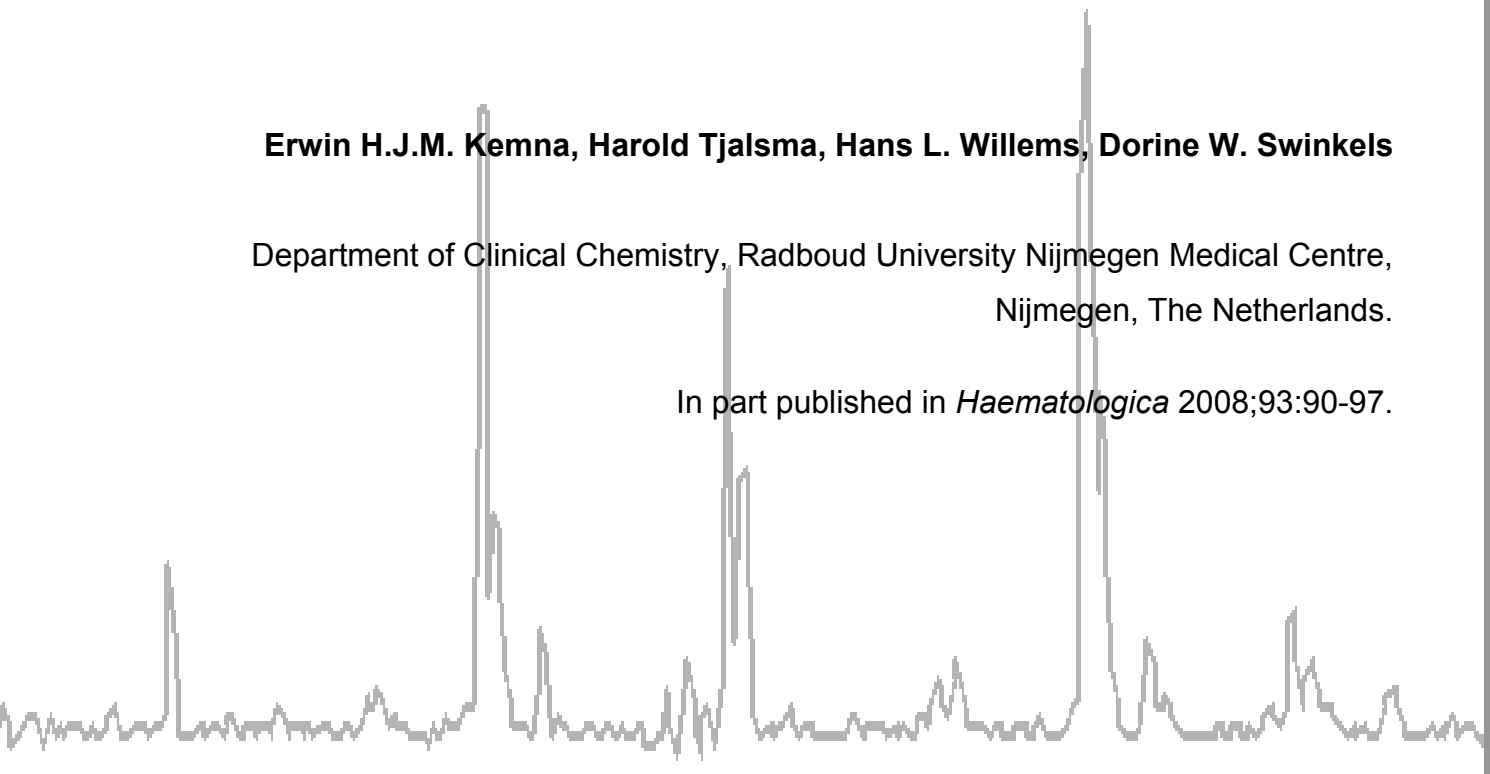

1. Iron

1.1 Iron: essential and harmful

Iron is an element that is interwoven with our daily environment because of its abundance in the earth's crust[1,2]. Its capacity to participate in one-electron transfer and acid-base reactions, explains why iron is indispensable and thus essential for life.

For survival and proliferation eukaryotic cells and most prokaryotic organisms require iron, as a constituent of oxygen binding heme proteins, iron-sulfur proteins, and proteins that use iron in other functional groups to carry out essential functions for cellular metabolism. Therefore, cellular iron deficiency stops cell growth and even might lead to cell death.

However, a side effect of the reactive properties of iron can also lead to cell damage. Fe^{2+} is capable of catalyzing the generation of highly reactive hydroxyl radicals from hydrogen peroxide, which is called the 'Fenton reaction'[3]; a condition that especially occurs during iron overload. Besides the functional role of radical formation in cell signaling pathways, these radicals damage cellular membranes, proteins, and DNA. In order to combine life with a potential toxic primal element, evolution has provided us with a large number of deeply integrated regulation systems and scavenger molecules that protect against iron mediated tissue damage, and by which the body is challenged to carefully control iron within its functional limits[4].

1.2 Maintenance of body iron homeostasis

In humans, approximately two third of the total body iron is utilized in hemoglobin, with the rest in myoglobin, respiratory enzymes, and stored as hepatic ferritin. As displayed in Figure 1A, body iron metabolism is based on a highly efficient system of iron conservation and recycling by which only up to a 10th of the daily need is replaced by duodenal absorption from diet. Excess iron is either not absorbed, or is retained in enterocytes. After approximately 2 days these cells are shed from the tips of the villi into the luminal intestinal contents. This apparently regulated iron uptake and the absence of a physiological excretion mechanism makes it impossible to release excessive iron in case of iron overload. Most of the 20 to 25 mg iron that is required daily in the bone marrow for erythropoiesis, is recovered from senescent erythrocytes by macrophages in the spleen and released into the circulation where it is transported throughout the body, bound to transferrin. In case of increased iron demand due to high erythropoietic activity of the bone marrow or iron depletion, intestinal iron absorption and iron recruitment from hepatic stores increases. The maintenance of body iron homeostasis requires mechanisms to control iron uptake and mobilization from stores, in order to meet erythropoietic needs, and for scavenging previously used iron. Therefore the communication between cells that consume iron and cells that acquire and store iron must be tightly regulated[4,5].

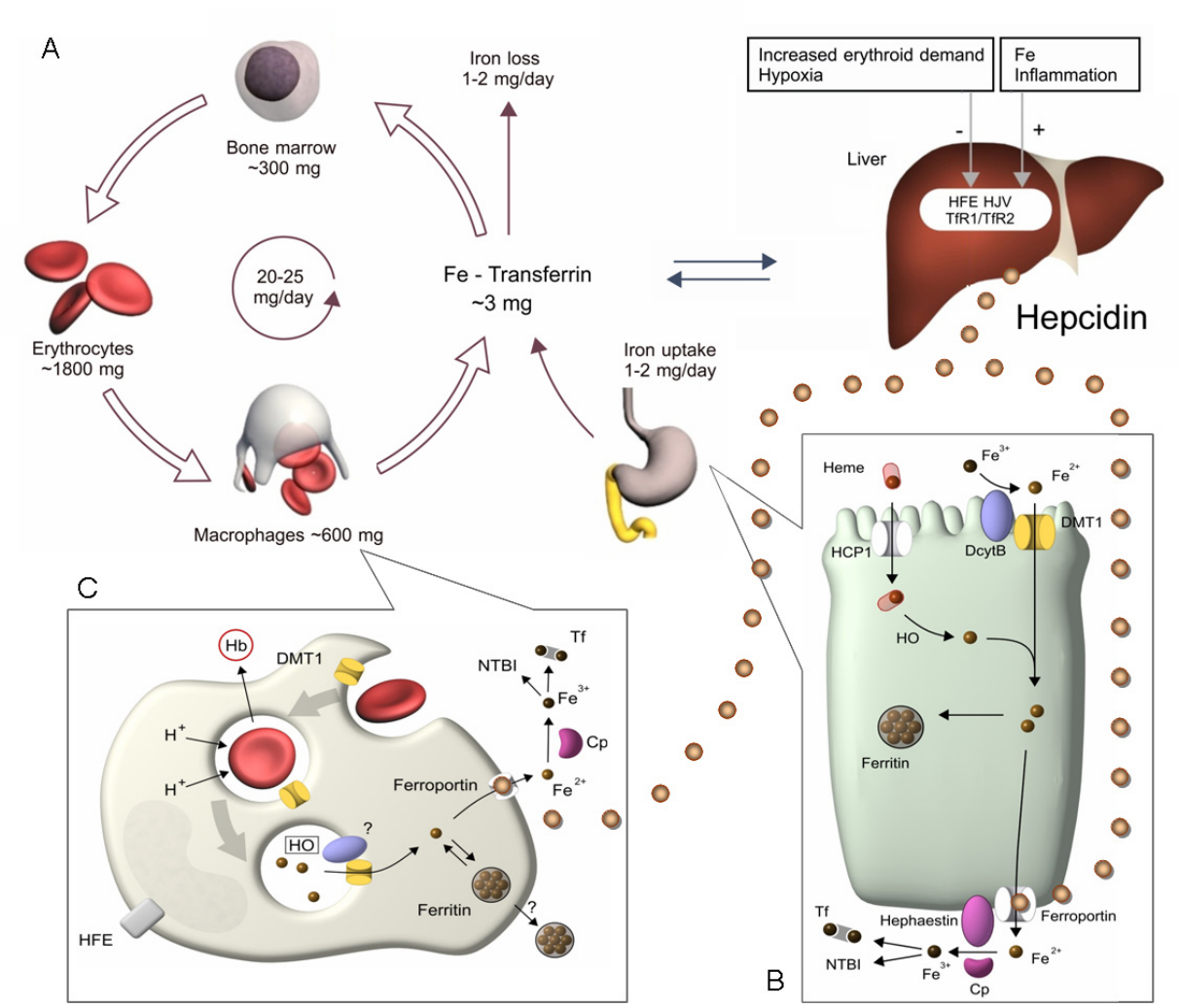

Figure 1. Iron uptake and recycling.
(A) Most of the utilized body iron is recycled from senescent erythrocytes by macrophages, and returned to the bone marrow for incorporation in erythroid precursors. The liver and reticuloendothelial macrophages function as major iron stores. 1-2 mg of iron is absorbed and lost every day. Only duodenal absorption is regulated by transporters such as DMT1 and HCP1, whereas iron loss occurs passively. The liver produced peptide hepcidin controls the plasma iron concentration by inhibiting iron export by ferroportin from enterocytes (B) and macrophages (C). This implicates that an increased hepcidin production leads to a decrease in plasma iron concentrations. Hepcidin expression is regulated by body iron stores, inflammation, erythroid iron demand, and hypoxia via regulation pathways involving expression of *HFE*, *TfR2,TfR1* and *HJV* genes. Details are discussed in the text and in figure 3. DMT1: divalent metal transporter 1; Hb: hemoglobin; HO: heme oxygenase; NTBI: non-transferrin bound iron; Tf: transferrin; Cp: ceruloplasmin; HCP1: heme carrier protein 1; DcytB: duodenal cytochrome B. Reproduced with permission in adapted form from Swinkels *et al* Clin Chem 2006[77]

As shown in Figure 1B, duodenal enterocytes absorb iron from the diet, where it is mostly present as ferric Fe^{3+} or as heme, a molecule that consist of a protoporphyrin ring that binds iron. After reduction on the apical surface of the enterocytes by duodenal cytochrome-b (Dcytb), iron enters the cell by divalent metal transporter-1 (DMT-1). Heme is reported to be absorbed by a recently identified receptor heme-carrier protein-1 (HCP-1) [6]- also involved in folate uptake[7]- and released from iron by heme oxygenase-1 (HO-1). Once in the cell, iron is stored by ferritin and in case of a

high body demand for iron released into the plasma by the sole known cellular iron exporter ferroportin. After oxidation by ferroxidase hephaestin (intestinal cells) or ceruloplasmin (nonintestinal cells), iron is loaded onto transferrin for transport in the plasma were it is picked up by TfR1 on the cell surface of cells in need of iron. Next to dietary iron uptake, iron is recycled from senescent erythrocytes by macrophages (Figure 1C). Also here, HO-1 plays an important role in extracting iron from heme in the cytosol whereas involvement of DMT-1 is suspected but not proven[8]. Recent reviews on cellular iron uptake, storage and export are given by Hentze *et al* and Dunn *et al* [4,9].

2. Hepcidin

2.1 The discovery of hepcidin

Although hepcidin was first discovered in human urine and serum, most data on hepcidin expression, regulation, structure and function were obtained by *in vitro* approaches and studies in mice, as reviewed in the next paragraphs. Hepcidin was initially isolated from plasma ultrafiltrate[10] and named liver-expressed antimicrobial peptide (LEAP-1). Almost simultaneously, it was isolated from human urine and named hepcidin after its hepatic origin and bactericidal effect *in vitro* [11]. The development of severe iron overload[12] by knocking out the gene in mice suggested hepcidin to be involved in iron metabolism, whereas this key role in regulation was stressed by the discovery of hepcidin mutations in patients[13]. The newly discovered peptide was found to be regulated by inflammation, iron stores[14], hypoxia and anemia[15].

2.2 Structure of hepcidin

The human hepcidin gene (*HAMP*; OMIM 606464), located on chromosome 19q13.1, encodes a precursor protein of 84 amino acids (aa). *In vitro* experiments in murine hepatic cell lines showed that the production and localization of pre-prohepcidin is assumed to be intracellular in the secretory pathway[16]. During its export from the cytoplasm, this full-length pre-prohepcidin undergoes enzymatic cleavage of a 20 aa N-terminal endoplasmic reticulum-targeting signal peptide, resulting in the export of a 64 aa prohepcidin peptide into the ER lumen[17]. Next, the 39 aa pro-region peptide is, most likely, posttranslational removed by a furin-like proprotein convertase[11,18] in the trans-Golgi network, resulting in mature bioactive hepcidin-25 (25 aa form). Park *et al*, identified in human urine also hepcidin-22 and hepcidin-20, which are N-terminally truncated iso-forms of hepcidin-25[11]. Our recent results confirm that in addition to hepcidin-25, the 20 aa iso-form is detectable in both human urine and serum, while the 22 aa iso-form can only be detected in urine[19]. These results support the hypothesis that both the 20 and 25 aa peptides are secretory cell products, whereas the 22 aa peptide is merely an urinary degradation product of hepcidin-25[20]. It is currently unclear whether hepcidin-20 is functional, whether it is produced by

hepatocytes or other cell types, if it originates from proteolysis of hepcidin-25 or from convertase-mediated differential processing of pro-hepcidin, and whether this event takes place before or after hepcidin is secreted by hepatocytes.

Structural analysis by NMR spectroscopy of human synthetic hepcidin revealed that this small 8 cysteine-containing peptide forms a hairpin-shaped molecule with a distorted β-sheet which is stabilized by four disulfide bridges between the two antiparallel strands[21]. One of the disulfide bridges is located in the vicinity of the hairpin loop which points to a possible crucial domain in the activity of the molecule[21] (Figure 2). The high cysteine content of the molecule, and therefore the disulphide interconnections are highly conserved among other species[11]. Structure-function *in vivo* (mice) and *in vitro* studies on synthetic hepcidin have shown that the iron regulating bioactivity is almost exclusively due to the 25 aa peptide, suggesting that the five N-terminal amino acids are essential to exert this activity[22,23]. The typical β-defensin structural conformation with amphipathic properties is a hallmark for antimicrobial activity[24], and *in vitro* experiments have shown that especially human hepcidin-20 exerts antibacterial and antifungal activity in a concentration range 10-fold higher than measured in healthy individuals[10,11]. Therefore, it is not clear whether *in vivo* hepcidin levels in the circulation or in urine of man can reach values in which it can be antimicrobial, and thus whether this function still is of biological importance or only rudimental of its evolutionary origin[25,26]. A recent report on inductively coupled plasma-mass spectrometry (ICP-MS) on human hepcidin-20 and -25, extracted from pooled urine samples showed co-purification of at least one iron molecule with hepcidin[27]. Modeling of a best-fit 3D structure of hepcidin with iron displayed significant differences with the previous reported synthetic hepcidin model[21]. These new insights suggest a conformational polymorphism for hepcidin as a regulatory mechanism for iron uptake as part of its role as regulator of iron homeostasis. Further research must reveal if this proposed iron binding property of hepcidin expands the regulation complexity of iron metabolism.

2.3 Mechanism of hepcidin activity

Recently, new light was shed on how hepcidin exerts its regulatory function on iron metabolism. It was reported to bind to the transmembrane iron exporter ferroportin[28-30] which is present on macrophages and the basolateral site of enterocytes (Figure 1B and C), but also in hepatocytes.

In vitro it has been demonstrated that next to binding, hepcidin subsequently induces the internalization and degradation of ferroportin[31]. It remains to be defined whether, as recently suggested[32], structural properties such as amino terminal Cu and Ni binding (ATCUN) motifs in the hepcidin-25 molecule play a role in this process (Figure 2). For cellular iron export, the cell depends on ferroportin as originally demonstrated in zebrafish[33]. Moreover, in mice, ferroportin deletion was incompatible

with life[34] whereas inactivation of the gene after birth leads to iron overload in hepatocytes, macrophages and enterocytes[34].

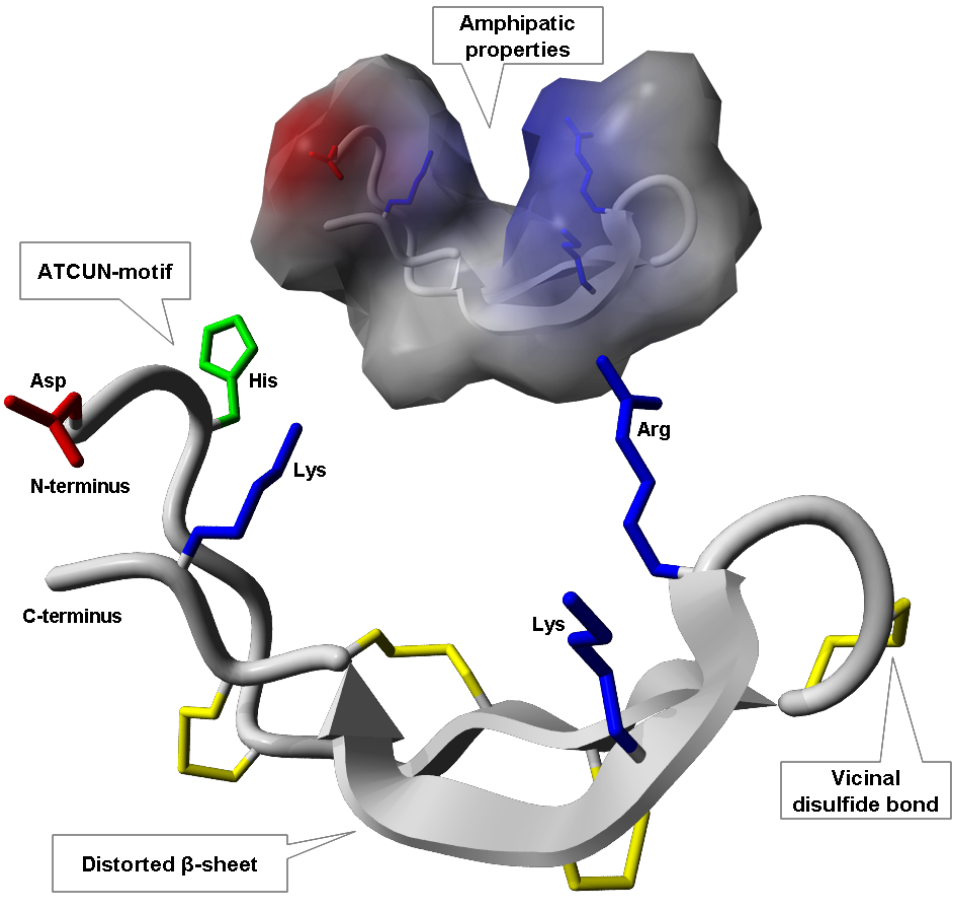

Figure 2. Molecule structure of human synthetic hepcidin-25.
Front: overview of the structure of hepcidin-25. Distorted β-sheets are shown as grey arrows, and the peptide backbone is colored gray. The disulfide bonds are colored yellow, highlighting the position of an unusual vicinal bond between adjacent cysteines at the hairpin turn. Positive residues of Arginine (Arg)and Lysine (Lys) are pictured in blue, the negative residue of Aspartic acid (Asp) in red, and the Histidine containing amino terminal Cu^{2+}- Ni^{2+}(ATCUN)-binding motif in the N-terminal region is colored green. Background: hepcidin-25 molecule displayed with solvent accessible surface that illustrates the amphipathic structure of the molecule. The molecule is colored gray, except for the side-chains of positive (blue) and negative (red) residues. Molecular graphics created with YASARA[128] (www.yasara.org) and PovRay (www.povray.org), with coordinates and factors obtained from the Protein Data Bank (www.rcsb.org ; PDB file code 1M4F).

Thus, by diminishing the effective number of these iron exporters on the basolateral membrane of the enterocytes or on macrophages, hepcidin suppresses iron uptake and release, respectively. This is in line with the phenotype of ferroportin disease in which iron accumulation is observed mainly in macrophages often in combination of anemia[35,36].

Next to this systemic liver hepcidin controlled reduction of iron uptake and release, there is also evidence for local production of hepcidin by macrophages under inflammatory conditions[37,38], or even production in fat cells[39] and cardiomyocytes[40].

These findings suggest different regulatory mechanisms to control iron imbalance in which hepcidin is involved.

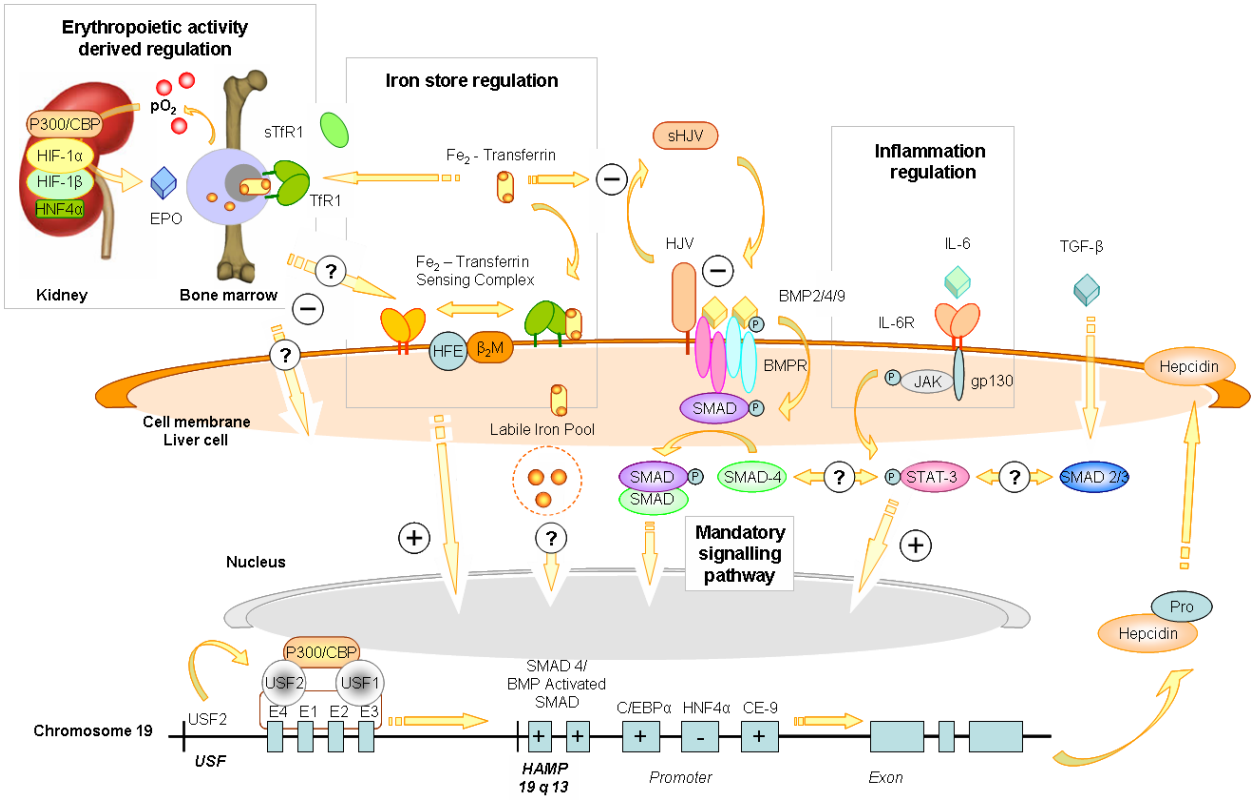

Figure 3. Model of pathways involved in hepcidin regulation.
Displayed are the three active regulation pathways (erythropoietic activity derived regulation, iron store regulation, and inflammation regulation) and a mandatory signaling pathway that together control the hepcidin production in the liver cell. Erythropoiesis is under control of hypoxia induced erythropoietin production by the kidney. The bone marrow "communicates" by a thus fare unknown factor that is likely to interfere with the iron store regulation. This store regulation involves the circulating amount of transferrin bound iron that competes with HFE in binding to TfR1 hereby promoting formation of the TfR2/HFE complex as an inducer of hepcidin production. Direct influence of intracellular iron from the labile iron pool on hepcidin regulation is not clear yet. The inflammation regulatory pathway is foremost induced by IL-6 and is suggested to act more dominant regardless the activity of the other pathways. The HJV controlled BMP/SMAD signaling pathway appears to be mandatory for the activity of the store and erythropoiesis derived regulation. The metabolic syndrome candidate genes *USF1* and *2* are in direct control of the *HAMP* gene, hereby suggesting a link between lipid, glucose, and iron metabolism. pO_2: partial oxygen pressure; HIF: hypoxia inducible factor; CBP: CREB binding protein; EPO: erythropoietin; HNF: hepatic nuclear factor; TfR: transferrin receptor; sTfR: soluble TfR; HFE: hemochromatosis iron protein; β2M: beta-2 microglobuline; HJV: hemojuvelin; sHJV: soluble HJV; BMP: bone morphogenetic protein; BMPR: BMP receptor; P: phosphate; SMAD: mothers against decapentaplegic homologue (*Drosophila*); IL: interleukin; IL-6R: interleukin-6 receptor; gp: glycoprotein; JAK: Janus kinase; STAT: signal transducer and activator of transcription; TGF: transforming growth factor; USF: upstream stimulation factor; E: E-box; *HAMP*: hepcidin anti microbial peptide gene; CE-9: conserved element 9.

3. Hepcidin at the nexus of various regulatory pathways

3.1 Upstream regulation

Momentarily four putative upstream regulatory pathways that control liver hepcidin production have been described: i) iron store-related regulation, ii) erythropoietic activity driven regulation, iii) inflammation related regulation), and iv) a mandatory signaling pathway. All are found to interact with liver cells to initiate the production of sufficient hepcidin for a proper maintenance of iron homeostasis[41-46]. In Figure 3 we depict a model of pathways involved in hepcidin regulation that builds upon recently acquired insights, in general derived from mice studies and *in vitro* cell culture work. The model is focused on three relevant sites involved in hepcidin regulation: kidney, bone marrow and liver cells. Notably, the iron efflux regulation in macrophages by hepcidin, just as skeletal muscles which express high levels of hemojuvelin are kept out of this picture. We will shortly discuss each of these 4 pathways in the following sections.

3.1.1 Iron Store-related Regulation

Information on the amount of iron in depot is communicated by a "store regulator" [5]. How this stores regulator acts upon the hepcidin producing liver cells is unclear, although *in vitro* work on interactions between transferrin and the membrane proteins HFE, and transferrin receptor (TfR) 1 and 2 has lead to a hypothetical model in which circulating iron bound to transferrin, affects the formation of a complex of TfR2, and HFE on the surface of liver cells. This regulation mechanism is clarified in Figure 3 and the corresponding legend. This complex is capable of increasing the hepcidin production by a thus far unknown intracellular signaling pathway[42,47].

3.1.2 Erythropoietic activity-driven regulation

An erythropoietic activity derived regulator is proposed to act as the communicator between the erythron and the liver[5]. In case of hypoxia or anemia, low oxygen pressure (pO_2) levels induce hypoxia inducible factor (HIF)-1α stabilization in kidney cells, which results in erythropoietin (EPO) production of the kidney. EPO increases the erythropoietic activity and thus the need for iron of the bone marrow, resulting in a fast iron mobilization from the stores. This results in an increased duodenal iron absorption by diminishing the circulating hepcidin concentration regardless the status of the iron stores. This suggests that the erythropoietic activity derived regulation interacts with the store regulator and is able to control its effect on hepcidin induction. A suitable candidate for this role, reflecting the erythropoietic activity of the bone marrow, might be found in the soluble transferrin receptor (sTfR) molecule[48]. By competing with TfR2 in the binding to the HFE-β_2 micro globulin

(β_2M) complex on the cell surface of hepatocytes[43,44,49,50] it is capable to interfere with the store regulator. However, to date this has not been proven[51].

3.1.3 Inflammation-related regulation

A third upstream regulator of hepcidin is controlled by infection and inflammation. This "inflammatory regulator" pathway has lately been shown to be predominantly induced by interleukin (IL)-6 followed by Janus kinase (JAK)/Signal transducer and activator of transcription (STAT)-3 signalling[45,46,52]. It is suggested that this pathway might act more independently from the other pathways[53-58] although results lack consensus on this matter[59]. Interactions between inflammation and HJV/BMP regulation through STAT-3 and SMADs as a result of TGF-β cell signalling[60-62], illustrate the complexity of the signalling cascades involved in hepcidin regulation[63,64].

3.1.4 Mandatory signalling pathway

A recent report hypothesized that the functional effect of both the store regulator and erythroid regulator fully depends on the activity of an additional pathway that is controlled by the glycosylphosphatidylinositol (GPI)-linked cell associated hemojuvelin (HJV). HJV has been suggested to maintain a mandatory regulation pathway by Bone Morphogenetic Protein (BMP)/SMAD signalling[41] in which SMAD4 seems to be essential[65]. Disruption of this pathway by HJV mutations cripples the functionality of both store and erythropoietic activity related regulation[66], hereby claiming a critical role in hepcidin production. Next to the membrane-linked HJV, the presence of a soluble form was reported to be detectable in human serum[53]. This soluble HJV (sHJV) is suggested to be a cleavage product of the membrane-anchored protein, and in some way under control of circulating iron[57]. In addition, *in vitro* experiments have shown that recombinant soluble hemojuvelin is capable of suppressing hepcidin mRNA expression. Together these data suggest an iron controlled binding competition between membrane-bound and sHJV that result in the control of hepcidin production[67,68]. However, many details of this mandatory hepcidin signaling pathway and its nexus with other regulatory pathways are still unknown.

3.2 Transcriptional regulation

Several transcription factors are reported as important for the Hepcidin promoter function such as C/EBPα[69], hepatic nuclear factor (HNF4α)[69], upstream stimulatory factor (USF)[70] and p53 [71] and probably cooperate to allow opening of the chromatin at the hepcidin locus and initiation of transcription.

Simultaneously, some of these factors are also mentioned in association with metabolic syndrome[72,73], alcohol metabolism-mediated oxidative stress[74] and hypoxia[75]. Few are even under cyclical control (discussed by Bayele *et al*[70]) and may

also cooperate with the SMAD proteins activated by the HJV/BMP pathways. Involvement of the von Hippel-Lindau (VHL)/HIF-axis is recently reported[76] as a possible regulation pathway related to erythropoietic activity. So far, nothing is known of the signaling pathway responding to the HFE-TfR2 interactions and its interference with ubiquitous or hepatic-specific transcription factors indicating that our understanding of this last step in hepcidin gene regulation is far from complete. Taken together, what once was considered as a regulation system with only a few roads now appears to be part of a complex regulatory network in which hepcidin is in fact a protein that has numerous irons in the fire. This complexity might preclude a clear prediction of the effects on mutations in upstream regulators of hepcidin. This is exemplified by the variable clinical penetrance of the homozygous C282Y alteration in the HFE-protein, which thus far remained largely unexplained[77].

4. Hepcidin kinetics

After hepcidin is secreted into the circulation by the hepatocytes and performing its regulatory role in cellular iron uptake and release, it exits the body with the urine. Its effect on the decline in serum iron levels in mice appeared to take place within 4 hours in a dose-dependent way that sustained for more than 48 hours[22]. Indirect hepcidin inductions by IL-6 or LPS in man displayed the same fast response in urinary hepcidin excretion[78,79], thereby acting like an acute phase protein with a peak value after 6 hours, followed by a steady decline. The clearance of hepcidin from the circulation appears to be a process that occurs within 24 hours, as demonstrated in mice injected with radio-labeled hepcidin[22]. Oral iron administration for 3 days in healthy human volunteers showed a significant increase in urinary hepcidin after 24 hours that disappeared in the following days despite the iron intake. The peak value in urine suggests a fast clearance of hepcidin from the circulation, with a paradoxical sustained inhibitory effect on iron uptake as displayed by the absence of change in iron parameters in the following days[78].

The fast appearance of hepcidin in urine illustrates the exceptional features of this peptide. Thus far tubular dysfunction or even reported tubular expression[80] of hepcidin as a causative factor in hepcidinuria has not yet been studied. It is interesting to investigate the role of the kidney tubuli in the urinary hepcidin excretion as in several disorders of iron metabolism disturbed excretion might be expected such as (severe) inflammation[81] and iron overload associated tubular dysfunction[82,83].

5. Hepcidin: from mice to man

Although highly homologous hepcidin genes have also been identified in many vertebrates including rodents like mice and rats, several species of fish, dogs and pigs, it should be noted that in mice two paralogous genes have been found from

which only hepcidin1 appears to be involved in iron metabolism[84-86]. As described in the previous paragraphs, mice have been a useful animal model to unravel pathways in iron metabolism, but amongst others these differences at the genetic level of hepcidin necessitates a careful extrapolation of results on hepcidin obtained in mice to that in man. Similarly, although *in vitro* systems allow a broad spectrum of interventions, the similarity to the human *in vivo* situation is restricted. Therefore, measurement of circulating levels of hepcidin in man is important to increase our knowledge on the role of hepcidin in different pathological conditions (Table 1A). More specifically, these insights might add to the definition of hepcidin as a differential diagnostic tool and therapeutic target in human disease.

5.1 Assessment of urinary and serum hepcidin in humans

Until recently, only few investigative tools were available to detect hepcidin in human studies. Hepcidin mRNA expression is mostly preferred in animal and cell culture studies, but obviously very sporadically used in human studies because of the need of invasive sampling. Immunochemical methods based on the use of specific anti-hepcidin antibodies, like in immuno-histochemical tissue staining, SDS-PAGE and Western Blot[87,88] is largely hampered by the limited availability of suitable antibodies. This can be attributed to the small size of hepcidin, the compact and complex structure of the molecule and the highly conserved sequence among species, complicating the elicitation of an immune response in host species.

In the next sections we will shortly describe and discuss the assays which are currently available to measure (pro)hepcidin in serum and/or urine.

5.1.1 Antibody-based hepcidin assay

To our knowledge, to date, only one antibody-based dotblot assay has successfully been used to (semi)quantify hepcidin in urine[78,79]. With the use of cation exchange chromatography, peptides are extracted from urine, eluted from the matrix, lyophilized, and resuspended in an acetic solution. Extracts are immobilized on a vinyl membrane by dotting, along with synthetic hepcidin standards. With the use of rabbit anti-human hepcidin primary antibodies[87], and goat anti-rabbit horseradish peroxidase as a secondary antibody, the dots are quantified after Chemiluminescence detection. Hepcidin quantity in each sample is normalized using urinary creatinine concentrations, and expressed as nanogram hepcidin per milligram creatinine. However, due to its laborious procedure, and the use of relatively large sample volumes, this assay is not optimal for high-throughput measurements in large clinical studies. Absence of a control for hepcidin losses in the pre-analytical phase of the analysis like in the protein extraction step and resuspension step, might regard this assay as semi-quantitative. The limited availability of suitable antibodies which are mostly non-commercially made hampers the optimization of antibody-based assays with guaranteed specificity. On the other hand, a hepcidin ELISA will greatly

enhance the accessibility of the analysis, but it is not likely to be discriminative for the different isoforms of hepcidin.
Next to bioactive hepcidin, the measurement of its precursor pro-hepcidin is reported with the use of a commercially available ELISA kit that uses antibodies directed against the pro-peptide region of the 64 aa precursor of hepcidin[89]. The diagnostic use of this assay is controversial because of the lack of clear correlations with hepcidin[19,79] and other iron related parameters[90-93]. Significant concentration differences only have been reported in ferroportin disease[94] or in combination with end stage renal disease (ESRD)[95].

5.1.2 Mass spectrometry-based hepcidin assay
Recently we described a surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS)-based assay that detects the three known isoforms of hepcidin[96]. More recently, analytical improvement has made this technique also suitable of measuring hepcidin-25 in serum[19]. Importantly, the procedure is fast; allowing the simultaneous analysis of large sets of human samples, but is difficult to robotize due to the on-spot procedure. The urinary Immobilized Metal Affinity Capture (IMAC)30 ProteinChip application has shown to correlate highly with the Normal Phase (NP)20 array application[19], which in turn strongly correlated with the immuno-dotblot assay[96]. Latest improvement of the IMAC30 application include both a procedure to prevent hepcidin losses during sample preparation and the introduction of a hepcidin analogue as internal standard, which corrects for the analytical variation and excludes the sample matrix influence and allows sensitive quantification of hepcidin in both serum and urine samples (D. Swinkels and C. Laarakkers, unpublished results).
The potency of MS-based hepcidin analysis is underscored by other recent reports[97,98]. Tomosugi and co-workers[97] reported serum hepcidin measurements with the use of SELDI-TOF MS, but with the use of diluted serum samples and a bioprocessor. Compared to the aforementioned direct on-spot method, this application allows the combination with robotics. It uses synthetic human hepcidin-25 to construct an external standard curve, but lacks the use of an internal standard and should therefore be regarded as semi-quantitative[97].
Another report on serum hepcidin analysis in only healthy subjects uses of liquid chromatography tandem mass spectrometry (LC-MS/MS)[98] with a non-hepcidin related internal standard. Both methods still need highly specialized equipment, and therefore do not readily improve accessibility. Comparative studies, the use of uniform internal standards and accuracy adjustment of all techniques available to date might increase the comparability of results in the future.
For the moment, mass spectrometry-based hepcidin measurements in serum and urine seem to be an attractive option to semi-quantify serum and urine hepcidin levels in clinical studies for research purpose only and in a small number of laboratories in the world.

Ongoing improvement of analysis techniques for hepcidin are essential to accurately assess levels of hepcidin and its isoforms in body fluids of different species, cell media and cell contents. This will contribute to the further unraveling of the hepcidin networks that are key for diagnostic and therapeutic approaches in man.

5.2 Insights from hepcidin measurements in man

The development and implementation of urinary and serum hepcidin assay's allowed several clinical studies that increased the existing knowledge on hepcidin levels in physiologic and pathophysiologic states. In the next paragraphs, these studies will be discussed in view of previous related *in vitro* approaches and analogous studies using mice as model organisms.

5.2.1 Pre-analytical factors and diurnal variation

First of all, evaluation of our MS-based hepcidin measurements in human serum and urine samples revealed a substantial influence of pre-analytical factors on especially urinary hepcidin and a strong diurnal variation of serum hepcidin levels[19]. In line of agreement, Murphy *et al*[98] found high between-day variation of serum hepcidin levels. Despite these variations we were able to differentiate various disorders of iron metabolism by the hepcidin values of sera and urines collected randomly throughout the day[19]. This indicates that the inter-individual variation in hepcidin levels is higher than the intra-individual variation. In mice, background influences like strain and gender have been shown to substantially modulate liver hepcidin mRNA's[99,100]. In a small set of samples Murphy *et al* found no major differences in serum concentrations from normal male and female women[98], which is in concordance with our findings in a different control group for both serum as urine values (data extracted from ref. 19). To the best of our knowledge urine and serum hepcidin levels have not been reported for non-Caucasian populations, and for children only once under pathological conditions[101].

5.2.2 Hepcidin regulation

The involvement of hepcidin in the induction of hypoferremia by inflammation was translated from mice to human studies after the introduction of the urinary dotblot and the SELDI-TOF MS assays, which also demonstrated the existence of the highly responsive LPS- IL-6- hepcidin axis as a link between innate immunity and iron metabolism[78, 79, 87].

Erythropoietic activity appeared to be a strong regulator of hepcidin levels, i.e. erythropoietin that stimulates erythropoietic activity has been shown to down-regulate liver hepcidin mRNA-expression in mice[102]. However, in the absence of erythropoietic activity, hepcidin expression is no longer suppressed[43,103].

The strong inverse association between erythropoietic drive and hepcidin production was also observed in various patients with congenital chronic anemias[104,105], which are characterized by low urinary hepcidin levels.
The influence of these strong regulators, together with the iron store status was recently studied in healthy controls and patients with iron metabolism disorders in which a relative hepcidin level was calculated applying a simple additive algorithm (Kemna *et al*, submitted). Based on actual measured transferrin saturation levels as parameter reflecting the iron store status, sTfR as indicator for erythropoietic activity, and C-reactive protein (CRP) for inflammation, the calculated relative hepcidin levels showed a strong correlation with the actual measured serum hepcidin levels in these patients using the SELDI-TOF MS method ($R = 0.756$, $P < 0.001$; results not shown). This simplified working model implies that hepcidin is dominantly regulated by these three main regulating pathways. These insights enable the construction of an additive graphic model that combines the nett effect of the erythropoietic activity and store regulation with the hepcidin inducing effect of inflammation in different iron metabolism disorders, as displayed in Figure 4 (page 126).

5.2.3 Hepcidin in hereditary hemochromatosis

Both SELDI-TOF MS and the dotblot method were used to measure hepcidin levels in juvenile cases of hemochromatosis, and were found to be extremely low[19,66,106,107]. Less severely decreased, but still clearly inappropriately low urinary hepcidin levels, were found in patients with TFR2 mutations using the urine dotblot method[108]. Similarly, decreased mRNA levels were seen in liver biopsies of HFE-KO or deficient mice and man[109-111], whereas levels ranging to almost normal values in serum and urine were found in HFE-hemochromatosis patients[19,87]. Although, the mechanism behind this observed variability in hepcidin values especially in classic hemochromatosis is not completely clear yet, the presence of iron overload at presentation and that of the increased erythropoiesis upon phlebotomy treatment are likely to contribute (B. van Dijk and D. Swinkels. Unpublished observations).
In ferroportin disease hepcidin concentrations appear to vary with the sequence variations of the gene and the way they influence the activity of the ferroportin protein[23,112,113]. Patients with 162delVal and N144H alterations in ferroportin were reported to give high hepcidin levels with a loss in function of ferroportin[19,106], whereas in cases of other variants with a gain of function the hepcidin levels are normal but relatively too low for the degree of iron loading in these patients[19,114].

5.2.4 Hepcidin in secondary iron overload

The dotblot and SELDI-TOF method were exploited to observe inappropriately low/normal hepcidin levels for the degree of iron load in thalassemic patients[19,104,106], which is in concordance with expression levels found in mice[115-118] and are thought to be the cause of increased iron uptake. Most of these patients are transfusion

dependent, thereby aggravating the iron burden. The lower hepcidin levels mostly found in thalassemia intermedia compared to major can be attributed to the higher degree of erythropoiesis expansion and lower degree of iron burden of the intermedia form[119]. Interestingly, in thalassemia patients it is shown that urinary hepcidin concentrations increase in response to transfusions after 3-4 days. This is most likely a result of relief of anemia which decreases the bone marrow demand for iron[104].

5.2.5 Hepcidin and renal anemia

SELDI-TOF MS measurement of serum hepcidin in end stage renal disease patients showed accumulated hepcidin levels that could be partly reduced by hemodialysis[97]. This accumulation suggests a contribution of hepcidin to the pathogenesis of renal anemia. In various kidney diseases, renal anemia occurs. Much of the etiology behind this anemia is still unclear, but is thought to be caused by reduced EPO production and is therefore mostly treated by administration of human recombinant erythropoietin (EPO) in combination with oral iron[120]. Other factors like hyperparathyroidism, aluminum toxicity, systemic inflammation, and impaired iron metabolism seem to be of minor importance[121]. Theoretically, in renal diseases hepcidin levels might be increased due to its decreased clearance and low grade inflammation described to be associated with end stage renal disease hereby decreasing the iron availability, but also might be influenced by concomitant iron deficiency[122].

5.3 Differential diagnosis and therapeutic implications

The outcomes of the human studies as described in the previous sections have implications for the use of hepcidin as a diagnostic and therapeutic tool. The fact that these implications are based on small series, sometimes even on a few cases, shows that large scale clinical validation still is needed to prove the power of hepcidin in differential diagnosis.

First, the circadian rhythm observed for serum might necessitate sampling protocols for hepcidin analysis that include standardization of time of blood withdrawal, similar to that for the assessment of serum iron levels or transferrin saturation.

Second, recognition of iron deficiency anemia (IDA) in the context of anemia of chronic disease (ACD) is currently performed with routine biochemical parameters such as, transferrin saturation, ferritin, CRP and less often soluble TfR (sTfR), zinc protoporphyrin and new erythrocyte indices which all have their own disadvantages[77,123-126]. In contrast to increased levels of hepcidin in ACD, both *in vitro* iron deficiency[15] and classic IDA in man are associated with low hepcidin levels[19,87] which makes hepcidin a potential marker for detection of IDA in ACD[77]. However, studies in anemic patients suffering from diseases such as rheumatoid arthritis, inflammatory bowel diseases, cancer, and end stage renal disease are needed to validate the potency of hepcidin measurements under these conditions.

Table 1. Hepcidin values in the various pathological conditions of mice and man.

A. Pathological conditions	Hepcidin[#]	Human	Animal	Reference
Elevated iron stores/ iron overload	↑	U		76,87,96
		mRNA		69,87
			mRNA (mice)	14,69,78
Iron deficiency/ hypoxia	↓↓	S		19
		U		19,87,96
			mRNA (mice)	15,102
Increased and/or ineffective erythropoiesis [†]	↓↓	S		19
		U		19,104,105,106
		mRNA		44,105
			mRNA (mice)	43,103,115-118
Anemia of chronic disease/ inflammation/infection	↑ / ↑↑	S		19,97
		U		19,78,87,96,101
		mRNA		87,133
			mRNA (mice)	14,15,55,56,59,78,133
Severe obesitas (BMI > 40 Kg/m^2)	↑	mRNA		39
Alcohol abuse	↓	mRNA		134
			mRNA (rat)	74,134
Liver disease[‡]	↑ / N / ↓	mRNA		127

B.Hereditary Hemochromatosis	Gene	OMIM type[§]	Hepcidin[#]	Human	Animal	Reference
Classic	*HFE*	1	↓	S		19
				U		19,96,87
				mRNA		110
					mRNA (mice)	12,109,111,113
Juvenile						
HJV-related	*HJV*	2a	↓↓	S		19,107
				U		19,66,107
					mRNA (mice)	58,129
Hepcidin-related	*HAMP*	2b	n.d.	U		106
					mRNA (mice)	12,86
TfR2-related	*TFR2*	3	↓	U		108
					mRNA (mice)	130,131,132
Ferroportin disease						
"Loss of function" phenotype	*SLC40A1*	4	↑	S		19
				U		19,106
				mRNA		112,113
"Gain of function" phenotype	*SLC40A1*	4	N	U		114
				mRNA		112,113

† After phlebotomy or in iron loading anemia's.
‡ Depending on status of inflammation, iron loading or fibrosis stage.
§ OMIM, Online Mendelian Inheritance in Man.
# ↓↓, strongly decreased; ↓, mildly decreased; N, normal; ↑, mildly increased; ↑↑, strongly increased. n.d., not detectable.
U, Urine; S, Serum

Third, hepcidin analysis might have a role as a screening, prognostic and monitoring test for hereditary hemochromatosis (HH), provided that abnormalities in liver functions[127], inflammation and a short interval between sample collection and phlebotomy are excluded.

As shown in Table 1, panel B, at presentation urinary hepcidin levels are low of even undetectable in all cases of juvenile hemochromatosis and likely to be moderately decreased or inappropriately low for the increased iron stores in TfR2 and HFE sequence variants. In our opinion assessment of hepcidin values might be instrumental to determine the likelihood of candidate genes involved in patients with non-HFE HH. This might reduce the workload and costs of the cumbersome procedures of screening for sequence variations in the multiple genes responsible for hemochromatosis[77]. Assessment of hepcidin levels or hepcidin/ferritin ratio's can be potentially useful in the prediction of biochemical or clinical penetrance of HFE-related HH as well as in monitoring of phlebotomy treatment, in which hepcidin levels below a certain threshold might become an indication to increase the phlebotomy interval.

Fourth, in iron loading anemias such as thalassemia, studies have suggested hepcidin or hepcidin/ferritin index values at the lower end of reference range as a result of suppressed hepcidin production due to high and less effective erythropoietic activity[103]. These findings may be relevant in the search for non-invasive measures of iron burden and improved therapeutic interventions for these often congenital diseases.

Finally, determination of hepcidin levels might turn out to be of value in the prediction of a response to EPO (and iron) treatment in patients with anemia's of chronic disease as well as in the monitoring of EPO treatment. To date, the forecast how patients will react on EPO treatment is complicated by the co-existence of several factors that contribute to anemia, such as inflammatory activity and liver toxic therapy.

Importantly, additional investigations are warranted to determine hepcidin levels in chronic kidney disease, end stage renal disease, upon hemodialysis and EPO and/or iron treatment in relation to iron and inflammatory status and blood counts. These studies will pave the way for novel diagnostic and more optimized therapeutic strategies in patients treated with EPO.

In conclusion, improved hepcidin assays are expected to increase insight in circulating hepcidin levels in various conditions and its kinetics. This knowledge aids in the development of hepcidin agonists and antagonists or the targeting of other proteins of the hepcidin regulatory circuitry pathways that will then be of value in the treatment of these iron related disorders.

6. Scope of this thesis

The major aim of this thesis was the development of a high through-put assay for hepcidin in order to study the regulation of this peptide, and to gain knowledge on the role of hepcidin in iron metabolism. Chapter 2 presents a novel urinary hepcidin assay exploiting surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS). This assay showed to have a strong correlation with the only pre-existing antibody-based dotblot assay. As described in chapter 3, further analytical improvement of this assay expanded the usability of hepcidin analysis also for serum samples and made it possible to determine associations with other iron related parameters, especially in patients with distorted iron metabolism. By using this serum hepcidin assay chapter 4 shows that insights on hepcidin regulation, foremost gained form *in vitro* cell culture and mice studies, can indeed be translated to the human *in vivo* model. Besides this, chapter 4 also describes an algorithm, based on biochemical serum parameters reflecting the main hepcidin regulators, which is capable to predict highly accurate the measured serum hepcidin levels.
Chapter 5, shows a time-course analysis of urinary hepcidin, serum iron and plasma cytokine levels in a human endotoxemia experiment that defines the temporal associations between Interleukin 6, hepcidin and iron during inflammation. Using this endotoxemia model, next to hepcidin the contribution of nitric oxide (NO) in the development of hypoferremia was assessed with the use of aminoguanidine as specific inducible NO synthase (iNOS) inhibitor during acute inflammation, as described in chapter 6.
Finally, the clinical use of hepcidin in the pre-screening of non-HFE hemochromatosis patients is described in chapter 7. These results indicate a potential role for hepcidin measurements in clinical practice which has to be explored more intensively in the near future.

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Chapter 2

Novel Urine Hepcidin Assay by Mass Spectrometry

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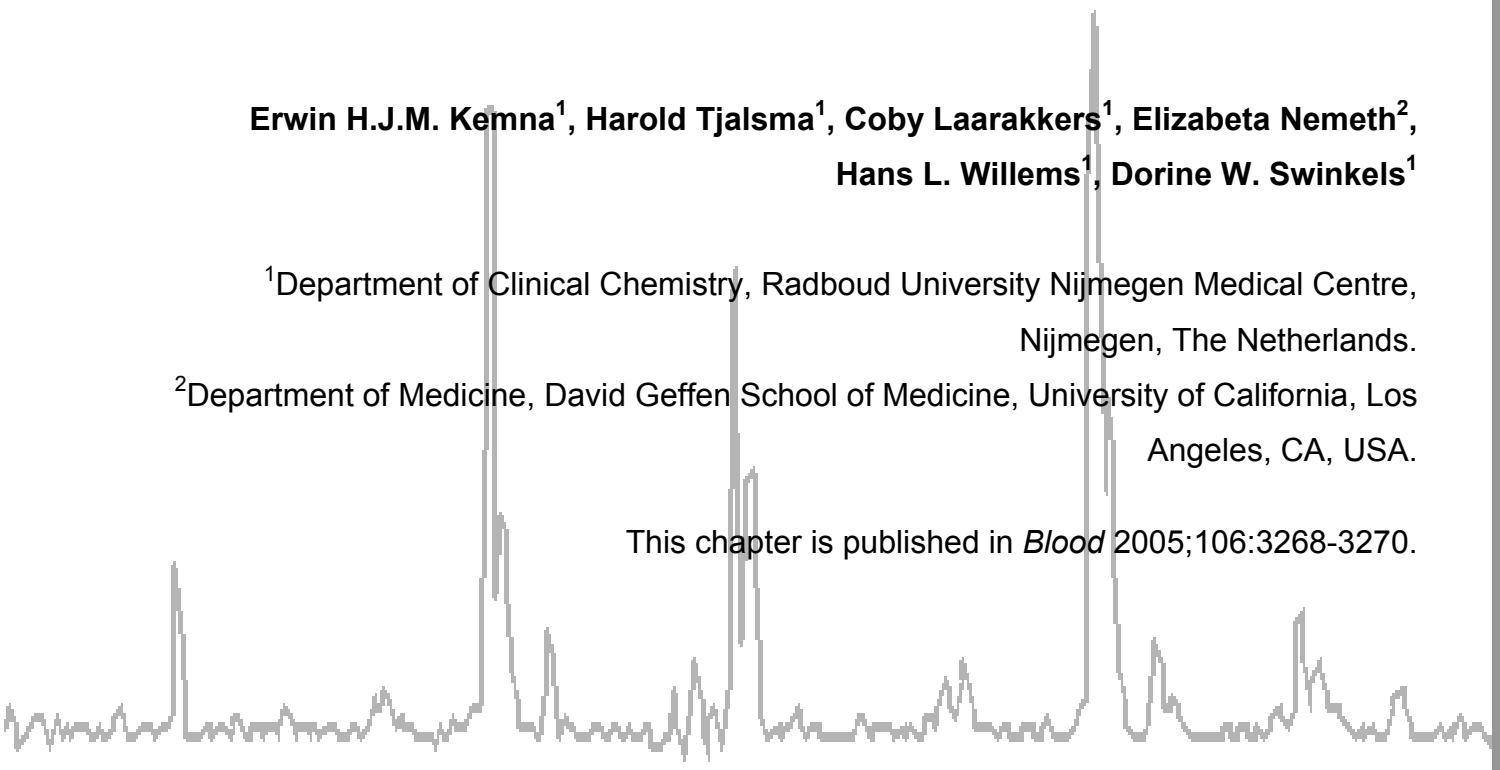


Abstract

The hepatic peptide hormone hepcidin is the central regulator of iron metabolism and mediator of anemia of inflammation. To date, only one specific immuno-dot assay to measure hepcidin in urine had been documented. Here we report an alternative approach for quantification of hepcidin in urine by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS).

Peptide peaks were detected corresponding to the 3 forms of hepcidin normally found in urine. The identity of the peptide peak equivalent to hepcidin-25 was confirmed using synthetic human hepcidin-25.

Validation of our MS data on samples with various hepcidin levels showed a strong correlation with previous immuno-dot assay results (Spearman R = 0.9275, *P*< .001). Most importantly, this hepcidin assay clearly discriminates between relevant clinical iron disorders.

In conclusion, this novel MS urine hepcidin assay is easy to perform and available to a wide audience. This enables the implementation of hepcidin measurements in large clinical studies.

Introduction

Hepcidin is a small, cysteine-rich cationic peptide produced by hepatocytes[1-3], secreted into plasma and excreted in urine. Hepcidin expression is induced by iron stores and inflammation[3] and suppressed by hypoxia and anemia[5]. Hepcidin is proposed to be the key regulator of iron metabolism and its discovery has changed our understanding of the pathophysiology of iron disorders. It now appears that hepcidin deficiency is the cause of most types of hereditary hemochromatosis and that hepcidin excess mediates anemia of inflammation[4]. Measurements of hepcidin concentrations could therefore be useful in diagnosis of iron disorders and would provide further insight into hepcidin regulation in vivo. However, assays for hepcidin detection and quantification in plasma or urine have not been generally available, and the development of reagents has been hampered by technical difficulties[6,7]. The development of immunochemical methods based on the production of specific anti-hepcidin antibodies is difficult due to the small size of hepcidin (25 amino acids), conservation between animal species[8] and the limited availability of the antigen as the production of synthetic hepcidin in its native conformation[9] or the isolation of hepcidin from urine[2] involves complex, time-consuming procedures. To date, only one immunochemical assay was successfully used to quantify urinary hepcidin in clinical studies[10].

We sought to develop a more widely available, high-throughput assay. Here we report a new quantification method for hepcidin in urine by the use of surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF

MS). This provides a fast assay with increased simplicity and accessibility that circumvents the difficulties encountered with antibody and antigen production. Moreover, this assay has also the potential to provide insight in the proportional contribution of the 3 known hepcidin isoforms that can be found in urine (hepcidin-20, -22, and -25).

Study design

Urine collection and storage

Approval was obtained from the Radboud University Nijmegen Medical Centre institutional review board for these studies. Informed consent was provided in accordance with the Declaration of Helsinki. From healthy volunteers second-morning urine was collected. Clinical urine samples from patients in the intensive care unit with severe sepsis (selection by protocol and APACHE [Acute Physiology and Chronic Health Evaluation] score) were collected by urine catheter. Samples from patients with (1) secondary iron overload (myelodysplasia [MDS]; World Health Organization [WHO] classification of refractory anemia [RA] or RA with ring sideroblasts [RARS] with blood-transfusion dependency), (2) iron-deficient anemia (hemoglobin level <7.5 mM and mean corpuscular volume [MCV] <85 fl), and (3) hereditary hemochromatosis (homozygous C282Y, in various stages of phlebotomy treatment, urine samples at least 1 week after the last phlebotomy) were collected during visits at the outpatient department of the Radboud University Nijmegen Medical Center, The Netherlands. Healthy volunteers and outpatients lacked clinical signs of inflammation. Freshly collected urine was centrifuged for 10 minutes at 2600g, and the supernatant was divided in aliquots and stored at – 20°C. Patient laboratory characteristics are shown in Table 1.

Table 1. Laboratory characteristics of patients and healthy volunteers who provided urine samples for the clinical validation

	Hemoglobin, nM	MCV, fL	Serum iron (Fe), µM	Fe/TIBC (TS), %	Ferritin, µg/L	CRP, mg/L
Normal, n=7	8.1 (7.8-9.1)	n.a.	20 (14-29)	32.8 (24.6-52.7)	80 (32-190)	<5 (<5-8)
Sec. Iron overload, n=8	5.6 (3.4-6.2)	n.a.	39 (29-63)	96.0 (77.1-100.0)	3088 (568-14 496)	<5 (<5-<5)
HH, n=7	8.7 (7.2-9.6)	n.a.	27 (9-39)	50.9 (16.1-93.5)	54 (12-2118)	<5 (<5-<5)
Iron def. Anemia, n=6	7.1 (3.3-7.4)	78 (69-84)	10 (2-11)	16.0 (2.4-19.6)	6 (2-11)	<5 (<5-6)
Endotoxemia model, n=6	8.1 (7.2-9.1)	n.a.	16 (4-22)	25.6 (8.0-38.6)	65 (15-219)	8 (5-33)
Sepsis, n=4	5.4 (4.9-6.2)	n.a.	1 (1-4)	7.1(3.8-17.4)	370 (280-505)	192 (124-227)

Data are expressed as the median with the range in parentheses.
HH indicates hereditary hemochromatosis; n.a., not analyzed; CRP, C-reactive protein; and TIBC, total iron binding capacity.

Protein chip preparation and SELDI-TOF MS measurements

The preparation procedure was based on protocols from Ciphergen and previous reports[11,12]. 8-spot hydrophilic Normal Phase chips (ProteinChip NP20; Ciphergen Biosystems, Fremont, CA), mimicking normal-phase chromatography with silicate functionality were used for their binding characteristics of proteins through hydrophilic and charged residues.

Urine samples were thawed, vortexed, and centrifuged for 10 minutes at 2600g. Urine supernatant (7 µl) was applied to the chip and incubated for 30 minutes in a humidity chamber. When protein overload was expected, a reduced sample volume was applied (minimum 1 µl). Spots were washed 3 times with 10 µl ultraPURE distilled water (Invitrogen, Breda, The Netherlands) and air-dried for 10 minutes. Finally, 0.8 µl of a saturated solution of sinapinic acid in 0.5% (vol/vol) trifluoroacetic acid and 50% (vol/vol) acetonitrile, used as energy-absorbing matrix (EAM), was applied to each spot surface, allowed to air-dry, and reapplied. Mass spectrometry was performed with a PBS IIc mass spectrometer (Ciphergen Biosystems).

Data were collected using the following settings: 2 warming shots at laser intensity 185 (not collected); collection of 50 shots at laser intensity 180 every 5 positions between 29 and 89; high mass 50 000 Da; detector voltage 2900V; detector sensitivity 9. The acquired mass range was from a mass-over-charge (m/z) ratio of 1500 to 10 000. External mass calibration was performed with synthetic human hepcidin (Peptides International, Louisville, KY). The bioactivity of this synthetic peptide was shown to be comparable to the synthetic hepcidin-25 used in the immuno-dot assay[13]. The mass of the synthetic hepcidin-25 from Peptides International was verified by matrix-assisted laser desorption/ionization (MALDI)-TOF MS (2787.80 m/z) and corresponded well with data from the supplier (2789 m/z), and previous reported values (2789 m/z) for hepcidin-25[2]. Peak annotation was performed with Ciphergen ProteinChip Software (version 3.2.0), after baseline subtraction and adjustment (fitting 8 times expected peak width). Peak intensity levels were normalized to urinary creatinine values and reported as Mega intensity/mmol creatinine. We found that changes in solvent and matrix (e.g. sample dilution) can influence the flight behavior of peptides during SELDI-TOF MS analysis[11]. Therefore, we aimed at a semiquantitative method for hepcidin quantification in urine.

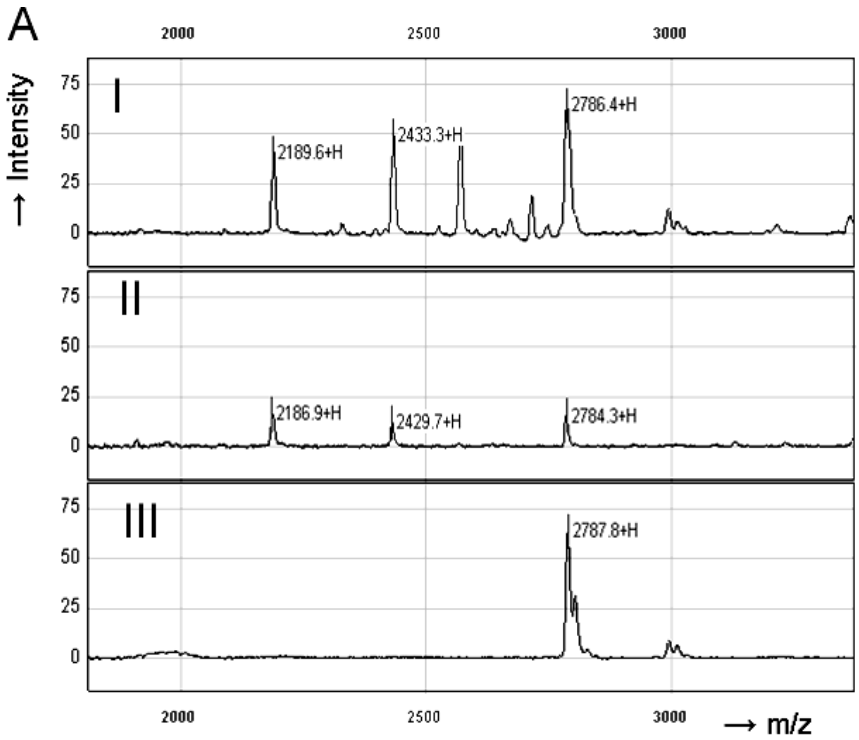
Statistic analysis

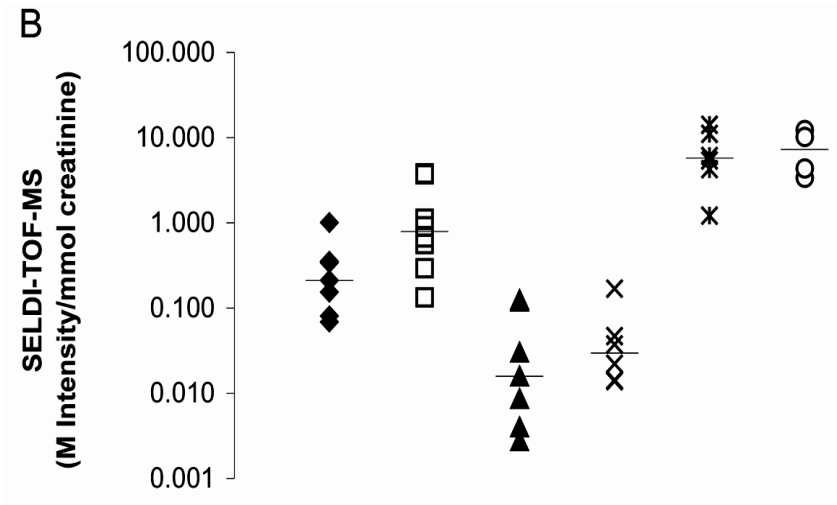
Analyses were performed with GraphPad Prism software (version 4.0; GraphPad Software, Inc, San Diego, CA). Correlation was tested by Spearman rank test. Group differences were tested for statistical significance by paired *t* test and Mann-Whitney *U* test.

Results and discussion

Hepcidin tracing by mass spectrometry

To investigate the feasibility of a mass spectrometry-based assay for the quantification of urine hepcidin, a pilot SELDI-TOF MS was conducted where the spectra of a patient with septicemia and a healthy volunteer were generated. Figure 1A I-II, shows in both spectra a clear peak at 2788 m/z that corresponds with the peak mass of 2789 m/z from the synthetic human hepcidin-25 peptide (Figure 1A III).





Group	Normal	Sec. Iron Overload	Hereditary Hemo-chroma-tosis	Iron Deficiency anemia	Endo-toxemia model	Sepsis
n	7	8	7	6	6	4
Median	0.211	0.794	0.016	0.030	5.752	7.269
Range	0.069 – 1.012	0.133 – 3.796	0.003 – 0.125	0.014 – 0.170	1.219 – 14.133	3.363 – 12.281

Figure 1. Urine hepcidin profiles by SELDI-TOF MS, and urine hepcidin levels in different iron metabolism disorders. (A) SELDI-TOF MS spectra from urine samples of a septicemia patient (I), healthy subject (II), and human synthetic hepcidin-25 (III). Urine hepcidin-25 peaks from spectra shown in panel I and panel II correlate with the reference human synthetic peptide (spectrum shown in panel III). The annotated peak masses correspond with hepcidin-20, -22, and -25.[2] (B) Urinary hepcidin excretion in patients with transfusion-induced iron overload (□), treated hereditary hemochromatosis (▲), iron deficiency anemia (×), endotoxemia after LPS injection(✳), and sepsis (○) compared with healthy subjects (◆). In each group the median is indicated.

Besides hepcidin-25, the urine spectra also show peaks that correspond with reported masses of the N-terminally truncated hepcidin-20 and -22 (respectively 2192 and 2436 m/z, as measured by MALDI-TOF MS)[2]. As expected, the intensities of the hepcidin peaks are strongly increased (about 3-fold) in the case of septicemia (Figure 1A I-II).

The results indicated hepcidin was detectable and quantifiable in urine samples by SELDI-TOF MS As the lack of commercially available peptides hampers the mass confirmation of the 20- and 22- amino-acid hepcidin forms, measurements will be based on the hepcidin-25 peptide until new insights on the 20- and 22- amino-acid peptides will approve a change in the data analysis protocol.

Validation of SELDI-TOF MS measurements

To validate SELDI-TOF MS measurements, we performed SELDI-TOF MS on urine samples from our previous study[14], in which hepcidin concentration was determined by the immuno-dot assay. The samples were from 10 volunteers injected with LPS who collected urine at 4 time points within a 22-hour time frame[14]. Statistic analysis showed a strong significant correlation between the two methods (Spearman R=0.9275, $P< .001$) and no significant differences between methods for each volunteer at each time point (Paired *t* test $P> .05$). These results prove that SELDI-TOF MS approach for urinary hepcidin measurements is comparable to the published immuno-assay method. In addition to providing accurate results, the assay is fast, simple and high-throughput, and therefore suitable for large experimental clinical studies.

Implementation in Clinical Practice

To investigate whether hepcidin quantification by mass spectrometry can distinguish between different clinical iron metabolism disorders, urine from patients with several iron-related diseases were used for SELDI-TOF MS measurements. Figure 1B shows that patients suffering from septicemia as well as those injected with LPS had significant elevated urinary hepcidin excretion compared with healthy subjects (Mann-Whitney *U* test, $P< .05$). Patients with iron deficiency anemia, and (partly) compensated hereditary hemochromatosis showed significant reduced hepcidin excretion compared with healthy subjects ($P< .05$). Patients with MDS with transfusion-induced iron overload, serum transferrin saturation values higher than 77%, and ferritin levels over 500 µg/L showed relatively increased but greatly varying hepcidin levels. This variety precludes differentiation of patients with secondary iron overload from healthy individuals ($P= .054$), while median difference with acute infection patients is still significant ($P< .05$). These results are consistent with previous reports on hepcidin levels in physiologic and pathophysiologic states[10,15-17]. In addition, the SELDI-TOF MS method would be suitable for differentiation between

(hepcidin induced) anemia of inflammation, and iron deficiency anemia where hepcidin excretion is physiologically reduced.
In conclusion, we present a novel mass spectrometry-based assay for the high-throughput measurement of hepcidin levels in urine. We anticipate that this will become an important tool to increase our insight in the role of hepcidin in iron metabolism-related disorders.

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Chapter 3

Mass Spectrometry-Based Hepcidin Measurements in Serum and Urine: Analytical Aspects and Clinical Implications

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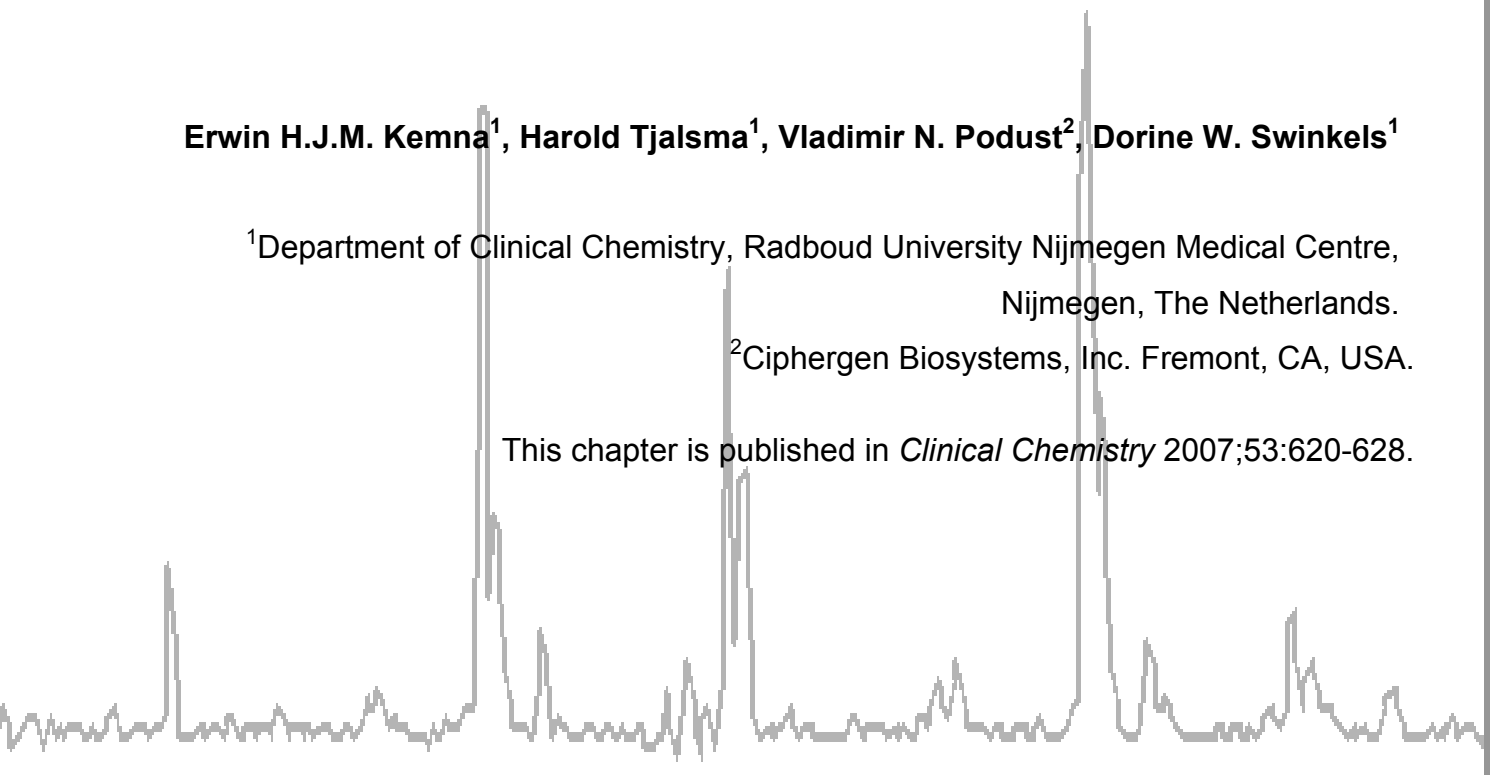


Abstract

Discovery of the central role of hepcidin in body iron regulation has shed new light on the pathophysiology of iron disorders. Information is lacking on newer analytical approaches to measure hepcidin in serum and urine. Recent reports on measurement of urine and serum hepcidin by surface-enhanced laser-desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) necessitate analytical and clinical evaluation of MS-based methodologies.

We used SELDI-TOF MS, Immunocapture, and tandem MS to identify and characterize hepcidin in serum and urine. In addition to diagnostic application, we investigated analytical reproducibility and biological and preanalytical variation for both serum and urine on Normal Phase 20 and Immobilized Metal Affinity Capture 30 ProteinChip arrays. We obtained samples from healthy controls and patients with documented iron-deficiency anemia, inflammation-induced anemia, thalassemia major, and hereditary hemochromatosis.

Proteomic techniques showed that hepcidin-20, -22, and -25 isoforms are present in urine. Hepcidin-25 in serum had the same amino-acid sequence as hepcidin-25 in urine, whereas hepcidin-22 was not detected in serum. The interarray CV was 15% to 27%, and interspot CV was 11% to 13%. Preliminary studies showed that hepcidin-25 differentiated disorders of iron metabolism. Urine hepcidin is more affected by multiple freeze-thaw cycles and storage conditions, but less influenced by diurnal variation, than is serum hepcidin.

SELDI-TOF MS can be used to measure hepcidin in both serum and urine, but serum requires a standardized sampling protocol.

Introduction

The iron-regulating peptide hepcidin is produced by hepatocytes and secreted into plasma[1-3]. Increased iron stores and inflammation induce hepcidin expression[3], whereas suppression occurs during hypoxia and anemia[4]. Although recognition of the central role of hepcidin in body iron regulation has increased our understanding of the pathophysiology of iron disorders[5,6], few investigative tools are available; these include an immunodot method for measuring urinary hepcidin[7] and a urinary hepcidin assay based on surface-enhanced laser-desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) for differentiating various disorders of iron metabolism[8]. A SELDI-TOF MS-based serum assay is under development for measuring hepcidin in serum[9], but this method requires optimization and evaluation to facilitate clinical investigation of hepcidin measurement in serum compared with urine and the biological variation of hepcidin in body fluids. In this study, we used SELDI-TOF MS, Immunocapture, and tandem MS to identify and characterize hepcidin in serum and urine.

Material and Methods

Study participants

Study participants included a control population of healthy volunteers (laboratory personnel), hereditary hemochromatosis (*HFE* C282Y homozygous) patients cross-sectionally selected from a family screening program (various stages of phlebotomy treatment), iron deficiency anemia patients (Hb ≤8.3 mmol/L men, ≤7.3 mmol/L women; mean corpuscular volume ≤80 fl; ferritin ≤10 µg/L), and thalassemia major patients treated with chelation therapy. The patients were recruited by their physicians during outpatient clinic visits (all in Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands, except for the thalassemia major patients, who were in Ospedale Sant'Eugenio, Rome, Italy). Endotoxemia samples from volunteers injected with lipopolysaccharide were selected from a human endotoxemia project[10]. Written informed consent was obtained from all study participants, according to the Declaration of Helsinki. Characteristics of the study participants are shown in Table 1.

Table 1. Characteristics of subjects who provided sample material for assay validation

	Control	Endotoxemia Model	Iron deficiency anemia	Thalassemia Major	Hereditary hemochromatosis
	n = 20	n = 28	n = 6	n = 5	n = 14
Hemoglobin (mmol/L)	8.7 (7.8 – 11.0)	n.a.	6.7 (5.0 – 8.3)	6.3 (6.1 – 7.3)	9.3 (7.5 – 12.0)
MCV (fL)	88 (84 – 93)	n.a.	77 (76 – 79)	86 (78 – 88)	90 (83 – 95)
Serum iron (Fe) (µmol/L)	19 (9 – 38)	18 (7 – 38)	6 (4 – 7)	52 (47 – 62)	22 (10 – 48)
Fe/TIBC (TS) (%)	35 (14 – 73)	40 (23 – 79)	8 (5 – 10)	98 (93 – 100)	56 (21 – 100)
Ferritin (µg/L)	60 (11 – 191)	120 (31 – 233)	6 (6 – 10)	784 (272 – 1710)	64 (28 – 1361)
CRP (mg/L)	< 5	9 (< 5 – 33)	< 5	< 5	< 5
U Hepc-25 (NP20) (M Int/mmol Cr)	0.52 (0.09 – 2.97)	6.16 (1.27 – 19.78)	0.01 (0.01 – 0.02)	0.21 (0.04 – 0.98)	0.15 (0.02 – 1.25)
U Hepc-25 (IMAC30) (M Int/mmol Cr)	1.45 (0.52 – 7.83)	12.97 (1.95 – 30.81)	0.10 (0.01 – 0.19)	0.59 (0.24 – 2.96)	0.66 (0.08 – 4.50)
S Hepc-25 (IMAC30) (M Int/L)	4.38 (0.58 – 9.95)	12.69 (4.11 – 27.33)	0.41 (0.35 – 0.60)	0.28 (0.18 – 2.42)	1.60 (0.19 – 9.39)

Data are expressed as the median with range in parentheses.
n.a. indicates not analyzed; CRP, C-reactive protein; TIBC, Total iron binding capacity; U Hepc-25, urinary hepcidin-25; S Hepc-25, serum hepcidin-25; M Int/mmol Cr, Mega Intensity/mmol creatinine.

In addition, collaborators from several medical centers in The Netherlands provided urine and serum samples from 3 patients with distinct forms of hereditary hemochromatosis not associated with the hemochromatosis (*HFE*) gene [Online Mendelian Inheritance in Man (OMIM) type 2a, homozygous G320V HJV mutation[11]; OMIM type 4, N144H-caused ferroportin disease[12]]. Characteristics of these patients are shown in Table 2.

Table 2. Urinary and serum hepcidin-25 levels in types of non-HFE hereditary hemochromatosis.

	Reference values	FPN N144H	HJV G320V (1)	HJV G320V (2)
Gender	m / f	m	m	m
Hemoglobin, mmol/L	8.1 – 10.7	n.a	8.3	8.7
MCV, fL	80 – 98	n.a	89	94
Serum iron (FE), µmol/L	10 – 25	17	47	53
Fe/TIBC (TS), %	30 – 60	26.6	78.3	96.4
Ferritin, µg/L	15 – 280	68*	60*	187*
CRP, mg/L	< 10	< 5	< 5	< 5
U Hepc-25 (NP20 array), M Int/mmol creatinine	0.09 – 2.97	4.56	0.01	0.01
U Hepc-25 (IMAC30 array), M Int/mmol creatinine	0.52 – 7.83	5.19	0.01	0.06
S Hepc-25 (IMAC30 array), M Int/L	0.58 – 9.95	10.09	0.58	0.40

n.a indicates not available; * phlebotomized values

We collected samples randomly between December 2005 and June 2006, at no specified time of day, except from the endotoxemia patients, from whom samples were collected according to a tight time schedule[10,13]. Urine and blood samples were centrifuged immediately after collection, divided into aliquots to avoid multiple freeze-thaw cycles, and stored at -80°C. We performed the hepcidin assay within 2 months after collection.

Laboratory measurements

Using an Abbott Aeroset analyzer, we measured total serum iron and latent iron binding capacity by the ascorbate/FerroZine colorimetric method, urine creatinine by enzymatic/colorimetric detection (Roche Diagnostics), and C-reactive protein by immunologic agglutination detection with latex-coupled polyclonal anti-C-reactive protein antibodies (Abbott Laboratories). We quantified serum ferritin by a solid-phase, 2-site chemiluminescent immunometric assay (Immulite 2000 and 2500, Diagnostic Products Corp.) and routine hematology characteristics by use of a Sysmex XE-2100 analyzer.

SELDI-TOF MS

We performed nonblinded hepcidin measurements by use of SELDI-TOF MS as previously reported[8]. In brief, a 5-µL sample was applied to Immobilized Metal Affinity Capture 30 (IMAC30), Normal Phase 20 (NP20), or cation exchange ProteinChip arrays (CM10), all equilibrated with appropriate buffers according to the manufacturer's instructions (Ciphergen Biosystems). Loaded arrays were incubated in a humidity chamber for 30 min and then washed according to the manufacturer's instructions and air-dried for at least 15 min. Finally, 1 µL of energy-absorbing matrix (EAM), made up of a 12.5 g/L solution of sinapinic acid in 500 mL/L acetonitrile (ACN) containing 5 mL/L trifluoroacetic acid (TFA), was applied twice onto each spot surface with the use of polymer-free polypropylene pipette tips and allowed to air dry

for 5 min. Mass-to-charge (m/z) spectra were generated using a Ciphergen Protein Biology System IIc TOF mass spectrometer at laser intensity 180 (NP20 and CM10) or 175 (IMAC30); detector sensitivity 9; high mass to acquire 50 kDa; optimization interval 1500 to 10 000 Da. External mass calibration was performed with a mixture of synthetic human hepcidin-25 peptide (2789.4 Da, Peptides International), hepcidin-22, and hepcidin-20 (2436.1 Da and 2191.8 Da, respectively; kindly provided by E. Nemeth, University of California, Los Angeles). Peak annotation was performed with Ciphergen ProteinChip Software version 3.2.0. From unpublished experiments we found that for serum and urine, normalization to total ion current (TIC) did not improve the hepcidin measurements and was therefore not applied in this study. Urine hepcidin measurements showed a relationship with TIC and total peptide content, which was predominated by hepcidin under the applied experimental conditions and instrumental settings (data not shown). Consequently, normalization of urine hepcidin values to TIC leads to loss of differentiation between samples. In contrast, due to the relatively stable protein content of serum samples, the serum hepcidin concentrations did not significantly change upon normalization to TIC (unpublished observations). Although the reabsorption and excretion characteristics of hepcidin are unclear, we used urinary creatinine to normalize all peak intensities for hepcidin-25 in urine. Normalization on creatinine is a prerequisite for comparison of urine hepcidin measurements because it is the best method to correct for the highly fluctuating concentration differences between urine samples. Relative concentrations were expressed as mega-intensity units per millimole of creatinine. Relative concentrations of serum hepcidin-25 were expressed as mega-intensity units per liter. Information on the assay can be found on: www.UMCN.NL/Hepcidin.

Purification of peptides from serum and urine

We first fractionated serum samples by use of spin columns containing Q HyperD F resin (Pall Corp.), eluted the flow-through fraction by centrifugation, and eluted bound proteins with buffers of pH 9, 7, 5, 4, and 3. We analyzed fractions by use of IMAC30 arrays. Every serum fraction or urine sample was adjusted to final concentrations of 5% ACN and 0.5% TFA and bound to PLRP-S beads (Polymer Laboratories Varian). Bound proteins were eluted with 5%, 10%, 20%, 30%, 40%, 50%, and 60% ACN solutions containing 0.1% TFA. We detected proteins in eluted fractions by profiling 1 µL of each fraction on an NP20 array using Protein Biology System IIc MS.

Identification of peptides by tandem mass spectrometry

We analyzed peptides of interest for a presence of disulfide bonds. Aliquots of 30% ACN fractions were air-dried on an NP20 array. Solution containing 10 mmol/L dithiothreitol (DTT) in 50 mmol/L ammonium bicarbonate, and the sample loaded arrays, was preheated to 70°C. Then we loaded 10-µL aliquots of the DTT solution onto the spots and air-dried them at 70°C. After complete evaporation of solutions and cooling to room temperature, we applied EAM. We acquired single MS spectra for

unreduced and DTT-reduced samples by use of a Q-STARXL tandem mass spectrometer (MS/MS; Applied Biosystems) equipped with a Ciphergen PCI-1000 ProteinChip Interface. We used the reduced samples to acquire MS/MS spectra. We subjected the ions of interest (m/z values of 2198 for hepcidin-20, 2442 for hepcidin-22, and 2796 for hepcidin-25) to collision-induced dissociation and submitted the results to the database-mining tool Mascot (Matrix Science) for peptide identification.

Immunocapture

We captured hepcidin from urinary samples by use of Protein G coupled to IDM beads (Ciphergen Biosystems) and polyclonal rabbit antihepcidin serum (generous gift from E. Nemeth and T. Ganz, University of California, Los Angeles). We performed all incubation steps at room temperature. Protein G beads were first incubated with rabbit antiserum diluted 20 times in 0.01 mol/L phosphate-buffered saline (PBS; Sigma-Aldrich Chemie BV) supplemented with 0.1% Triton-X100 (PBS-Tx). We washed the beads 3 times with PBS-Tx to remove unbound serum proteins and resuspended the beads in urine, diluted 20 times in PBS containing a final concentration of 0.25 mol/L NaCl and 0.1% Triton-X100 ($PBS_{0.25}$-Tx). We washed the beads 6 times with $PBS_{0.25}$-Tx to remove unbound and nonspecifically bound proteins. Finally, we eluted bound proteins with 500 mL/L ACN containing 3 mL/L TFA. To obtain profiles of Protein G-captured proteins, we applied eluates to a CM10 array equilibrated with 0.1 mol/L ammonium acetate (pH3) and incubated them for 30 min in a humidity chamber. To obtain reference spectra, we diluted untreated urine samples once in equilibration buffer before on-spot incubation. Spots were washed, allowed to air-dry, and followed by sinapinic acid application.

Analytical assay characteristics

We performed spot-to-spot precision for hepcidin-25 on NP20 and IMAC30 ProteinChip array with 2 human urine samples with medium and high hepcidin concentrations. Both samples were applied onto the first 6 positions of the 8-spot array, followed by addition of the EAM. The last 2 spots were used for control samples. For serum we followed the same procedure for a single sample.

We used the same urine and serum samples for a chip-to-chip reproducibility study. We collected single measurements for serum and urine on NP20 and IMAC30 array types from randomized chip positions for 10 days (8 days for serum application on NP20 arrays). Every day a new sample aliquot was thawed, and freshly prepared EAM was applied. From these data, we calculated means, SDs, and CVs.

We measured linearity of peak intensities by dilution of the urine and serum samples used for the reproducibility tests with human urine or serum (dilution samples) from a patient with hepcidin concentrations below those detectable by SELDI-TOF MS. Immediately before sample application, we diluted 1 to 18 µL of sample in a polypropylene microcentrifuge container to a 20 µL end volume with the dilution sample. After pipette mixing, 5 µL of the diluted sample was spotted on the array.

We created standard curves of synthetic hepcidin-25 for both serum and urine applications. After dissolving the lyophilized peptide in distilled water (100 µmol/L), the peptide was diluted 4500-fold with dilution sample for serum or urine (22.2 nmol/L). Dilutions of 18, 16, 12, 8, 4, 2, and 1 µL of the 22.2 nmol/L solution with the dilution sample to the final volume of 20 µL yielded a standard curve ranging from 22.2 to 1.11 nmol/L.

Preanalytical and biological interferences

We evaluated the influence of multiple freeze-thaw cycles for both serum and urine using IMAC30 arrays. Five sera and 4 urine samples from different participants and different intensity concentrations underwent 4 freeze-thaw cycles. After every cycle, we analyzed hepcidin batchwise by single measurement.

We studied the existence of a circadian rhythm for hepcidin in serum and urine by performing a 24-h time-course in 3 healthy volunteers (1 man, 2 women). Blood and urine was collected every 3 h for analysis of routine iron measurements and serum and urine hepcidin (IMAC30 array), starting at 6:00 AM with fasting blood and urine samples, after which the fasting state was ended. All samples were processed as described above and stored at -80°C before analysis (batchwise by single measurement).

Statistic analysis

We performed statistical analyses with GraphPad Prism software, version 4.0. Pearson correlation was used for comparison studies. *P* values < .05 were considered significant.

Results

Hepcidin identification and characterization

The presence of hepcidin-25 in a human urine[8] and serum[9] has been demonstrated. Our current investigation revealed that, in addition to hepcidin-25, hepcidin-20 can be verified in both urine and serum with the use of a purified synthetic reference human hepcidin peptide, whereas hepcidin-22 can be identified only in urine (Figure 1A). This supports the hypothesis that both the 20- and 25-amino acid peptides are secreted in the circulation after production in hepatocytes, whereas the 22-amino acid peptide merely is a urinary degradation product of hepcidin-25[14].

In addition to mass tracing, we investigated immunocapture of all 3 known forms of hepcidin from urine. Immunoaffinity assays using polyclonal rabbit antihepcidin antibodies [the same as those used in hepcidin dotblot assay[7]] showed that the 3 peaks annotated as hepcidin-25, -22, and -20 can be specifically captured from a human urine sample of a healthy individual (Figure 1B).

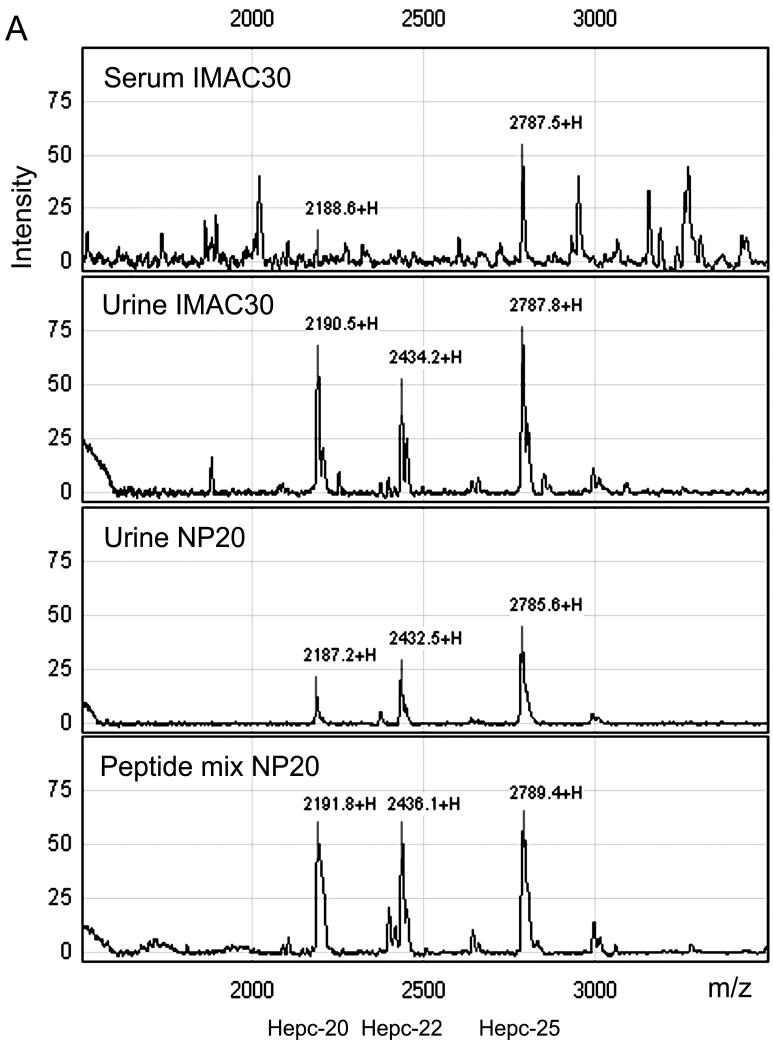
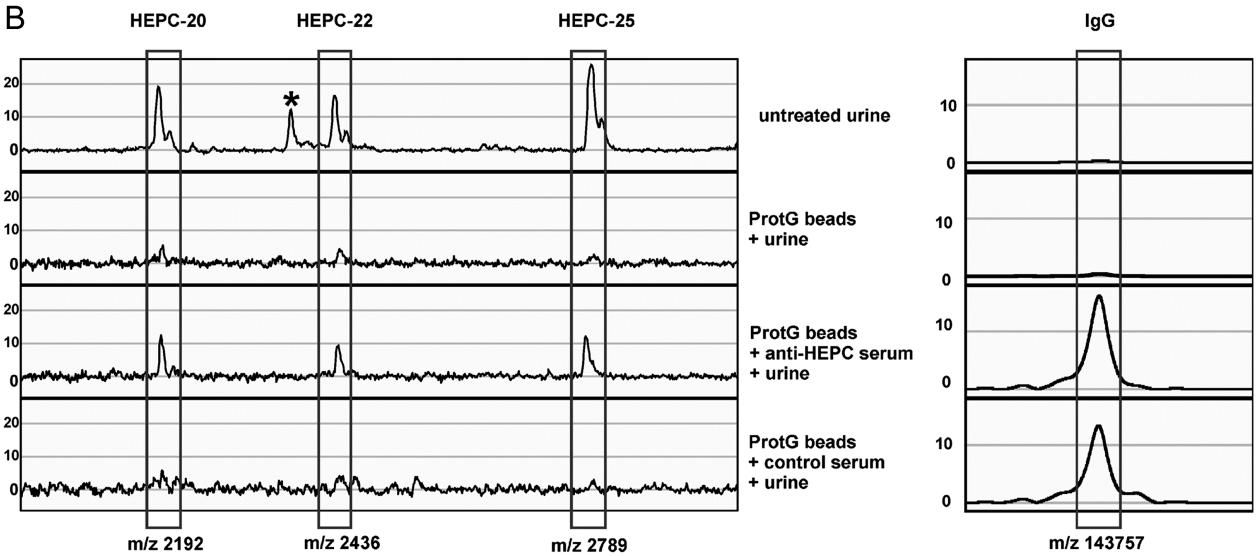


Figure 1. Hepcidin identification and characterization.

(A), SELDI-TOF MS profiles in serum and corresponding urine samples from a participant with an inflammation response after lipopolysaccharide injection. The annotated peak masses matched to the masses in the reference peptide mix that contained purified synthetic human hepcidin-20, -22 (E. Nemeth, University of California, Los Angeles) and -25 (Peptides International). (B), Immunocapture of urine hepcidin. Urine samples from a healthy individual were incubated with Protein G beads, loaded with either a polyclonal antihepcidin rabbit antibody or control rabbit IgG. Immunocaptured proteins were analyzed by SELDI-TOF MS, and protein profiles were compared with the protein profile of an untreated urine sample (upper panel). Peaks corresponding to 3 forms of urine hepcidin[8], and those corresponding to rabbit IgGs are indicated. A peak with m/z 2436 that is present in untreated urine, but not present in the captured fractions is marked ("*"), as it shows that the capture of the three hepcidin forms was specific. Notably, on-spot incubation of the same urine sample with antihepcidin antibodies bound to Protein G-loaded arrays, did not yield detectable hepcidin concentrations (our unpublished observations).



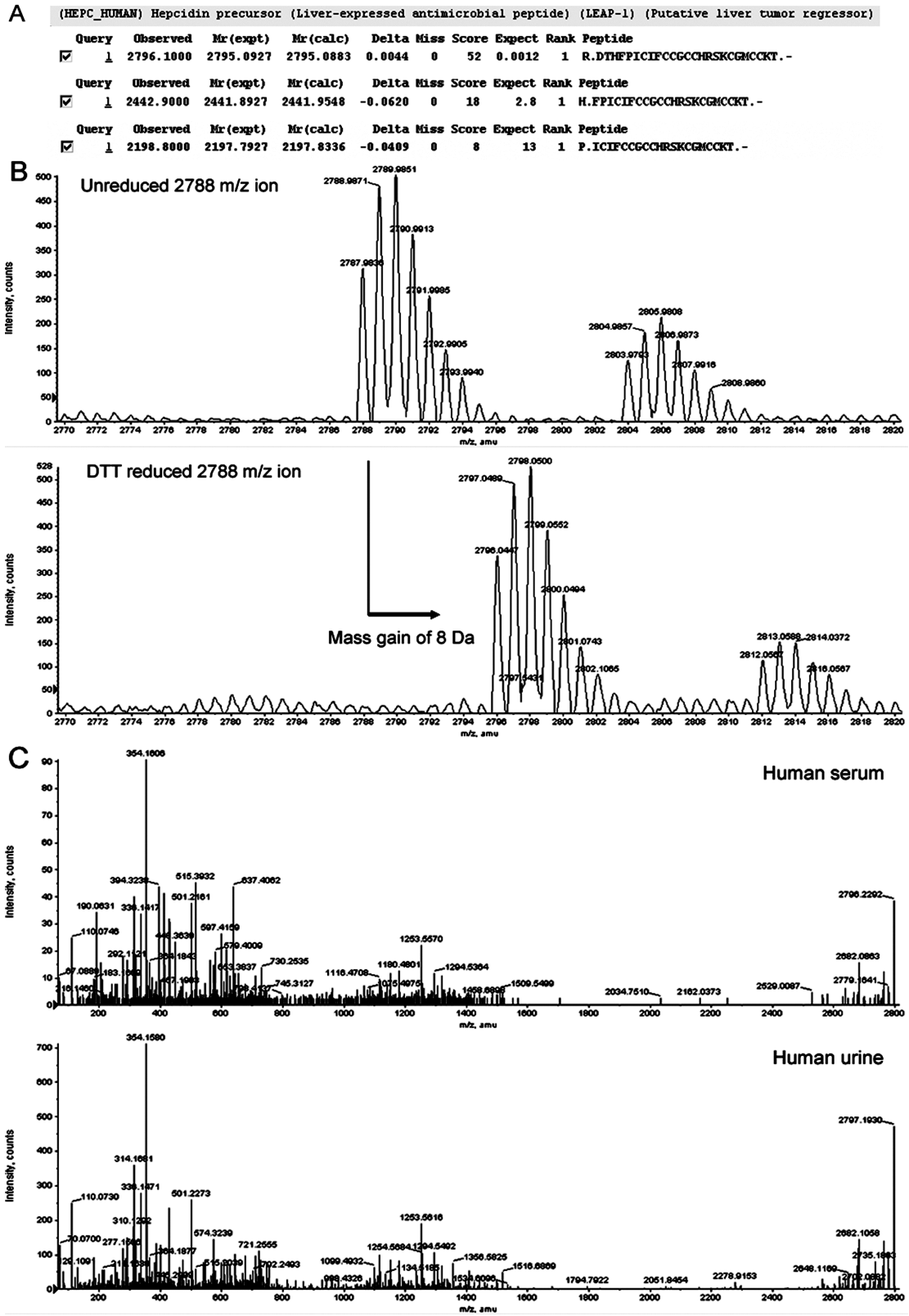


Figure 2. Hepcidin identification by tandem MS.
(A): Mascot search results of MS/MS data for DTT-reduced human urinary samples. The ions with m/z of 2796, 2442, and 2198 were identified as human hepcidin-25, -22, and -20, respectively. Identical result was obtained for the ion with m/z of 2796 from reduced human serum sample (not shown). (B): Single MS profile of human urine sample before and after reduction with DTT. A mass shift of 8 Da indicates the reduction of 4 disulfide bonds. (C): Aligned MS/MS spectra for the ions with m/z of 2796 from human serum and urine indicate that both parent ions correspond to the same peptide hepcidin-25.

MS/MS analysis of peptides in a urine sample from an endotoxemia patient confirmed that the peaks with m/z of 2198, 2442 and 2796 corresponded to hepcidin-20, -22, and -25, respectively (Figure 2A). Similarly, we identified the m/z 2796 peak in serum as hepcidin-25. Notably, single MS spectra of urine and serum samples displayed a mass shift of +8 Da for all hepcidin forms after reduction with DTT, as exemplified for urine hepcidin-25 (Figure 2B). This observation confirms the presence of 4 disulfide bonds in these peptides which is a typical characteristic of hepcidin[15]. Finally, the MS/MS spectra for serum and urine peptides with m/z of 2796 displayed mostly the same fragment ions, strongly suggesting that both parent ions corresponded to the same peptide hepcidin-25 (Figure 2C).

Analysis of hepcidin-25 on NP20 and IMAC30 ProteinChip arrays

Because the biological roles of hepcidin-20, and -22 in iron metabolism are still unclear[2,14], hepcidin-25 is used for optimization of serum and urine hepcidin measurements. To evaluate the ProteinChip array type on the performance of the hepcidin-25 assay, we analyzed 73 urine and serum sample pairs from controls and patients with various iron metabolism disorders using the previously reported NP20 and IMAC30 arrays[8,9]. Although hepcidin concentrations measured using NP20 arrays are lower than those measured with IMAC30 arrays, both arrays correlate for the urinary application (R = 0.928, $P < .001$; Pearson correlation; Figure 3A). Sensitivity was higher with the use of IMAC30 compared to NP20 arrays, likely because of highly specific binding of hepcidin to the IMAC-Cu surface. In contrast to the IMAC30 arrays, NP20 arrays did not bind detectable concentrations of serum hepcidin-25; therefore, we could not perform correlation analyses for serum and urine on NP20 arrays. In contrast, IMAC30 arrays showed a significant correlation (R = 0.822, $P <.001$; Pearson correlation; Figure 3B).

Chip-to-chip variation of the urine application for IMAC30 ranged from 22% at a high intensity of 48 to 27.5% at a lower intensity of 20 (Table 3), whereas NP20 variation reached 34% for the same samples. Precision was also better for urine hepcidin measurements using the IMAC30 chip (CVs 11% and 13%) compared with the NP20 chip (CVs of 16% and 23%) (Table 3).

For serum, the reproducibility was strongly affected by differences in protein binding capacity of both arrays. Intensities measured from the same sample differed, from an intensity of 5 with relatively high CVs on NP20 array to an intensity of 50 with low CVs on IMAC30 array.

We checked the linearity of the urinary and serum hepcidin application on IMAC30 array by measuring dilution series of urine and serum samples from an inflammation patient (single measurements). Figures 4A and B show a high degree of linearity for both urine and serum (R = 0.993 and 0.971, respectively). Intensity values >55 seem to deviate from linearity, perhaps because of MS detector saturation. Therefore, an intensity value of 55 was considered to be the upper level of detection.

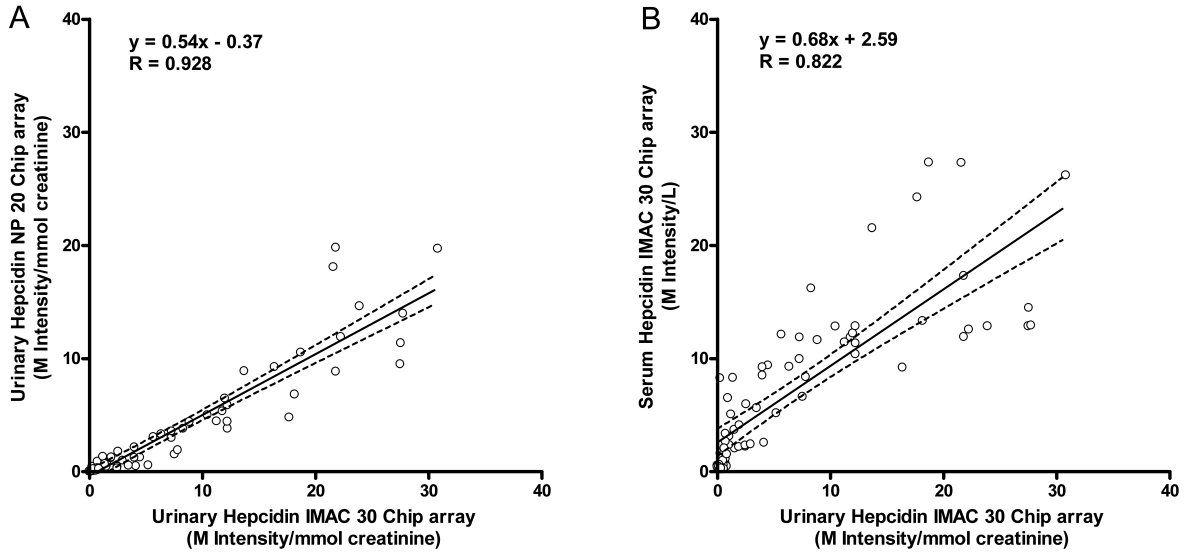


Figure 3. Detection of serum and urine hepcidin-25 on NP20 and IMAC30 arrays.
Correlation of urinary hepcidin-25 values as measured by NP20 and IMAC30 ProteinChip arrays (A), and coupled serum and urine hepcidin-25 concentrations measured on IMAC30 array (B). The *solid line* represents the regression line, and the *dotted line* indicates the 95% CI of the regression line. It should be noted that the apparent increased scatter in absolute variation on high hepcidin values compared to low values, as presented in panel A of this figure, is because absolute concentrations are used in this plot. Relative average for the high values is not more scattered than the low-end values (data not shown), which is in concordance with the CV values shown in Table 3. As it is currently unknown whether and how much hepcidin is reabsorbed in the tubulus, we cannot indicate whether this aspect contributes to the scatter in panel B of this figure.

Table 3. Reproducibility of hepcidin-25 analysis by SELDI-TOF MS in NP20 and IMAC30 chip arrays in both serum and urine.

Urine application		NP20		IMAC30	
Chip-to-chip*		m/z	Intensity	m/z	Intensity
Level I	Mean	2788.48	13.10	2788.65	19.84
	SD	0.94	4.37	1.96	5.46
	CV (%)	0.03	33.4	0.07	27.5
Level II	Mean	2788.37	41.55	2788.45	47.84
	SD	0.89	10.00	1.64	10.50
	CV (%)	0.03	24.1	0.06	22.0
Spot-to-spot**		m/z	Intensity	m/z	Intensity
Level I	Mean	2787.13	15.23	2788.53	17.83
	SD	1.00	2.62	1.55	1.97
	CV (%)	0.04	16.5	0.06	11.0
Level II	Mean	2787.88	40.61	2788.24	40.89
	SD	0.53	9.47	1.00	5.25
	CV (%)	0.02	23.3	0.04	12.8

* Ten-day single measurement with randomized chip position.
** One chip (6 replicates).

Serum application		NP20		IMAC30	
Chip-to-chip*		m/z	Intensity	m/z	Intensity
Level I	Mean	2788.40	5.26	2788.02	51.96
	SD	1.41	1.73	1.56	7.76
	CV (%)	0.05	32.9	0.06	14.9
Spot-to-spot**		m/z	Intensity	m/z	Intensity
Level I	Mean	2787.54	4.27	2788.33	43.89
	SD	0.60	1.14	0.59	5.06
	CV (%)	0.02	26.6	0.02	11.5

* Eight-day single measurement (NP20 array) and 10 days single measurement (IMAC30 array) with randomized chip position.
** One chip (6 replicates).

The construction of a calibration curve with synthetic human hepcidin-25 showed that both NP20 as IMAC30 arrays are capable of producing a 6-point calibration curve based on urine matrix (Figure 4C and D). Again, intensity values >55 deviated from linearity and therefore were considered as exceeding the upper level of detection. Construction of the same concentration interval on IMAC30 array in a serum matrix showed hepcidin-25 only for a concentration of 22.2 nmol/L and above (results not shown).

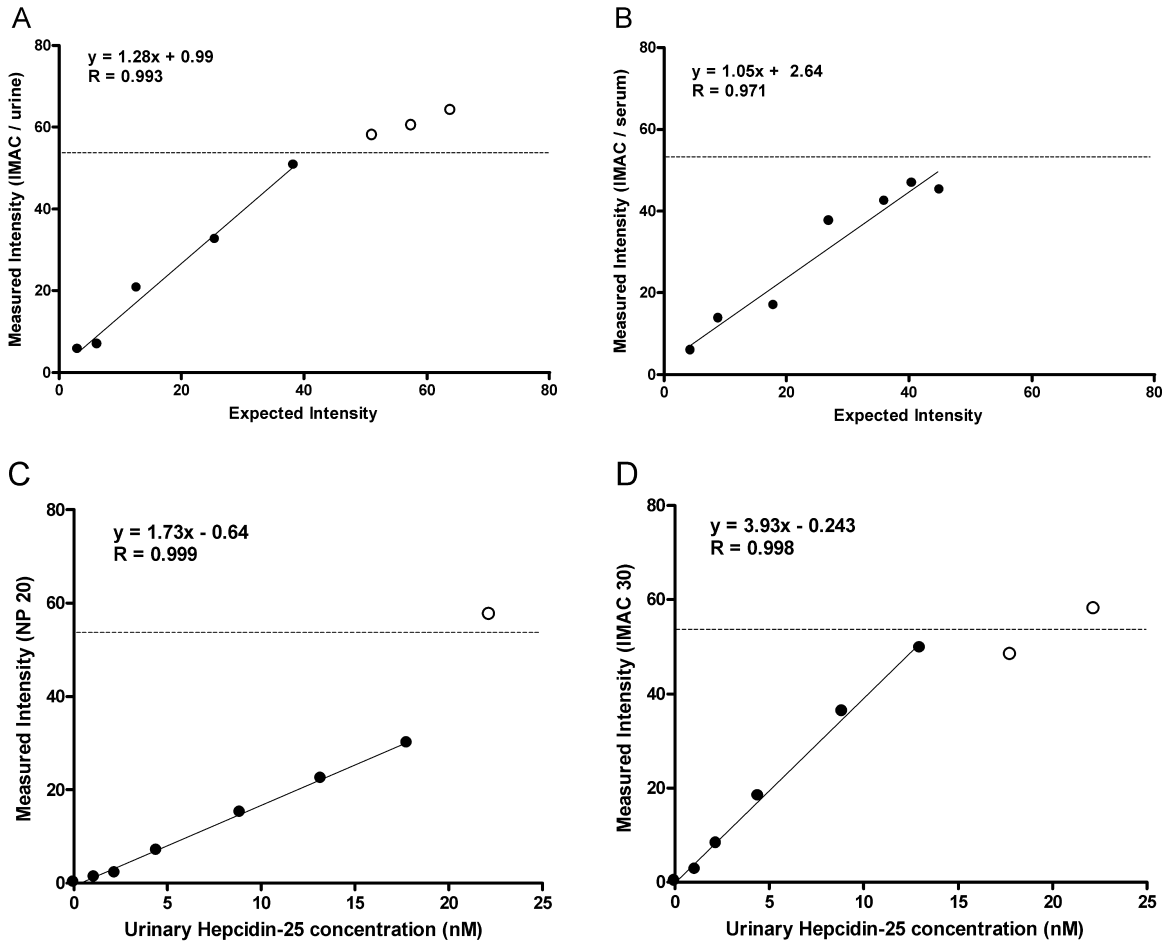


Figure 4. Linearity study of human hepcidin-25 in urine samples.
Dilution curve of human urinary inflammation sample (A), and human serum sample (B) on IMAC30 array.
Calibration curve of human synthetic hepcidin-25 in urine samples on NP20 array (C), and IMAC30 array (D). The *dotted line* indicates upper limit of accurate detection (intensity level of 55). Values above this level were excluded from analysis and are shown as open circles (O).

Diagnostic application

We categorized urine and serum sample pairs into 5 groups after clinical diagnosis. Figure 5 shows that, despite the variation within each group and a slight overlap, differentiation between inflammation-induced and iron deficiency anemia is clear for both serum and urine hepcidin concentrations measured on IMAC30 array. The results are comparable with the results obtained with NP20 and consistent with

previous reports[7,8]. The broad ranges of the thalassemia group and the hemochromatosis group are in accordance with the individual heterogeneity in genetic makeup, extent of anemia, and treatment[16-18].

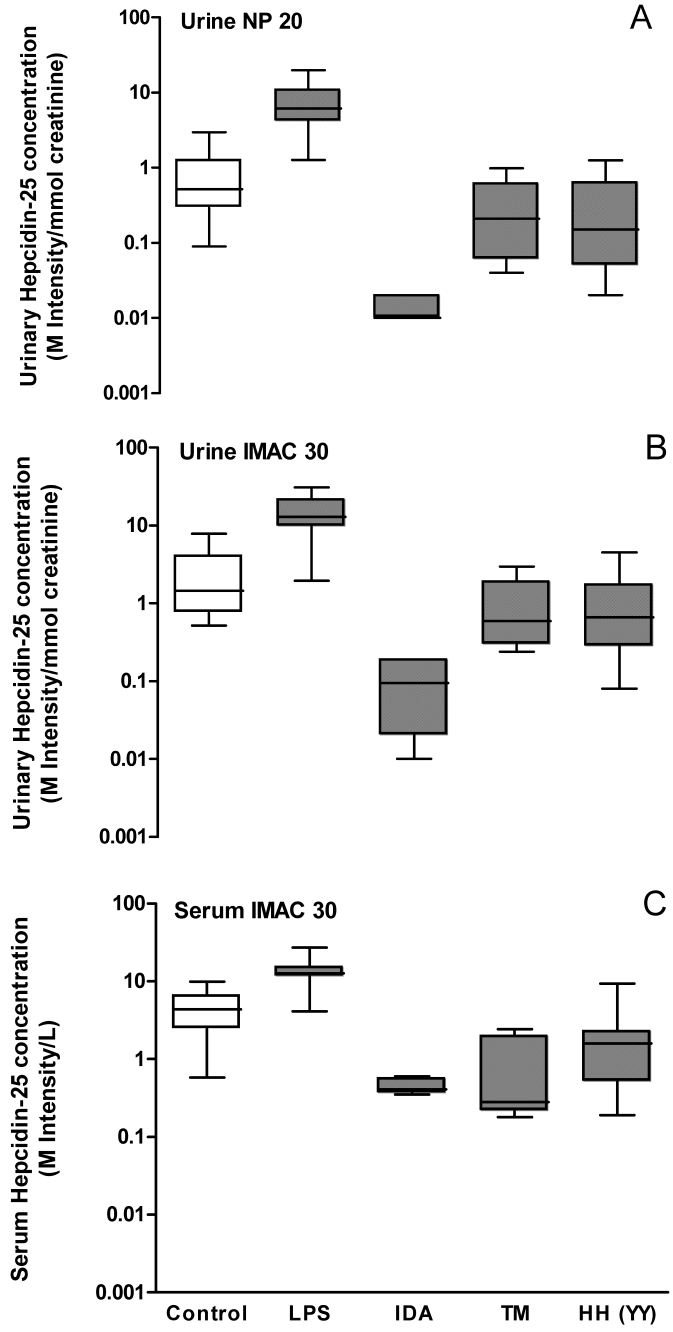


Figure 5. Urinary and serum hepcidin-25 concentrations in selected clinical populations.
Urinary hepcidin-25 concentrations measured on NP20 array (A), and IMAC30 array (B), and serum hepcidin-25 measured on IMAC30 array (C). LPS, volunteers injected with LPS (6 h after injection); IDA, iron deficiency anemia patients; TM, thalassemia major patients; HH(YY), C282Y homozygous hemochromatosis patients. Box plots show 25th and 75th percentile with median for every group. Error bars represent minimum and maximum values. Characteristics of each population are shown in Table 1.

In this study we found serum and urine hepcidin concentrations close to zero in 2 brothers treated for juvenile hemochromatosis (OMIM type 2a), and values in the upper level of the reference range for a patient treated for ferroportin disease (OMIM type 4; Table 2). These data confirm the potential of hepcidin analysis in prescreening for the presence of non-*HFE* Hemochromatosis.

Preanalytical and biological determinants that influence hepcidin-25 measurements in serum and urine

Decrease in hepcidin concentrations in serum samples due to multiple freeze-thaw cycles is of minor importance compared to those in urine (Figure 6). Preliminary results from urine samples stored at -80°C and -20°C during a 6-month course showed that (*a*) hepcidin was stable only at -80°C and (*b*) addition of protease inhibitor phenylmethylsulfonyl fluoride[19] had no beneficial effect (unpublished observations).

Hepcidin concentrations in serum follow a clear circadian rhythm, such that the concentrations were 2- to 6-fold higher at 1500 than at 0600. Urinary concentrations, however, show a more blunted response, and thus less diurnal variation. The course of serum iron shows an inverse association with serum and urine hepcidin values (Figure 7).

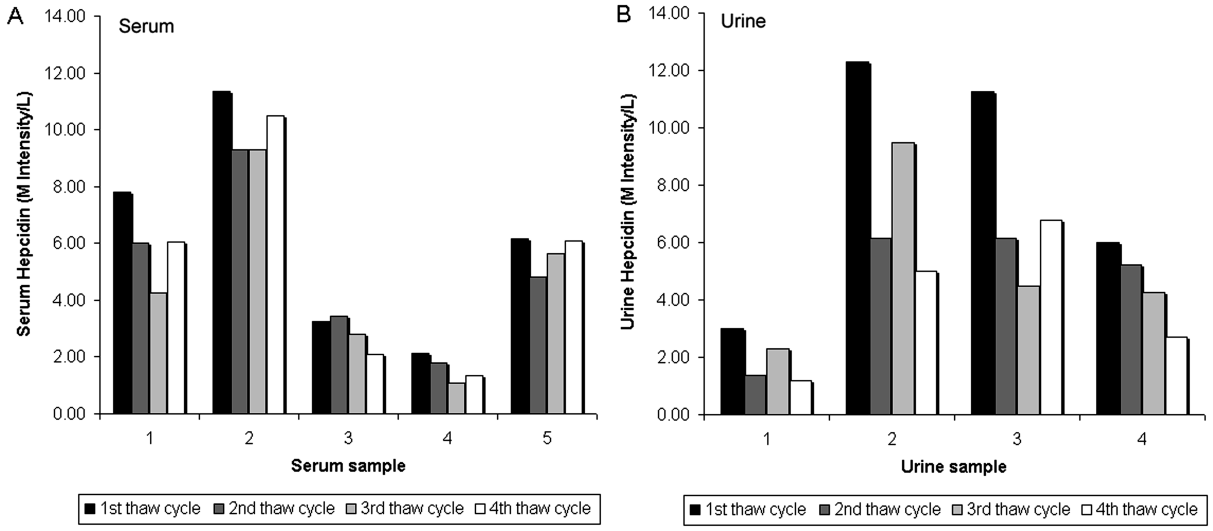


Figure 6. Influence of multiple freeze/thaw cycles on hepcidin reproducibility.
Serum hepcidin (A), and urine levels (B) of different subjects measured after a freeze/thaw cycle for four times. Both serum and urine are expressed in Mega Intensity per liter in order to compare the influence of variation directly on measured MS signal. For serum analytical variation is probably a more important factor than the effect of multiple freeze/thaw cycles.

Discussion

The IMAC30 array-based urinary hepcidin assay correlates significantly with the previously described urinary hepcidin MS application using NP20 arrays[8] and showed greater sensitivity and reproducibility, resulting in improved analytical performance. The increase in protein-binding capacity of the IMAC30 array makes it particularly suitable for serum analysis and measurement of low hepcidin concentrations in urine.

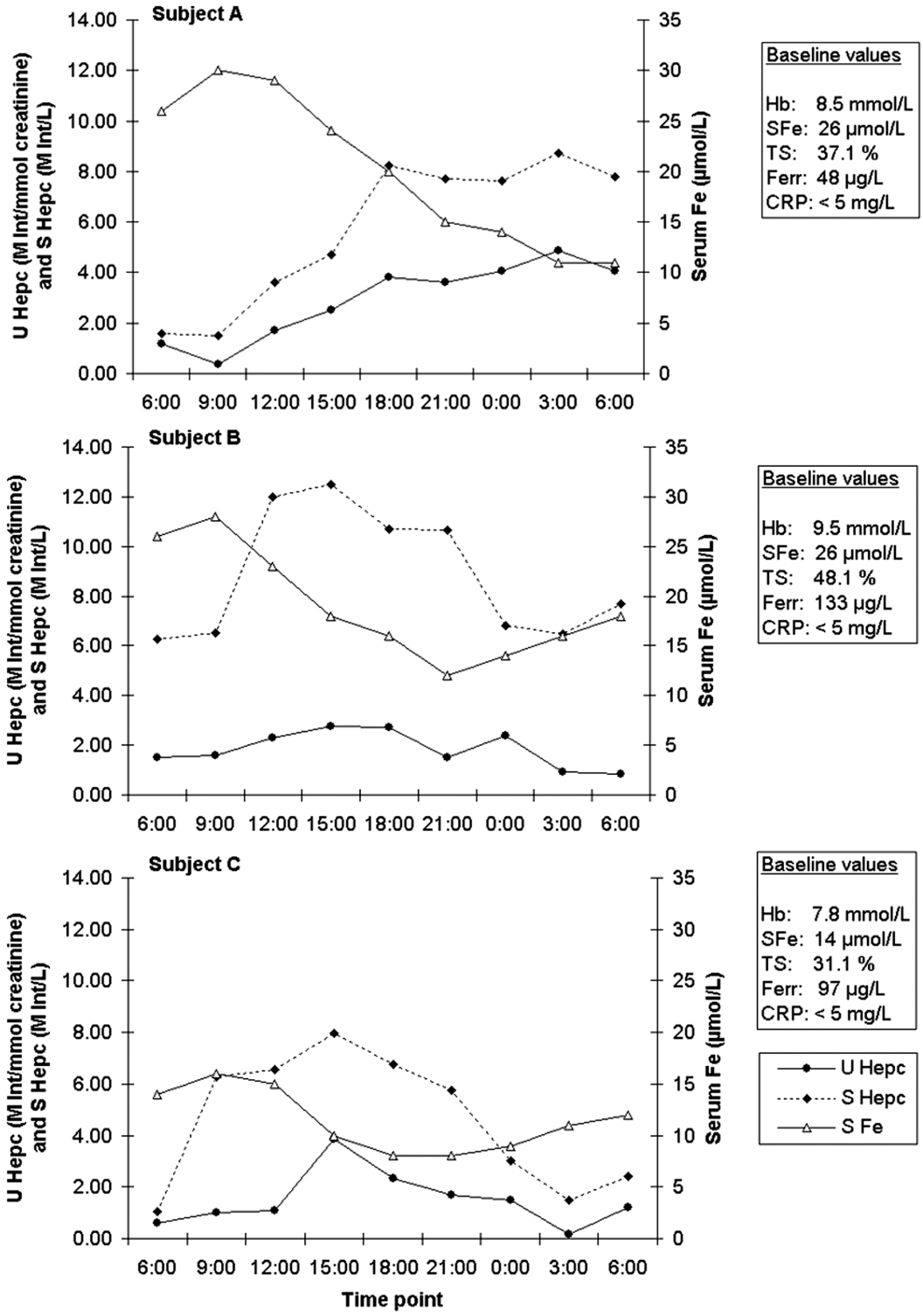


Figure 7. Circadian influence on urinary and serum hepcidin-25 concentrations.
Urinary and serum hepcidin concentrations, and serum iron concentration in 3 healthy volunteers during a 24 hour time-course (Person A and C are pre-menopausal females, person B is male).
Circadian influence is seen on all three parameters. Individual differences are visible on amplitude and frequency pattern, and relative concentration difference between urinary and serum hepcidin. Note that in subject A CRP-concentrations increased to 10 mg/L in the last 6 hr of the time-course, which suggests that an inflammatory process may have contributed to the persistent increase in hepcidin and the decrease in serum iron during this period.

The total assay variation includes analytical variation, biological or diurnal variation, and preanalytical variation, all contributing to the widening of ranges of the clinical utility clusters. In the future, we plan to routinely perform duplicate measurements and investigate isotope dilution[20] or hepcidin derivatives as internal standards to reduce analytical variability.

Biological variation in hepcidin concentrations due to a circadian rhythm correlated inversely with daily variations in serum iron concentrations[21], in accordance with previous reports that transferrin receptors 1 and 2 on the outer hepatocyte membrane act as sensors of circulating iron and TS, thereby linking low serum iron with increased hepcidin synthesis[22,23]. Circadian rhythm may also lead to important variation in outcome if sampling time is not standardized, thereby contributing to the wide variation in hepcidin concentrations of the control population (Fig 5C). Sampling according to protocol led to decreased variation in hepcidin concentrations within the lipopolysaccharide group. The low variation within the iron deficiency anemia group is likely to be due to the dominant down-regulating influence of an iron-deficient erythropoiesis on hepcidin that prevents circadian increases.

Variation in hepcidin results is also attributable to preanalytical conditions. According to our preliminary results, urine samples are more susceptible than serum to variation from multiple freeze-thaw cycles, which should be avoided, and urine samples should be stored at -80°C as soon as possible[24,25].

Measurement of serum and urine hepcidin by the same technique, and under the same circumstances, enabled comparison of serum with urinary values. Although their association was strong, confirming the previously reported correlation between hepcidin mRNA expression in liver cells and urinary hepcidin excretion[26], differences in serum en urine composition prohibit absolute comparison of analytical characteristics such as binding competition on the array surface and flight behavior during SELDI-TOF MS analysis[27]. Aspects such as ionization efficiency or ion suppression also can play a role and should be investigated in future studies. Meanwhile, difference in specimen behavior precludes calculations on the kinetics of hepcidin release by hepatocytes and its excretion from the blood into the urine. Construction of a calibration curve by addition of the same amount hepcidin-25 to dilution-serum or -urine confirms this effect, leading to speculation that a binding protein in serum prevents the binding of free hepcidin on the chip surface at low concentrations[27]. To circumvent differences in binding characteristics or possible matrix interferences, methodological approaches such as plasma fractionation[27] or the use of magnetic reverse-phase beads[28] have to be investigated for utility on hepcidin measurements.

Our reported SELDI-TOF MS method detected all 3 isoforms of hepcidin, improved urinary hepcidin analysis, and enabled measurement of serum hepcidin by use of IMAC30 array. We show for the first time a high correlation of concentrations measured in corresponding serum and urine samples. Selection of the optimal body fluid for analysis, however, is influenced by preanalytical, analytical, and biological

variations that effect serum and urine differently. Urine is more vulnerable to multiple freeze-thaw cycles and storage temperatures other than -80°C, but less influenced by diurnal variation. Serum is more susceptible to circadian variation, and therefore standardization of sampling time is needed in clinical studies with serum. Serum is likely to be more sensitive than urine, however, for monitoring short-term kinetics of body hepcidin concentrations. Therefore, the specimen of first choice depends on study design and the practical aspects such as availability of materials and equipment. The ability to measure hepcidin in both serum and urine confirms that the learning process on hepcidin characteristics, kinetics, and clinical utility has only just begun.

Acknowledgments

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Chapter 4

Regulation of Hepcidin: Insights from Biochemical Analyses on Human Serum Samples

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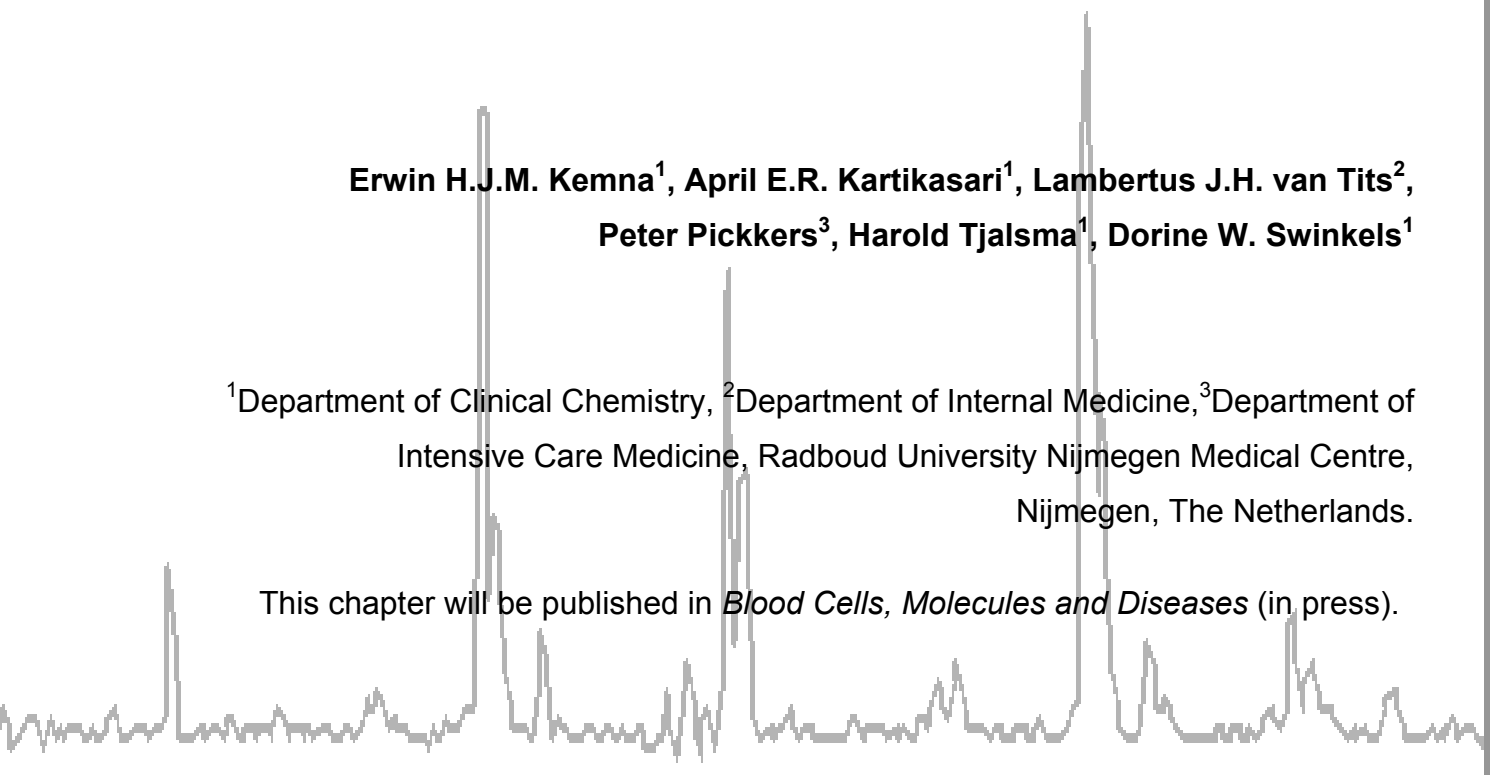


Abstract

Knowledge of hepcidin regulation is foremost gained by *in vitro* studies. We aimed to translate this knowledge into the human *in vivo* situation.
We measured serum markers as transferrin saturation (TS), soluble transferrin receptor (sTfR), and C-reactive protein (CRP) in parallel with hepcidin and prohepcidin in patients with iron metabolism disorders and controls. To assess sTfR as erythropoietic activity-associated factor in hepcidin regulation, we studied its influence on hepcidin expression in HepG2 cells.
sTfR highly associated with erythropoietic activity that strongly interfered with the iron store regulation of hepcidin. HepG2 expression results display an inverse association between hepcidin and sTfR. Inflammation was strongly related to increased hepcidin levels regardless of the iron store and erythropoietic activity status. In contrast, prohepcidin failed to correlate to any other parameter.
These studies verify that previous conclusions based on *in vitro* studies on hepcidin regulation are also likely to apply to human patients. This is underscored by a simple algorithm, based on parameters reflecting the main regulating pathways, that accurately predict the actual measured hepcidin levels. Future studies are needed to validate the combined utility of this predictive algorithm together with actual measured hepcidin levels in clinical diagnosis.

Introduction

The maintenance of body iron homeostasis requires mechanisms for the control of iron uptake and mobilization from stores, in order to meet erythropoietic needs, and for scavenging previously used iron. Therefore, the communication between cells that consume iron and cells that acquire and store iron must be tightly regulated[1]. Recent studies indicate that it is the liver produced peptide hormone hepcidin that plays this important role in the regulation of systemic iron homeostasis[2,3] by directing the intestinal absorption and macrophage release of iron via the sole iron exporter ferroportin[4,5].
Recent work has identified three putative upstream pathways (i.e., iron store related, erythropoietic activity, and inflammation related) as well as a mandatory signaling pathway, which are in a way presumed to be interconnected. These pathways are all found to interact with liver cells to control the production of sufficient hepcidin for a proper maintenance of iron homeostasis[6-11]. Figure 1A (page 127) depicts a simplified model of hepcidin regulation that builds upon these recently acquired insights.
Most of the evidence for this upstream regulatory model is obtained by molecular *in vitro* work and mice models applying recombinant techniques. Due to the

experimental set-up, such knowledge can not be directly transferred to the complex situation of aberrant iron homeostasis in human patients. In the present study, we aim to delineate these regulatory processes of hepcidin in humans with well-defined distortions of iron metabolism. This was done by the measurement of the serum iron indices, sTfR, and CRP, in healthy controls and in patients with documented iron deficiency anemia, acute inflammation, and thalassemia major. In separate *in vitro* experiments, we assessed the role of sTfR as an erythropoietic activity-associated factor in relation to hepcidin expression in the human hepatocytes cell line HepG2. As outcome parameters of the aforementioned regulatory processes we assessed serum hepcidin and prohepcidin by our recently developed mass spectrometry (MS) assay for serum[12], and a commercially available prohepcidin enzyme-linked immunosorbent assay (ELISA) kit, respectively. Additionally, as a proof of principal, we developed an algorithm based on measured TS, sTfR and CRP levels by which hepcidin production can be estimated (Figure 1B, page 127) as a possible tool in clinical diagnosis along with actual measured hepcidin levels.

Materials and Methods

Study participants

Iron Deficiency Anemia patients (n = 6; Hemoglobin male ≤ 8.3 mmol/L, female ≤ 7.3 mmol/L; MCV ≤ 80 fl; ferritin ≤ 10 µg/L), were recruited during outpatient clinic visits at the Radboud University Nijmegen Medical Centre. Thalassemia Major patients (n = 5) treated with iron chelation therapy and multiple blood transfusions were recruited in Ospedale Sant 'Eugenio, Rome, Italy. Samples obtained during the systemic inflammation response following experimental endotoxemia (n = 16) were collected from a human endotoxemia project[13] after a single LPS injection on each of five consecutive days in healthy volunteers to mimic sub-chronic inflammation. Samples used were collected at day 5; 6 hours after LPS injection. Healthy volunteers were taken as controls (n = 20). Approval was obtained from the Radboud University Nijmegen Medical Centre institutional review board and written informed consent was obtained according to the Declaration of Helsinki. Samples were collected randomly between December 2005 and June 2006, all throughout the day, with exception of the endotoxemia samples that were sampled according to a tight time schedule. Thalassemia samples were collected at least 24 hours after the last chelation therapy administration.

Laboratory measurements

Total serum iron, latent iron binding capacity, and CRP levels were measured by Aeroset (Abbott Laboratories, Abbott Park, IL); serum ferritin was measured by Immulite 2000 and 2500 (Diagnostic Products Corporation, Los Angeles, CA).
Routine hematology parameters were determined using flow-cytometry (Sysmex XE-2100, Goffin Meyvis, Etten-Leur, The Netherlands).

sTfR was immunonephelometrically quantified with the use of polystyrene particles coated with monoclonal antibody specific to human sTfR on a BN II System (Dade Behring Marburg GmbH, Marburg, Germany).
Serum non-transferrin bound iron (NTBI) levels were analyzed by a method based on iron mobilization and detection with iron chelators[14]. The assay uses oxalate as mobilizing agent, Gallium (III) as blocker of vacant transferrin sites, and Fl-aTf to provide the fluorescence signal detectable with a fluorescence plate reader[15].
Serum pro-hepcidin concentration was measured by an enzyme-linked immunoassay (Lot 12K096-2; DRG Diagnostics, Marburg, Germany).
Serum hepcidin-25 measurements by surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) MS were performed as previously reported[12] with the use of IMAC30 ProteinChip arrays (Ciphergen Biosystems, Fremont, CA). Mass-to-charge (m/z) spectra were generated using a Ciphergen Protein Biology System IIc TOF mass spectrometer. Peak annotation was performed with Ciphergen ProteinChip Software (version 3.2.0). Concentrations of serum hepcidin-25 were expressed as mega intensity units per liter.

Hepcidin prediction by measured TS, sTfR, and CRP levels
Based on the known main regulators (Figure 1, page 127) we created an algorithm to predict relative hepcidin levels in relation to the levels found in controls. By using sTfR (mg/L) and TS (%) as biochemical erythropoietic activity and the iron store, respectively, and CRP (mg/L) as indicator of inflammation, the following algorithm was defined:

Equation 1

$$(TS\text{-}sTfR)+CRP=Hepcidin$$

It contains the interconnection of the iron store and the erythropoietic activity with the latter as suppressor of hepcidin induction, and inflammation as a more independent regulator on top of the other two.
The outcome of the algorithm is expressed as a relative value. Therefore the measured values of each parameter are transformed by use of equations 2,3 and 4 before importation in the algorithm (equation 1):

Equation 2

$$TS=\frac{(TS_i - TS_{ME_{Control}})}{TS_{ME_{Control}}}$$

Equation 3

$$sTfR=\frac{(sTfR_i - sTfR_{ME_{Control}})}{sTfR_{ME_{Control}}}$$

Equation 4

$$CRP=\frac{(CRP_i - CRP_{ME_{Control}})}{CRP_{ME_{Control}}}$$

i: individual value; $ME_{Control}$: median value of the control group

The algorithm does not correct for the differences in the relative contribution of the respective parameters to the hepcidin levels and therefore only provides a rough estimation.

HepG2 Cell culture

The human hepatocyte cell line, HepG2 (American Type Culture Collection, Manassas, VA) was cultured in a humidified 37°C incubator with 5% CO_2 using PC-1 serum-free medium (Cambrex, Walkersville, MD). Because HepG2 are slowly dividing cells, to mimic *in vivo* conditions optimally, confluent hepatocytes were treated with 10% serum of 15 different subjects (5 controls, 5 patients with iron deficiency, and 5 patients with thalassemia major, respectively), for 78 hours, prior to the analysis of hepcidin expression levels.

RNA isolation and real-time quantitative polymerase chain reaction

Isolation of total RNA from the serum-treated HepG2 cells and subsequent cDNA synthesis were performed as described previously[16]. Real-time polymerase chain reactions (PCR) of human hepcidin transcripts and, for normalization of expression, the housekeeping gene hypoxanthine phosphoribosyltransferase (HPRT)[16], were performed in a 25 µl total reaction using TaqMan Universal Master Mix (PE Applied Biosystems, Foster City, CA). After enzyme activation for 10 minutes at 95°C, 40 two-step cycles were performed (30 seconds, 95°C; 1 minute, 60°C) in an ABI Prism 7700 (PE Applied Biosystems). All primers and probes were purchased from PE Applied Biosystems (Hepcidin, assay no. Hs00221783_m1; HPRT, assay no. 4310890E).

Statistical analysis

Analyses were performed with GraphPad Prism software (version 4.0) (GraphPad Software, Inc, San Diego, CA). Group differences were calculated by non-parametric 1-way analysis of variance (ANOVA) with Dunn´s post-hoc test. Group comparison was estimated by Pearson correlation test. P values < 0.05 were considered significant.

Results

sTfR, TS, CRP, hepcidin and prohepcidin levels in disorders of iron metabolism

sTfR, TS and CRP are markers of erythropoietic activity, iron store and inflammation, respectively, representing putative upstream pathways of hepcidin regulation. These variables were measured in the serum of controls, patients with iron deficiency anemia, thalassemia major, and volunteers exposed to 2 ng/kg *E. coli* endotoxin to induce acute systemic inflammation.

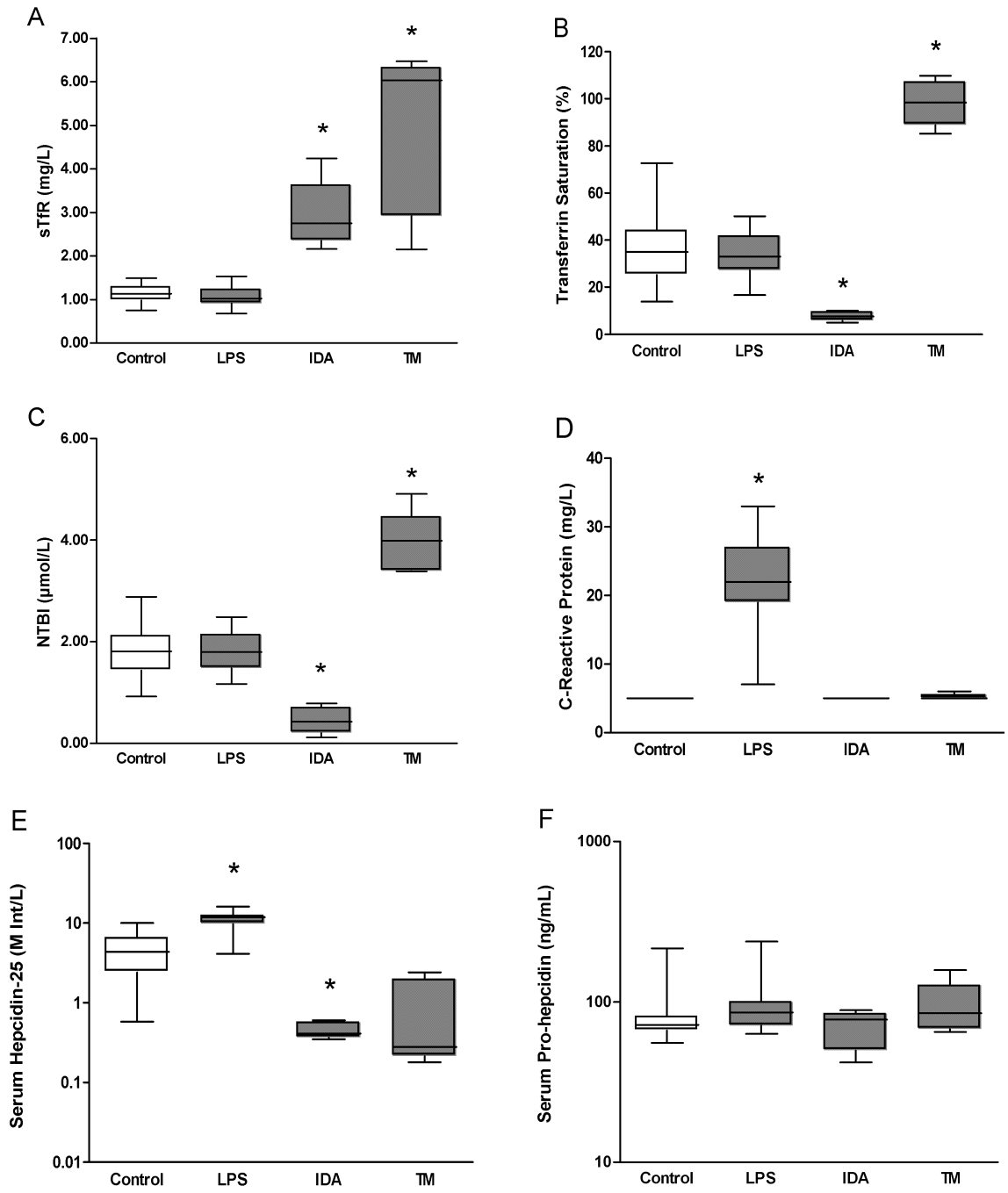


Figure 2. Laboratory results of biochemical parameters representing regulatory pathways of hepcidin. Box plots show 25 and 75 percentile with median, and error bars represent minimum and maximum values. * indicates significant difference from control group ($p < 0.05$; non-parametric 1-way ANOVA with Dunn's post-hoc test). LPS: healthy volunteers injected with LPS; IDA: iron deficiency patients; TM: Thalassemia Major patients treated with blood transfusions and iron chelators.

We found that sTfR concentrations were strongly increased in both iron deficient and thalassemia major patients (Figure 2A), while the endotoxin-treated group displayed no difference compared to controls. TS was found to be around 100% in thalassemia major and less than 10% in iron deficiency anemia, while the endotoxemia group showed average levels that did not differ from control values (Figure 2B). As shown in Figure 2C, for NTBI levels similar differences between the groups were observed as

for TS levels. Correlation analysis illustrates a strong association between TS and NTBI (R = 0.958; *P*<.001, data not shown).

CRP levels were only elevated in the endotoxin-treated group (Figure 2D), which showed the highest serum hepcidin values (Figure 2E) with a non-significant decrease in serum iron levels (data not shown). Hepcidin levels are highest during inflammation, patients with iron deficiency and thalassemia major show decreased hepcidin levels (Figure 2E). In contrast to hepcidin, serum prohepcidin levels displayed no significant difference between all the groups tested (Figure 2F), although the iron deficiency anemia patients showed a tendency to the overall lowest values, and the endotoxin-treated group the overall highest values. Furthermore, prohepcidin level did not correlate to serum hepcidin level or any other parameter tested in this study (results not shown).

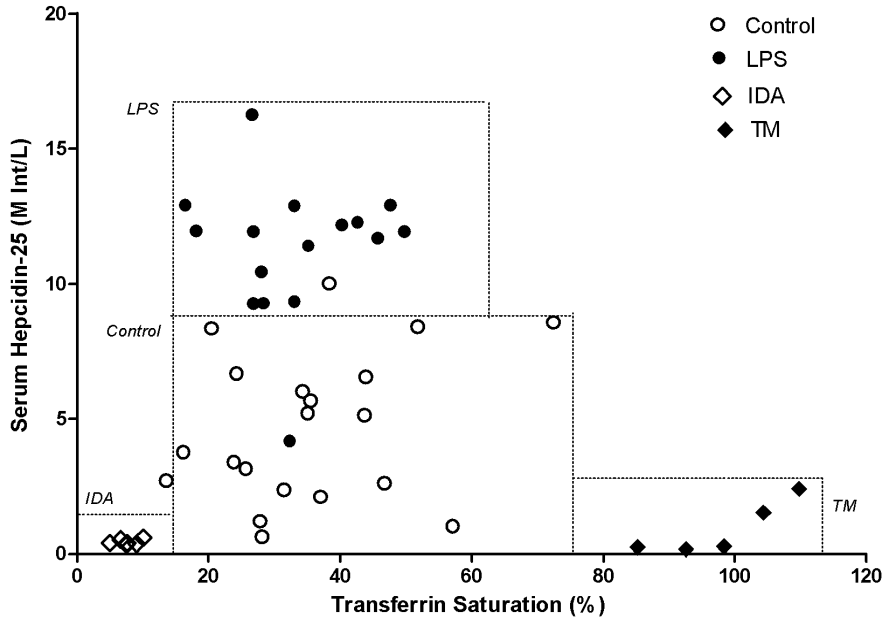
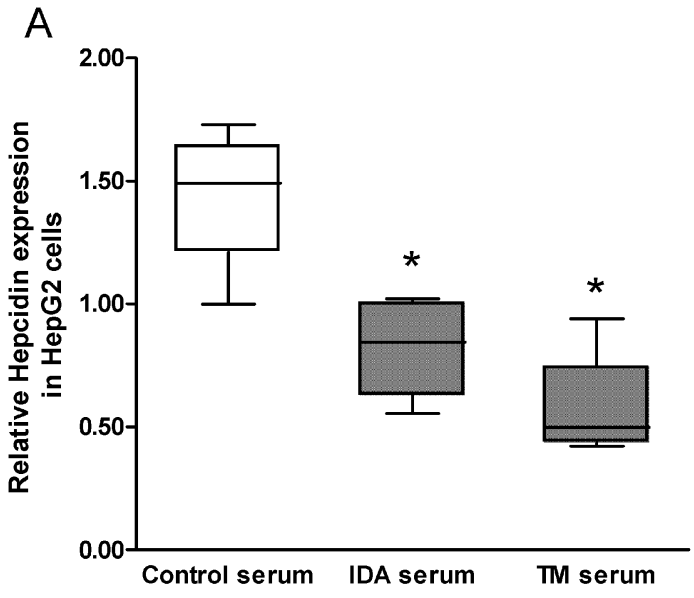


Figure 3. Serum Hepcidin-25 levels in relation to transferrin saturation levels in various iron metabolism disorders. Bracket lines give a rough indication of clusters of disorders of iron metabolism of similar etiology. Compared to the control population, TM patients show a small inter-individual variation of low hepcidin levels, even in the presence of fully saturated transferrin levels. IDA patients consistently show very low serum hepcidin levels that seem to be apparent for TS levels of 15% and below. The LPS group shows TS values within the reference range, but their hepcidin levels highly exceed those of the controls which suggests that inflammation acts as an additional regulating factor on top of the store regulation.

Serum hepcidin in relation to TS

To visualize possible interplay of the erythropoietic and inflammatory regulators with the store regulator, serum hepcidin concentrations were displayed in relation to TS values for every specific group measured (Figure 3). In the presence of physiological iron stores in the control group, serum hepcidin levels range from 0.58 to 9.95 M Intensity/L, whereas in case of deficient iron stores (IDA), hepcidin levels were consistently low. However, the relationship between TS and hepcidin became less

apparent for patients with thalassemia major and volunteers injected with endotoxin. Specifically in thalassemia major, the serum hepcidin levels appeared to be too low for the highly elevated TS levels. Furthermore, in these patients inter-individual differences in hepcidin levels are reduced in comparison to the control group. The endotoxin group displayed slightly lower serum iron levels with a similar variation in TS as the reference group but higher levels of serum hepcidin.



	Control (n=5)	IDA (n=5)	TM (n=5)
TS (%)	28 (14 - 44)	8 (5 – 10)	98 (85 – 110)
Ferritin (µg/L)	48 (22 - 109)	6 (6 – 10)	784 (272 - 1710)
CRP (mg/L)	< 5	< 5	< 5 (< 5 - 6)
sTfR (mg/L)	0.92 (0.82 – 1.28)	2.64 (2.16 – 4.24)	6.03 (2.15 – 6.47)
NTBI (µmol/L)	1.78 (1.24 – 2.14)	0.46 (0.12 – 0.79)	3.99 (3.39 – 4.91)

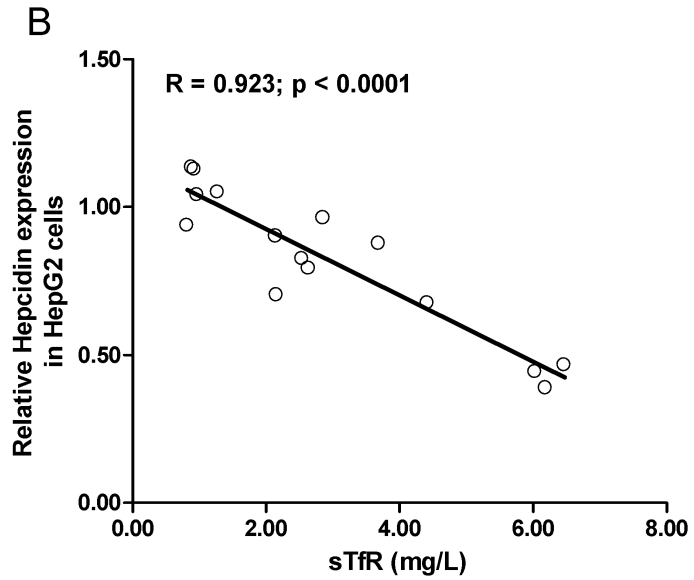


Figure 4. Hepcidin mRNA expression in HepG2 cells in relation to sTfR concentrations of human sera.
Panel A: Relative hepcidin expression measured in HepG2 cells after 72 hr incubation with 10% serum of 5 healthy controls, 5 iron-deficient patients (IDA), and 5 thalassemia major patients (TM). Laboratory characteristics of the used sera are displayed as median (range). Expression levels in IDA- and TM-treated cells is significant lower than in the control cells (* indicates $p < 0.05$). Panel B shows a strong association between the expressed hepcidin levels and the sTfR levels in the sera added.

sTfR as erythropoietic activity derived regulation factor

To assess the relation of sTfR as erythropoietic activity-associated factor with hepcidin production, we studied the influence of serum addition on hepcidin expression in the human hepatocytes cell line HepG2. Compared to controls, sera from IDA and TM patients containing elevated sTfR levels, significantly decreased hepcidin expression (Figure 4 A). The relative hepcidin expression values strongly correlated to the measured sTfR values (R = 0.923; *P* < .0001; Figure 4B).

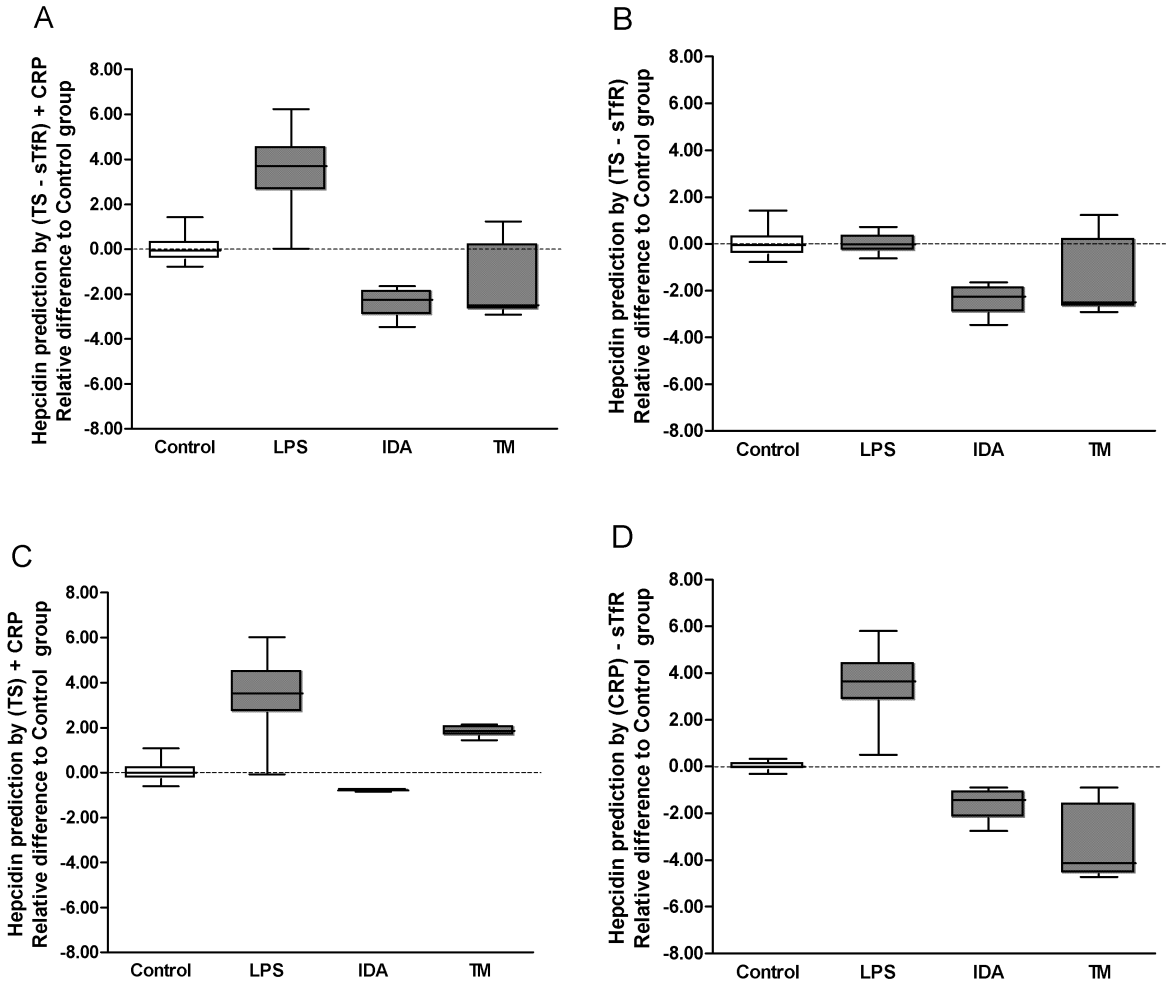


Figure 5. Hepcidin prediction by an algorithm based on measured TS, sTfR, and CRP levels. Levels display the relative difference to the control group. Box plots show 25 and 75 percentile with median, and error bars represent minimum and maximum values. Dotted line indicates relative level of control population. LPS: healthy volunteers injected with LPS; IDA: iron deficiency patients; TM: thalassemia major patients treated with blood transfusions and iron chelators. A) Predicted hepcidin levels calculated by an algorithm containing all regulators (TS – sTfR) + CRP. B) Predicted hepcidin levels calculated by reduced algorithm (TS – sTfR) simulating the effect of inflammation (CRP). C) Predicted hepcidin levels calculated by reduced algorithm (TS) + CRP simulating the effect of erythropoiesis (sTfR). D) Predicted hepcidin levels calculated by reduced algorithm (– sTfR) + CRP simulating the effect of the iron store (TS).

Hepcidin estimation by measured TS, sTfR, and CRP levels

As our data suggest that erythropoietic activity, iron store and inflammation are the major determinants for hepcidin expression in humans, we evaluated whether a simple algorithm based on measured TS, sTfR, and CRP levels could predict the measured

hepcidin levels. Figure 5A shows that the predicted hepcidin levels closely resemble the actual measured levels in the studied groups as depicted in Figure 2E. Linear regression analysis resulted in a strong significant correlation between the calculated and measured serum hepcidin levels (R = 0.756, *P* < .001; results not shown). By alternating omission of one of the parameters from the equation, we were able to assess the effect of each regulator on the out come level of hepcidin under different conditions (Figure 5B-D). When CRP was left out of the equation, this only influenced the hepcidin levels in the endotoxin-treated group (Figure 5B), whereas omission of sTfR considerably affected both the iron deficiency anemia and thalassemia major group. TS omission most of all changed the level of the thalassemia major group (Figure 5D).

Discussion

In vitro work and animal studies indicate that iron stores, erythropoietic activity and inflammation are involved in regulation of hepcidin production. Insights in the present study are based on a few small groups of patients with distinct iron disorders. Samples were randomly collected throughout the day and therefore might be influenced by diurnal rhythm. This is especially the case for parameters such as serum hepcidin thereby affecting its potential to differentiate between disorders of iron metabolism[12]. Nevertheless, we observed differences in serum hepcidin levels between various diseases indicating that the inter-patient variation exceeds this confounding aspect of the intra-individual circadian variation.

We defined an algorithm based on serum TS (iron store), sTfR (erythropoietic activity) and CRP levels (inflammation) to predict hepcidin levels and evaluated the algorithm in patients with different iron metabolism disorders. Based on today's knowledge that the Hemojuvelin (HJV)-related pathway is more mandatory than regulatory and the inaccessibility of a marker of this pathway such as soluble HJV[17] resulted in an algorithm that only comprises the three main regulatory pathways. The choice to select TS instead of ferritin as reflecting parameter of iron store is based on its proposed direct interaction with TfR1 and TfR2 as part of the iron sensor complex, and the strong correlation between levels of hepcidin predicted by an algorithm containing TS values and that in which TS is replaced by log ferritin values (R = 0.978; *P*< .001; results not shown). Although the selected parameters all have their own shortcomings as measures of the putative regulatory pathways of hepcidin, together with the absence of weight ascribed to any of the regulators in this preliminary phase, the algorithm based on these widely available and routine used laboratory indices predicts the serum hepcidin values with a high accuracy. This suggests that although hepcidin is regulated by a complex network, these three regulatory "highways" set the level of hepcidin production and that under different conditions fine tuning might occur by many "side roads" and "interconnections". Larger studies should validate the

robustness and suitability in clinical differentiation of this algorithm in combination with actually measured hepcidin levels.

The effect of erythropoietic regulation of hepcidin is shown to be especially strong for iron deficient and thalassemia patients[18,19]. Hepcidin levels in these patients are substantially decreased in the presence of clearly elevated levels of serum sTfR[20]. The relative wide range in sTfR concentrations in the thalassemia patients (Figure 2A) suggest individual differences in erythropoietic drive. Combined with differences in iron burden, this might explain the variation in serum hepcidin levels[21] as displayed in Figure 2E. Our algorithm corroborated these findings. Also our *in vitro* results expand recent reports indicating sTfR as possible candidate for the erythropoiesis related regulator[9,22]. Although the results are no proof of a causal relation because of the presence of other serum components, they show a strong association between the down-regulated hepcidin expression in hepatocytes cell line and increased sTfR levels from patient serum added to the cell medium. The contradiction between these results and recently published absence of an effects of sTfR on iron absorption and hepcidin expression in mice[23] and the presence of other potential candidates[24] show that more research is needed in the quest for the communicator between the iron consuming bone marrow and hepcidin producing liver cells.

Elevated iron stores, marked by increased iron saturation of transferrin is predicted to induce hepcidin production in order to decrease iron absorption and demonstrated as such in healthy volunteers treated with oral iron[25]. In the present study, the elevated TS values in thalassemia major patients were not associated with increased hepcidin levels. The anemia driven erythropoietic regulation clearly overruled the iron store regulation by decreasing hepcidin production[8,22,26]. Results from the algorithm indeed display a strong counter activity of iron store and erythropoietic regulation in these patients.

Inflammation-induced hepcidin production appears to occur by a rather dominant pathway as is illustrated by the high hepcidin levels measured in the endotoxin-treated group in the presence of slightly decreased serum iron levels. To the best of our knowledge human *in vivo* studies on the interaction of the inflammatory hepcidin regulatory pathways with that of the mandatory HJV/SMAD, iron store and erythropoietic networks have not been done. *In vitro* investigations, however, on the role of the HJV/SMAD pathway in case of inflammation showed that human Hep3B cells pre-treated with HJV small interfering (si)RNA were capable of a 4-fold hepcidin induction in response to IL-6, equal to control cells[27]. Next to this, *HJV*$^{-/-}$ mice were able to induce hepcidin after LPS, IL-6, or TNFα injections, although the response was less compared to controls[28]. Overall, the results of several mice studies point in the direction that inflammation also acts independently from the iron store and erythroid pathways[29-31]. We observed that in two iron deficient human volunteers (values: serum iron 7-7 µmol/L; ferritin 7-8 µg/L; TS 10.5-10.8%), the extent of endotoxemia-mediated hepcidin induction from low baseline values was similar to the relative induction in subjects that were not iron deficient (unpublished data). Taken together, our human *in*

vivo data are in agreement with previous reported molecular *in vitro* work and mice studies and indicate that inflammation increases hepcidin production in such a way that it can be added on top of the level set by the other regulators.
Our current as well as previously reported data[15] show a strong correlation between TS and NTBI which indicates that if NTBI regulation of hepcidin is involved, its influence is closely related to the iron store regulator. Further research must reveal the differential roles of NTBI and TS as upstream store regulators of hepcidin.
In conclusion, in the present study we show that with the use of biochemical serum analyses we were able to i) give insight in the interrelation of inflammation, erythropoiesis, and iron store regulating processes of hepcidin; ii) verify that previous conclusions based on mouse models of aberrant iron homeostasis are likely to apply to human patients; iii) indicate sTfR as a potential candidate for regulation of hepcidin production, and iv) create an algorithm that is suitable to estimate the contribution of the main regulators to the serum hepcidin levels during various conditions in humans *in vivo*. Future studies have to demonstrate the clinical utility of this predictive algorithm along with the actually measured hepcidin levels in a clinical setting.

Acknowledgments

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Chapter 5

Time-course Analysis of Hepcidin, Serum Iron, and Plasma Cytokine Levels in Humans Injected with LPS

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Abstract

Hepatic peptide hormone hepcidin is the key regulator of iron metabolism and the mediator of anemia of inflammation. Previous studies indicated that interleukin-6 (IL-6) mediates hepcidin increase and consequent hypoferremia during inflammation. Here we used an in vivo human endotoxemia model to analyze the effects of lipopolysaccharide (LPS) as a more upstream inflammation activator. The temporal associations between plasma cytokines, hepcidin levels, and serum iron parameters were studied in 10 healthy individuals after LPS injection. IL-6 was dramatically induced within 3 hours after injection and urinary hepcidin peaked within 6 hours, followed by a significant decrease in serum iron. Serum prohepcidin showed no significant change within a 22-hour time frame.

These in vivo human results confirm the importance of the IL-6-hepcidin axis in development of hypoferremia in inflammation and highlight the rapid responsiveness of this iron regulatory system.

Introduction

Anemia of chronic disease occurs in patients with acute and chronic immune activation and represents an important clinical problem. It is a condition that has also been termed “anemia of inflammation” and that is thought to be mediated by hepcidin[1], a small, cysteine-rich cationic peptide, produced by hepatocytes[2-4]. Furthermore, hepcidin is proposed to be the key regulator of iron metabolism. Hepcidin overexpression in patients with hepatic adenomas[5] or in transgenic mice[6] resulted in severe iron-refractory microcytic anemia. Conversely, hepcidin deficiency in humans[7] or mice[8] has been associated with severe iron overload. Hepatic hepcidin expression is suppressed by hypoxia and anemia[9], and induced by iron stores, and inflammation[4].

The induction of hypoferremia by inflammation is commonly seen in many infectious diseases. However, the mechanism remained unknown until the involvement of hepcidin was demonstrated in mice injected with bacterial lipopolysaccharide (LPS) or turpentine oil[4,9]. Importantly, hepcidin-deficient mice did not develop hypoferremia after turpentine injection[9]. In humans, increased urinary hepcidin levels were detected in patients with chronic infections or severe inflammatory diseases[10]. In human hepatocyte cultures, hepcidin expression was induced after direct exposure to LPS or medium from LPS-activated human monocytes, and this response could be ablated by the addition of anti-interleukine-6 (anti-IL-6) antibodies. Furthermore, IL-6 infusion in human volunteers rapidly induced hepcidin and hypoferremia[11], whereas IL-6 knock-out mice injected with turpentine failed to increase hepcidin and develop hypoferremia.

In the present study, we used an in vivo human endotoxemia model to study the temporal associations between different plasma cytokines, urinary hepcidin, and serum iron. We demonstrate the existence of a highly responsive LPS-IL-6-hepcidin axis linking innate immunity and iron metabolism.

Study design

Research subjects

After approval from the local ethics committee was received, 10 healthy individuals (4 man, 6 women; mean age 21; range 18-24 years) gave written informed consent to participate in this study. Individuals who were taking prescription drugs (except for oral contraceptives) or aspirin or other nonsteroid anti-inflammatory drugs were excluded. All research subjects were HIV- and hepatitis B-negative and had not had any febrile illness in the 2 weeks preceding the study. For 10 hours prior to the experiment, research subjects refrained from caffeine, alcohol and food. Approval for these studies was obtained from Radboud University Nijmegen Medical Centre's institutional review board.

Study protocol

Research subjects were intravenously injected with a bolus of 2 ng/kg body weight *Escherichia coli* O:113 LPS (United States Pharmacopeial Convention, Rockville, MD) between 8 and 9 AM. Blood and urine samples were taken just before LPS injection and serially thereafter at regular time intervals up to 22 hours. In the hour prior to the LPS administration research subjects were prehydrated with 1.5 L glucose (glc) 2.5% NaCl 0.45%. During the experiment, research subjects received 150 mL/h glc 2.5% NaCl 0.45%. Serum iron parameters, ferritin, C-reactive protein (CRP), prohepcidin, urinary creatinine, plasma cytokines, and routine hematology parameters were determined at the Radboud University Nijmegen Medical Centre, The Netherlands. Urines were preserved with 0.05% sodium azide, and shipped frozen to UCLA, Los Angeles, CA., for urinary hepcidin measurement.

Laboratory measurements

Total serum iron and latent iron binding capacity (LIBC) were measured using ascorbate/FerroZine colorimetric method, and urine creatinine was measured by colorimetric detection with picric acid (Roche Diagnostics, Mannheim, Germany). CRP was measured using immunologic agglutination detection with latex-coupled polyclonal anti-CRP antibodies (Abbott Laboratories, Abbott Park, Illinois), all measured by Aeroset (Abbott Laboratories, Abbott Park, Illinois). The serum ferritin was measured by a solid-phase, 2-site chemiluminescent immunometric assay (Immulite 2000, Diagnostic Products Corporation, Los Angeles, CA).

Routine hematology parameters were determined using flow cytometry (Sysmex XE-2100, Goffin Meyvis, Etten-Leur, The Netherlands).

Tumor necrosis factor-α (TNF-α), IL-6, IL-1β, IL-12, IL-10 and interferon-γ (IFN-γ) were measured in one batch, using a multiplex Luminex Assay[12] (Luminex, Austin, TX).

Serum prohepcidin concentration was measured by enzyme-linked immunoassay using a commercially available kit (DRG Diagnostics, Marburg, Germany).

Urinary hepcidin assay was performed as previously described[11]. Cationic peptides were extracted from urine using CM-Macroprep (BioRad Laboratories Inc., Hercules, CA). Hepcidin concentrations were determined by an immunodot assay. Urine extracts equivalent to 0.1 to 0.5 mg of creatinine were dotted on Immobilon-P membrane (Millipore, Bedford, MA) along with a range of synthetic hepcidin standards (0 to 80 ng). Hepcidin was detected using rabbit anti-human hepcidin[10] antibody with goat anti-rabbit horseradish peroxidase (HRP) as a secondary antibody. Dot blots were developed by the chemiluminescent detection method (SuperSignal West Pico Chemiluminescent Substrate, Pierce Chemical, Rockford, IL) and quantified with the Chemidoc cooled camera running Quantity One software (Bio-Rad Laboratories). Hepcidin quantity in each sample was normalized using urinary creatinine, and urinary hepcidin levels were expressed as nanograms of hepcidin per millimole of creatinine.

Statistical analysis

Statistical analyses were performed with GraphPad Prism software (version 4.0). Differences were tested for statistical significance by 1-way repeated measurements analysis of variance (ANOVA) or 1-way ANOVA.

Results and discussion

Injection of 2 ng/kg LPS induced a hypoferremic effect (Figure 1A) already detectable 6 hours after injection and reaching a 57% fall in serum iron after 22 hours. Importantly, the changes in urinary hepcidin preceded serum iron decrease (Figure 1B). Maximal hepcidin excretion was detected at 6 hours after injection, after which the levels started declining but were still higher than preinjection levels at 12 to 22 hours. This time course of hepcidin induction and serum iron decrease was similar to the one observed in volunteers infused with IL-6[11]. Hepcidin was recently shown to regulate cellular iron efflux in vitro by binding to the iron efflux channel ferroportin and inducing its internalization and degradation[13], and this mechanism could explain the rapid development of hypoferremia observed in research subjects who had been injected with LPS.

Daily, macrophages export around 20 mg of iron through ferroportin, and the iron is taken up largely by the developing erythrocytes in the bone marrow. However, the

plasma transferrin compartment contains only 2 to 4 mg of iron, which therefore must turn over every few hours. Accordingly, blocking macrophage iron efflux would be expected to decrease plasma iron concentration within hours.

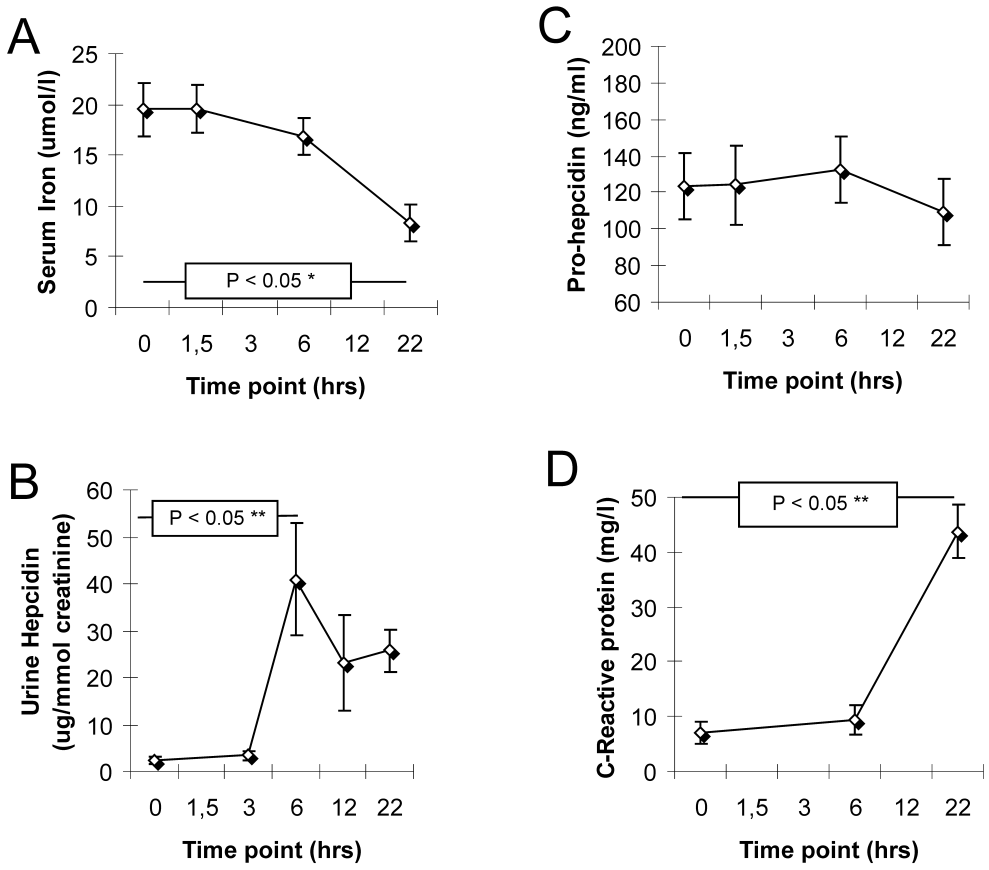

Figure 1. Laboratory measurements. Serum iron (A), urinary hepcidin (B), serum prohepcidin (C), and CRP (D) were measured in 10 healthy volunteers more than 22 hours after LPS injection. Each point represents the mean ± SEM. Significant differences are indicated (*1-way repeated measurements ANOVA; ** 1-way ANOVA).

Hepcidin is also known as a type II acute-phase protein[10] and, in chronic inflammatory conditions, increased hepcidin levels correlate with increased ferritin levels. We measured acute-phase reactants CRP and ferritin, in research subjects injected with LPS. CRP increased after LPS injection, but the time course lagged after hepcidin, with maximum levels detected 22 hours after injection (Figure 1D). Serum ferritin levels increased only slightly after 6 hours (results not shown) and stayed within normal reference intervals (10 to 150 µg/L) up to 22 hours, indicating that ferritin acute-phase response is probably delayed in comparison to hepcidin. The rapidity of the hepcidin response could be related to its proposed role as an inducer of hypoferremia that would restrict the flow of essential iron to infecting microbes, and slow their multiplication in tissues. This host response could be particularly valuable during the earliest phases of infection, before other components of the innate and adaptive immunity are fully mobilized[14].

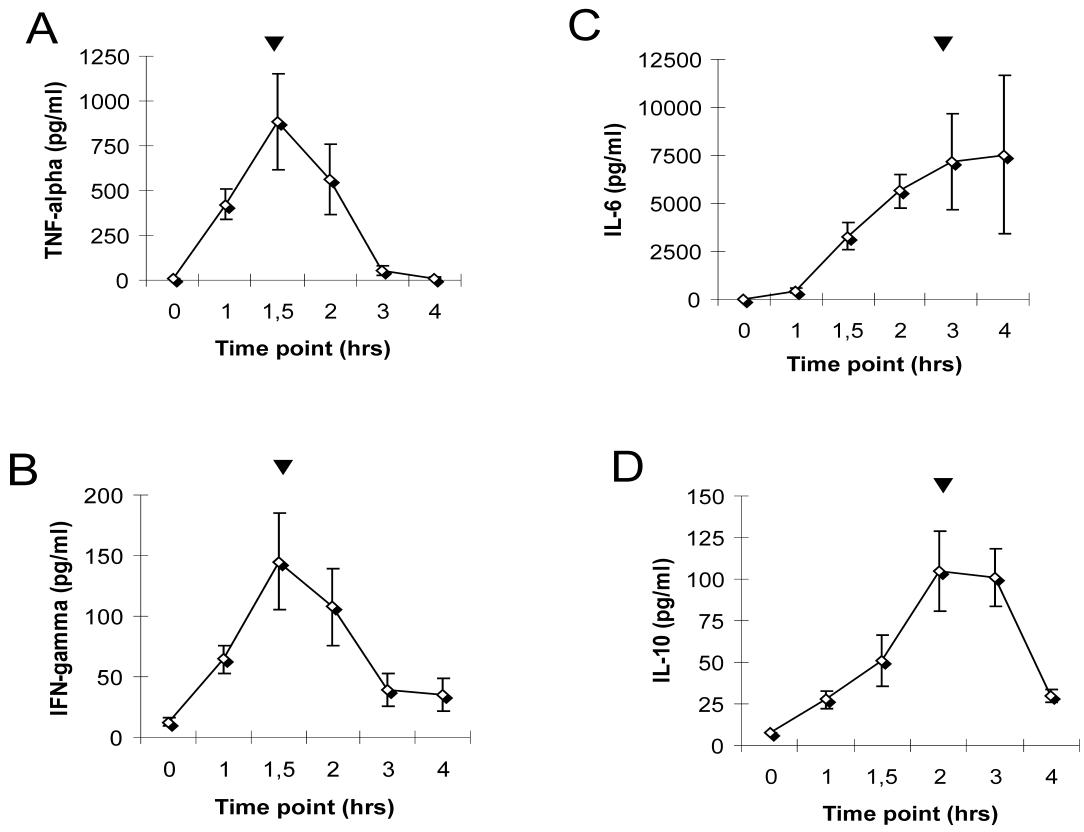

Figure 2. Plasma cytokine levels. Plasma levels of TNF-α (A), IFN-γ (B), IL-6 (C), and IL-10 (D) were measured in 10 healthy volunteers more than 4 hours after LPS injection. Each point represents the mean ± SEM. Peak values are indicated (▼).

LPS injection in human volunteers induced a cytokine response characteristic of inflammation[15]. After an early and transient induction of the proinflammatory cytokines TNF-α, and INF-γ, the acute-phase response was boosted by a dramatic increase in IL-6, which peaked at 3 to 4 hours after LPS injection (Figures 2 A-C). IL-1β expression, on the other hand, showed no significant changes within 4 hours, and IL-12 was undetectable in all research subjects (results not shown). Literature shows that anti-inflammatory cytokines, like IL-10, are able to counteract the proinflammatory IL-1β and IL-12 production[15]. In this study we observed a transient increase in IL-10 that peaked at 2 to 3 hours after injection (Figure 2D), which might explain the mentioned cytokine suppression. The time course of IL-6 increase in relationship to hepcidin induction and serum iron decrease coincides with that observed in research subjects injected directly with IL-6[11].

Serum prohepcidin levels showed no significant change within the 22-hour period (Figure 1C). Previous reports on serum prohepcidin measurements also showed lack of correlation with other iron parameters and only minor concentration differences between various patient populations with disturbed iron metabolism[16]. It remains to be determined if the lack of correlation between the urinary hepcidin and serum prohepcidin measurements is due to technical limitations of serum assays or if serum prohepcidin concentration does not reflect inflammation or iron metabolism changes.

In conclusion, our in vivo human endotoxemia model highlights the role of hepcidin at the interface between host defense and iron regulation, and further supports the importance of
IL-6-hepcidin axis in the development of hypoferremia and anemia of inflammation.

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Chapter 6

Nitric oxide does not contribute to inflammation-induced hypoferremia in humans.

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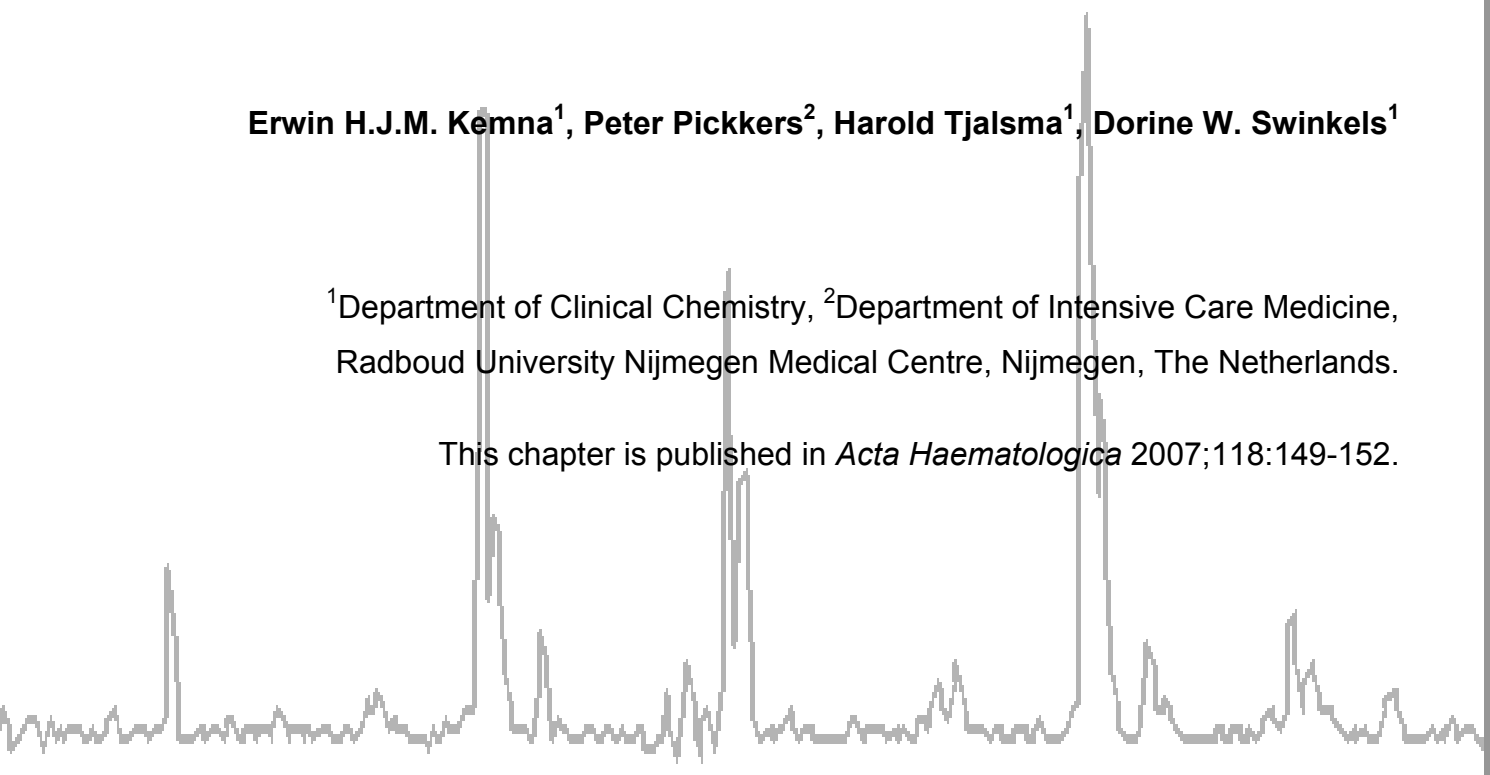

Abstract

Recent studies indicate an important role of hepcidin in the redistribution of iron between the intra- en extra-cellular body compartments during inflammation. *In vitro* studies suggest that nitric oxide (NO) is also involved in cellular iron shifts.

In order to value the effect of NO on iron metabolism *in vivo*, we examined human volunteers injected with lipopolysaccharide in the absence (LPS group, n = 6) and presence (LPS + AG group, n =6) of selective inducible NO synthase (iNOS) inhibitor aminoguanidine. We compared the course of iron parameters, cytokine profiles and urine hepcidin levels between both groups.

These studies showed that C-reactive protein, Tumor necrosis factor-α, and Interleukin (IL)-10, urinary hepcidin excretion levels and circulating iron levels showed no significant differences between the LPS and LPS + AG groups. These data indicate that, compared to hepcidin, NO plays no role of significance in iron homeostasis during systemic inflammation.

Introduction

A highly prevalent clinical problem that occurs in patients with acute and chronic immune activation is the development of a condition termed ‘anemia of inflammation’. As an important host defense strategy, mammals use the high vulnerability of infecting microbes for iron starvation by iron sequestration into iron-binding proteins and locations less accessible[1]. A currently accepted model in which hepcidin takes a central place as inducer of hypoferremia during inflammation[2] indicates that cytokines like IL-6 and IL-1β play a role as upstream activators of hepcidin production[3-6]. Hepcidin blocks the iron exporter ferroportin in macrophages and enterocytes[7], leading to hypoferremia, and eventually causes anemia as a negative clinical side effect.

Besides hepcidin, Nitric Oxide (NO) as a product of inducible NO synthase (iNOS), also plays an important role in various pathophysiological mechanisms during systemic inflammation and sepsis[8]. Until now, only *in vitro* experiments have shown that intracellular activation of the iron responsive element 1 (IRP1) by NO is demonstrated to promote an increase in cellular uptake from transferrin-bound serum iron[9,10]. To our knowledge, interactions between NO and hepcidin have not been reported.

Mediators such as lipopolysaccharide (LPS) and the proinflammatory cytokines IL-1β, IL-2, IL-6, Tumor necrosis factor-α (TNF-α), and Interferon-γ are able to induce iNOS[11], which is expressed in a variety of cells, including vascular endothelial cells, smooth muscle cells, different parenchymal cells and macrophages[12]. However, the role of NO in shifting iron from the extra- to the intracellular compartment during acute inflammation and therefore its contribution to the development of hypoferremia

under these conditions is controversial because of the discrepancy in results of different *in vitro* studies[13-16].
In order to value the contribution of NO to iron regulation *in vivo* during inflammation, we utilized an experimental endotoxemia model in human volunteers in whom LPS was injected followed by an infusion of the selective iNOS inhibitor aminoguanidine (AG)[17], of which we have previously demonstrated that it significantly inhibits NO production during endotoxemia[18]. Besides serum iron, inflammation parameters, together with plasma cytokine levels and urine hepcidin levels, were compared with results obtained during human endotoxemia without iNOS inhibition[5].

Study design

Subjects

After approval from the Radboud University Nijmegen Medical Centre institutional review board and the local ethics committee, healthy, nonsmoking subjects gave written informed consent according to the declaration of Helsinki to participate in this study. Those who were taking prescription drugs, aspirin or other nonsteroid anti-inflammatory drugs were excluded (except for oral contraceptives).
All subjects were HIV and Hepatitis B negative. They did not have a febrile illness in the 2 weeks preceding the study. Ten hours prior to the experiment, all subjects refrained from caffeine, alcohol and food and showed no significant difference in dietary intake of nitrate in the days before the experiment (data not shown). The subjects who received LPS in the absence of AG were previously described[5].

Study protocol

In the hour prior to the LPS administration, all subjects were prehydrated with 1.5 liters glucose 2.5% NaCl 0.45%, and during the experiment subjects received 150 mL/h glucose 2.5% NaCl 0.45%.
Between 8.00 and 9.00 AM, all test subjects were intravenously injected with a bolus of 2 ng/kg body weight LPS (*Escherichia coli* O:113; United States Pharmacopeia Convention, Rockville, Md., USA). In addition, the subjects in the LPS + AG group received an intravenous loading dose of 5 mM AG (Clinalfa, Switzerland) 1 hour after the administration of LPS, followed by a maintenance dose of 1.5 mM/h during 4 hours. We previously reported that with use of this protocol, a significant reduction in urinary NO metabolite production, with an additional reduction in iNOS mRNA expression in cells isolated from urine was achieved[18]. To monitor the onset and alterations of endotoxin-induced symptoms, subjects were asked to score the severity of nausea, headache, shivering, muscle pain and back pain every 30 minutes. Symptoms were scored on a scale ranging from 0 (symptom not present) to 5 (worst ever experienced). Scores were added up to form a “total symptom score”.

During the experiment, all subjects were hospitalized for 24 hours at the Intensive Care Research Unit. Blood samples were taken just before LPS injection and serially thereafter at regular time intervals up to 22 hours. Urine samples were collected between 0-3 hr, 3-6 hr and 6-22 hr after LPS injection. Samples were centrifuged immediately after collection, divided into aliquots to avoid multiple freeze-thaw cycles and stored at –80°C. Hepcidin measurements were performed within 2 months after collection.

Laboratory measurements

Routine chemistry parameters -- total serum iron, latent iron-binding capacity, urine creatinine and C-reactive protein (CRP) -- were measured by Aeroset (Abbott Laboratories). Cytokine levels were analyzed batch-wise using a multiplex Luminex Assay (Luminex, Austin, Tex., USA).

Urinary hepcidin measurements by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) on NP20 ProteinChip array (Ciphergen Biosystems, Fremont, Calif., USA) were performed as previously reported[19]. Peak annotation was established with Ciphergen ProteinChip Software (version 3.2.0).

Statistic analyses

Values are presented as means with standard error of the mean (SEM). Statistical analyses were performed with GraphPad Prism 4.0 software (GraphPad Software Inc., San Diego, Calif., USA). Differences between groups were tested for statistical significance by 2-way analysis of variance (ANOVA) with repeated measurements using SPSS 12.0.1 (SPSS Inc., Chicago, Ill., USA). $P < 0.05$ was considered significant. Because of the between-batch variability for all cytokine measurements and the analytical variability of the urinary hepcidin assay, for statistical analysis, relative values were used (expressed as percentage increase/decrease in relation to baseline values) but displayed as absolute values in Table 1.

Results and discussion

The role of NO in the development of hypoferremia remains controversial as, until now, only some *in vitro* experiments have suggested that activation of IRP1 by certain redox species of NO promote an increase in cellular uptake from transferrin-bound serum iron[10]. In order to evaluate the effect of NO on the iron metabolism *in vivo*, we compared iron parameters, cytokine profiles and urine hepcidin levels in human volunteers injected with LPS in the absence and presence of the selective iNOS inhibitor AG. Demographic characteristics are shown in Table 1.

Table 1. Demographic and laboratory characteristics of study groups.

	LPS (n = 6)		LPS+AG(n = 6)		LPS versus LPS + AG[b] (p value)
	Baseline	Peak value	Baseline	Peak value	
Sex, male:female	1:5		3:3		
Age, years					
Mean	20		22		
Range	18 - 24		20 - 25		
Vital Characteristics					
Body temperature, °C	36.6 ± 0.2	38.1 ± 0.3*	36.2 ± 0.1	37.7 ± 0.2*	0.879 (ns)
Total symptom score	0	4.3 ± 0.6*	0	5.3 ± 2.1*	0.855 (ns)
Inflammatory parameters					
TNFα, pg/mL[a]	≤ 8	860 ± 244*	≤ 8	349 ± 91*	0.099 (ns)
IL-6, pg/mL[a]	12 ± 4.0	6,449 ± 1,944*	≤ 8	1,283 ± 358*,+	0.033
IL-10, pg/mL[a]	≤ 8	89 ± 17.6*	≤ 8	142 ± 50*	0.609 (ns)
CRP, mg/L	8 ± 3.5	43 ± 6.8*	≤ 5	33 ± 3.6*	0.545 (ns)
WBC, × 10^9 /L	6.3 ± 0.5	14.5 ± 1.6*	4.8 ± 0.3	12.8 ± 1.1*	0.655 (ns)
Iron related parameters					
Urinary Hepcidin-25, M Int/mmol creatinine[a]	0.20 ± 0.09	6.16 ± 2.18*	0.84 ± 0.20	19.67 ± 5.65*	0.903 (ns)[c]
Serum iron, µmol/L	21 ± 3.4	6 ± 0.6[d,*]	17 ± 2.9	10 ± 1.8[d]	0.222 (ns)

Data are presented as means ± SEM (absolute values). WBC: white blood cell count; ns: not significant.
[a]Statistic analysis for group differences, and differences between distinct time points was performed on relative values (in relation to baseline values).
[b]Difference between LPS and LPS+AG group for the whole curve by 2-way ANOVA with repeated measures.
[c]Based on relative cumulative hepcidin-25 excretion per mmol urinary creatinine values during 22 hr time-course.
[d]Nadir value instead of peak value.
*P < 0.05 versus baseline value (paired sample t-test).
[+]P < 0.05 versus LPS group (independent t-test).

To our knowledge, direct influence of NO on cytokine induction is not reported and AG seems to be incapable of suppressing cytokine induction[20]. However, it should be noted that other pro- and anti-inflammatory cytokines like TNF-α and IL-10 (Table 1) and the acute phase protein CRP (Figure 1A) showed equal responses in both groups. In addition, increase in body temperature, total symptom score, and white blood cell count during the experiments were also comparable (Figure 1; Table 1). In contrast, the IL-6 response was weakly reduced in the LPS + AG group (Table 1), although the reason for this is unclear. Taken together, these results show that AG does not influence the inflammatory response during endotoxemia.

Given the power of these experiments, no statistically significant levels were reached on serum iron and hepcidin excretion by the use of NO inhibitor AG (Figure 1B, C). These results suggest that the absence of a difference in hepcidin excretion between groups results in a similar reduction in iron levels. Taken together, inhibition of iNOS and subsequent diminished NO levels appear to have no effect on hepcidin production and do not lead to a difference in circulating iron levels.

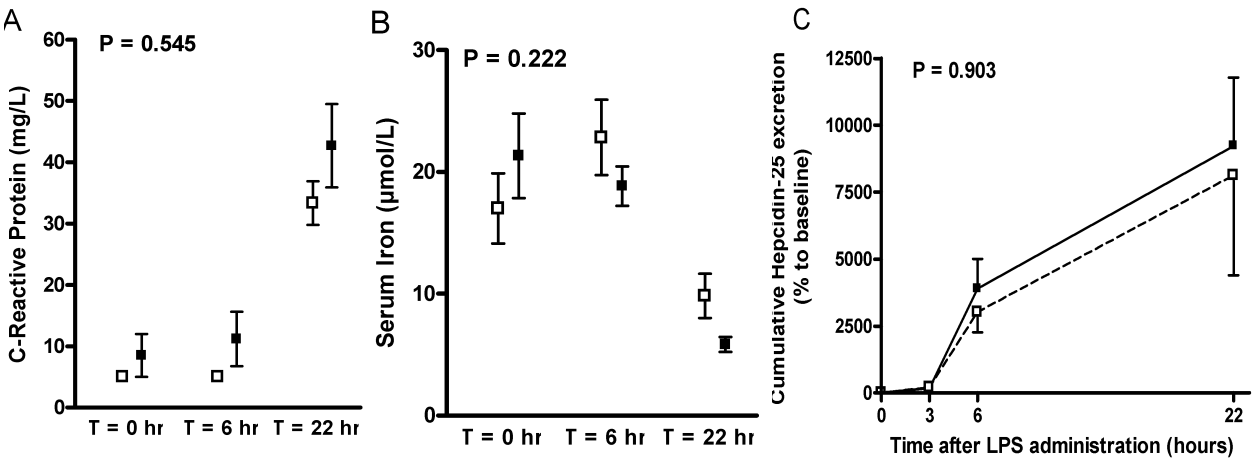

Figure 1. Laboratory results at different time points after injection of LPS with or without AG as an iNOS inhibitor. Baseline and peak values of CRP (A) and serum iron (B) are shown together with relative cumulative hepcidin-25 excretion in urine (C). The values of humans injected with LPS are compared in the absence ■, and presence □ of AG. C: The values in the absence of AG are connected by a continuing line, whereas the values in the presence of AG are connected by a dotted line. For CRP and serum iron each point represents the mean ± SEM, whereas for hepcidin-25 excretion, values are expressed as cumulative relative mean to baseline ± SEM. Data were analyzed by 2-way ANOVA with repeated measures over the complete curve and significant differences between both groups are indicated as $P < 0.05$.

Interestingly, during acute inflammation, hypoferremia is likely to be foremost regulated by macrophages[9]. It is possible that the systemic inflammation induced by LPS infusion is too mild to induce iNOS in the macrophages. It was recently demonstrated that iNOS was not induced in peripheral blood mononuclear cells during human endotoxemia[21]. Also, in a previous study, Annane *et al.*[22] did not detect iNOS in cells or vessels of the systemic circulation in patients with septic shock caused by cellulitis, but only at the site of infection suggesting a compartmentalized induction of iNOS. These studies, together with the data of our present report, suggest that NO does not contribute to hypoferremia during systemic inflammation in humans.

In summary, our data show that iNOS-mediated NO production has no significant role in the induction of hypoferremia during acute and systemic inflammation *in vivo* in humans. This study confirms the profound role of hepcidin in inducing hypoferremia during acute inflammation.

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Chapter 7

Effect of the New HJV-L165X Mutation on Penetrance of HFE

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Abstract

After hemochromatosis was mapped to chromosome 6 by linkage to *HLA* in 1976, two Dutch families with non-*HLA*-linked forms of hemochromatosis were reported. Indeed, since the identification of the HFE gene at least four other genes were found to be involved in hemochromatosis. We now report on one of these families with hemochromatosis 'unlinked' to *HLA.*

Twenty family members were investigated for serum iron indices and urine hepcidin levels and for mutations in the *HFE* and hemojuvelin (*HJV)* genes. A new homozygous truncating mutation of the *HJV* gene was found to be responsible for one case of severe juvenile hemochromatosis. In this family, the characteristic C282Y variant in HFE was also common. The presence of the *HJV* mutation on one allele does not lead to elevated serum iron indices and decreased urinary hepcidin levels in heterozygous or homozygous HFE-related hemochromatosis. Therefore, it does not appear to be a modifier of HFE linked hemochromatosis. This finding is in accordance with recent findings on the position of HJV in the iron network, which is distinct from that of HFE.

Introduction

Iron overloading in hereditary hemochromatosis (HH), if left untreated, may lead to serious complications, such as skin pigmentation, arthropathy, diabetes mellitus, hypogonadism and other endocrinopathies, liver cirrhosis, liver cancer, and cardiomyopathy [1,2]. Treatment usually consists of frequent phlebotomies. In the 1970s HH was recognized as an autosomal recessive disorder linked to the short arm of chromosome 6 close to *HLA-A* [3]. Feder and co-workers identified the hemochromatosis (*HFE*)-gene (previously called *HLA-H* gene) in 1996. In the vast majority of cases the mutation is a single base change that results in the substitution of tyrosine for cysteine at position 282 of the HFE protein (C282Y) [4]. However, we now know hemochromatosis to be genetically heterogeneous. Next to HFE-related hemochromatosis (Online Mendelian Inheritance in Man (OMIM) type 1), two additional adult forms are now known to be due to mutations in the *transferrin receptor 2* (*TfR2*, OMIM type 3) and *ferroportin* (SLC40A1, OMIM type 4) genes, respectively [1,5]. Juvenile hemochromatosis (JH) is an autosomal recessive disease that affects young patients and leads to severe clinical complications. JH results from mutations of either the hemojuvelin (*HJV*) gene (OMIM type 2A) or the hepcidin antimicrobial peptide (*HAMP)* gene (OMIM type 2B) [1,6,7]. The *HAMP* gene encodes for hepcidin, a small peptide hormone, produced by the liver, and critical for the maintenance of body iron homeostasis through blocking ferroportin-mediated iron export in intestinal endothelial cells and macrophages [8]. Urinary hepcidin levels have been found to be low in HFE [9] and TfR2 [10]mutated adult HH patients, and to be

extremely low or even undetectable in HJV and HAMP mutated juvenile cases [9,11,12], respectively.

The majority of subjects homozygous for the HFE-C282Y-alteration present with abnormal iron parameters [13], whereas only a small number will manifest clinical features [1,5,14-17]. Thus, iron loading in HFE-C282Y homozygosity appears to be susceptible to the effect of environmental and genetic modifiers. Knowledge of modifiers may enable us to better predict those HFE-C282Y homozygous patients with an increased risk to develop severe iron overload and, consequently, clinical complications [18]. The influence of factors such as excessive alcohol intake [19,20], viral hepatitis [21] dietary iron [22], or body mass index in women [23] likely explains part of this phenotypic heterogeneity in HFE-C282Y homozygosity. Support for *HJV* as a genetic modifier comes from a small number of studies [24,25].

In 1977, two Dutch families have been reported to be at variance [26] with the hypothesis launched by Simon *et al* [3] and Bomford *et al* [27] suggesting that the HH gene is situated on the short arm of chromosome 6 near the HLA-A locus. One of these families, on which we report here, turned out to harbor both *HFE* and non-*HFE*-related forms of HH. In this extended family [28], we have now identified a new truncating mutation in the *HJV* gene.

These findings provide us with the opportunity to investigate whether inactivating mutations in *HJV* may have influenced the penetrance of *HFE*-C282Y homozygosity.

Patients, materials and methods

Study population and data assessment

The investigated family has already been described in 1975 [28], 1976 [29] and 1977 [30]. Findings on liver and serum iron in the early seventies are copied into Table 1. The index case in our study (III-54) was born in 1956 and diagnosed in 1972 with increased serum iron values and heavy iron accumulation in the hepatocytes [28]. Subsequently 16L blood could be withdrawn without a fall of serum iron was noted. There was no apparent consanguinity between his parents or grandparents [28]. Late 2005, he provided contact information on family members from three branches, in which the presence of clinical hemochromatosis was apparent. The institutional review board approved this study and informed consent was obtained from all participating individuals (N=20). Participants filled out a self-administered questionnaire on demographics, lifestyle factors, general medical history, and medical history for HH and family structure, including morbidity and mortality. Non-fasting blood and urine samples from all subjects were collected between 7 and 9 p.m. on the same day. Blood samples were analyzed for the *HFE* and *HJV* genotypes, current transferrin saturation (TS) percentages, and ferritin values. In urine, creatinine and hepcidin levels were determined. We additionally asked patients undergoing phlebotomies to estimate how many phlebotomies were carried out, since

in these individuals TS and ferritin values are uninformative as indicators for the severity of the disease.

Table 1. Descriptive data on iron parameters of the relatives, sorted by HJV- and HFE-genotype.

					Current				1970s	
Code[a]	Sex	Year of Birth	HFE genotype	HJV genotype	TS, %	Ferritin µg/l	Urinary hepcidin, M Int/mmol creat	Iron removed/Age[b], g/years	TS, %	DFO-test[c] Mmol
B III-54 [de]	M	1956	Ht	Ho	87	56	0.04 [i]	1.02	93	159 [j]
B III-7 [e]	F	1943	Ho	Ht	53	76	2.50	0.23	53	32.3
B III-12 [ef]	F	1951	Ho	Ht	71	36	0.60 [i]	0.53	89	32.9
B II-12 [e]	M	1924	Ho	Ht	60	96	0.83	0.37	86	107 [k]
B III-85 [e]	M	1950	Ho	Ht	21	30	0.10 [i]	0.36	83	36.9
B III-86 [g]	F	1953	Ho	Ht	58	147	1.56	0.12	85	45.0
B III-2	F	1937	Ht	Ht	30	304	2.98	n.a.	44	19.0
B III-5	F	1940	Ht	Ht	27	185	6.12	n.a.	38	28.0
B III-10	F	1947	Ht	Ht	29	107	2.91	n.a.	40	17.1
B III-48	M	1943	Ht	Ht	34	190	8.30	n.a.	43	19.1
B III-50	M	1945	Ht	Ht	23	30	0.04	n.a.	35	31.2
B III-52 [f]	F	1949	Ht	Ht	46	62	0.75	n.a.	46	17.6
B III-87	M	1956	Ht	Ht	23	351	6.32	n.a.	55	22.0
B III-3 [f]	F	1938	Ho	Wt	65	354	1.93	0.89	87	46.0
B III-88 [eh]	M	1959	Ho	Wt	39	53	0.31	0.30	57	21.1
B III-89 [eh]	M	1959	Ho	Wt	51	87	1.06 [i]	0.28	54	23.2
B III-1	F	1936	Ht	Wt	24	275	5.35	n.a.	41	16.8
B III-4	M	1939	Ht	Wt	18	310	7.77	n.a.	45	28.8
B III-11	F	1949	Ht	Wt	24	56	2.07	n.a.	38	29.5
B III-9 [f]	M	1946	Wt	Wt	58	821	5.13	n.a.	40	17.1
Reference values					<50		0.52-7.83 [l]	n.a.	<50	<30.3
Men						15-280				
Women<50 years						6-80				
Women>50years						15-190				

Ho,homozygous for the mutation; Ht,heterozygous for the mutation; Wt,wildtype for the mutation. N.a.,not applicable. CRP (<10 mg/l), Hb (M:8.1-10.7 mmol/l; W:7.3-9.7 mmol/l), MCV (M:84-103 fl; W:85-104 fl) and ALAT (<45 U/l) are within reference values for all individuals, except for male II-12 who's Hb was fairly high (11.5 mmol/l) and for male III-50 whose ALAT was slightly increased (48 U/l). [a] These are the codes as used before[2], roman numerals indicate the generation. [b] Grams of iron removed were calculated by multiplying the number of phlebotomies by 0.20 g (assuming 500 ml whole blood was withdrawn per phlebotomy). [c] Urinary iron concentration in 24 hrs after administration of 1000 mg Desferrioxamine intramuscular (DFO-test).[d] Proband; the proband in the studies in the early 70s was his sister (Ba, III-51[3]) who was born in 1947 and died in 1998 (1970s: TS: 78%; DFO-test: 376 µmol; Liver iron content: 64.2 mmol/100 gram dry weight). [e] Currently on maintenance therapy with phlebotomies. [f] Alcohol consumption>15 units per week. [g] Phlebotomized in the past.[h] III-88 and III-89 are monozygotic twins.[i] Date of last phlebotomy less than 4 weeks in the past or unknown.[j] Liver iron content: 32.4 mmol/100 gram dry weight.[k] Liver iron content: 17.1 mmol/100 gram dry weight.[l] Based on the range of values observed in 20 healthy individuals [9].

Iron parameters

The serum ferritin was measured by a solid phase, two-site chemiluminescent immunometric assay (Immulite 2000, Diagnostic Products Corporation, Cirrus). Using an Abbott Aeroset analyzer, we measured total serum iron and Unsaturated iron binding capacity (UIBC) by the ascorbate/FerroZine colorimetric method (Roche Diagnostics). Serum TS (%) was calculated as follows: [serum iron/(serum iron + UIBC)] x 100%. The patient estimated the number of phlebotomies and grams of iron removed were calculated by multiplying the number of phlebotomies by 0.20 g (assuming 500 ml whole blood was withdrawn per phlebotomy). For standardization purposes this number was divided by the age of the patient (iron removed/age)[31].

Since hepcidin levels could be a useful parameter for assessing the severity of HH [1], we measured urine hepcidin by a recently described surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) method with the use of IMAC30 ProteinChip arrays [9].

Genetic testing

HJV-genetic testing results were obtained from routinely used genetic tests. Amplimers were designed to incorporate all known coding sequences and exon-intron boundaries and 5'and 3' untranslated region of the *HJV* gene. For sequencing, we used the sets of primers reported by Lee *et al* [32]. However, we developed new primers for exon 3; therefore all primers used are reported in Table 2 (GenBank NT-004434 / gi 88943080).

PCRs were performed in 25-µl reaction volume containing 30 ng genomic DNA, 2.5 µL PCR II buffer, 2.5 µL $MgCl_2$ (25mM), 0.5 µL dNTPs (2.5 mM), 0.1 µL of each primer (50 µM) and 1 unit Amplitaq Gold (Applied Biosystems, The Netherlands). PCR reactions were performed in a GeneAmp Thermal Cycler 9700 (Applied Biosystems, The Netherlands) under the following conditions: 95ºC 10 minutes, 35 cycles of 95ºC 30 seconds, 60ºC 45 seconds, 72ºC 1 minute, and a final step of 72ºC 7 minutes. Before sequencing, PCR products were run on 2 % agarose gel and purified using ExoSAP-IT (Amersham Biosciences, Sweden). Products were sequenced using the Big Dye Terminator (version 3) cycle sequencing kit (Applied Biosystems, The Netherlands) and analyzed on an ABI 3730 automatic sequencer (Applied Biosystems, The Netherlands).

To investigate the novel *HJV* gene alteration, a restriction fragment length polymorphism (RFLP) analysis was developed using 2202F and 2603R primers and the restriction enzyme HpyCH4V® (New England Biolabs). In case of the mutation one of the restriction sites disappears. The presence of this newly discovered mutation in family members and in 53 randomly chosen controls was investigated.

Table 2. Primers used for amplification of the *HJV* gene

HJV	Primer sequence	Fragment size, bp
HJV Ex 1 F	GTACTCTGGCCAGCCATATACT	286
HJV Ex 1 242R	CGAGAGACATCCAAGTAGGTGT	
HJV Ex 2 1297F	ATCTCCCCAAATTCCAGTCTG	359
HJV Ex 2 1655R	ACATAGCAGCCTACCCTCTAG	
HJV Ex 3a 1935F	GCAAACTACACTCCGATAGAG	319
HJV Ex 3a 2253R	GCTGGATCATCAGGTCTTCG	
HJV Ex 3b 2202F	GACCTCGCCTTCCATTCG	402
HJV Ex 3b 2603R	GAATCTCATGAGGTGGATCGG	
HJV Ex 4a 2929F	TAGTCCTGCATCTCTACTTGG	394
HJV Ex 4a 3322R	TGCAGGTCCTGTTCAGCTG	
HJV Ex 4b 3136F	ATGGAGGTGACCGACCTGG	374
HJV Ex 4b 3509R	AGCTGCCACGGTAAAGTTGG	
HJV Ex 4c 3257F	GCTCTCCTTCTCCATCAAGG	430
HJV Ex 4c 3686R	AAACTAGTAATGGGACTGATGG	
HJV Ex 4d 3624F	TCTGGGCTCTTTGTTCTGTG	407
HJV Ex 4d 4030R	GTCTTCTGCTTTCAGCTCTTG	
HJV Ex 4e 3944F	ATAAGTTTAGAGGTCATGAAGG	404
HJV Ex 4e R	GCCCTCTTTCAGTGGAGTG	

Primers as reported by Lee et al. [32] were used, except for exon 3 primers.

Statistical analyses

We compared serum iron indices, the estimated quantity of iron removed (iron removed/age in g/year) by bloodletting and urine hepcidin values between groups of genotypes using Mann-Whitney U-tests.

Results

Hepcidin levels as a first screen for the presence of HH in the index case

We decided to evaluate hepcidin levels in a patient (index case: III-54) with unexplained severe early onset hemochromatosis. This is according to the strategy proposed previously in applying the urinary hepcidin analysis as a screening test for the presence of HH and its various OMIM types [1]. We found urinary hepcidin-25 levels of 0.04 Mega Intensities / mmol creatinine (M Int/mmol creat) in the index case (detectable but extremely low). This value is clearly below the range observed for HFE-mutated cases (0.08-4.50) and well below the reference range (0.52-7.83)[9]. This led us to sequence the *hemojuvelin (HJV)* and *HAMP* (hepcidin) genes.

Discovery of a novel HJV mutation

We observed a novel homozygous mutation in exon 3 of the *HJV* gene. The 494T>A transversion led to a premature stop codon at position 165 of the HJV protein: L165X. The position of this alteration is depicted in Figure 1. In addition, the index case is also a carrier of HFE-C282Y. Mutations in the *HAMP* gene were absent.

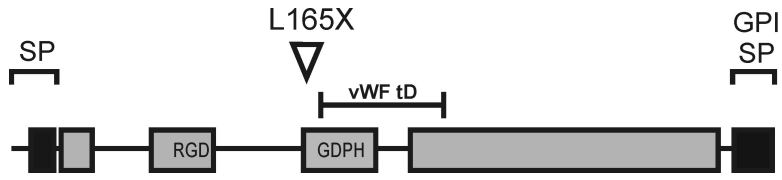

Figure 1. Position of L165X truncating mutation in HJV. Schematic representation of functional protein domains and the position of the L165X truncating mutation in HJV. Amino-terminal secretory signal peptide (SP) and carboxyl-terminal GPI signal peptide (GPI SP), which are removed during the cell surface sorting of HJV are indicated by black boxes. Grey boxes indicate HJV domains showing homology with repulsive guidance molecule domains. The position of an RGD motif, possibly involved in receptor-ligand interactions[33], and a partial von Willebrand factor type D (vWF tD) are shown. Although autocatalytical processing of the GDPH sequence (between Asp and Pro residues) has been documented, HJV remains one molecule after this cleavage by one or several disulfide bridges between both polypeptides[34]. Note that the truncated HJV-L165X product lacks the GPI anchor sequences for cell surface retention.

Family study

Since HH was common in this family, we decided to investigate relatives of our index case for mutations in the *HFE* gene and the novel *HJV* mutation. Past and current descriptives are shown in Table 1. In the family tree the mutational status of the HFE-C282Y and the HJV-L165X alteration are shown (Figure 2, page 126). Homozygosity for the HJV-L165X alteration was only present in the proband, while

heterozygosity was common among his relatives (allele frequency: 14/40=35.0%). The novel HJV-L165X alteration was absent among 53 population controls. Furthermore, the HFE-C282Y mutation was observed frequently in this family (allele frequency: 27/40=67.5%). Phlebotomies were reported only in individuals homozygous for either the HJV-L165X (N=1; proband) alteration or the HFE-C282Y (N=9) alteration and never in heterozygous persons (Table 1).

Hepcidin assay

Hepcidin-25 was measured in urine samples from investigated family members (Table 1). As a reference we used the range of hepcidin-25 values obtained from normal controls (range as measured in 20 persons: 0.52-7.83 [9]). The older brother from our index case also had very low hepcidin levels, which does not seem to be explained by his heterozygosity for both the HJV-L165X and the HFE-C282Y mutation (III-50, Figure 2, page 130). However, it may be associated with his relatively low ferritin levels that may reflect low iron stores, but that had no effect on hemoglobin levels yet. There are two other relatives with low hepcidin values (III-85 and III-88); both of whom are diagnosed with *HFE*-related HH. There are 6 family members who reported being diagnosed with HH with hepcidin levels in the normal range. Finally, one individual was found with a relatively high hepcidin level (III-48), without a clear explanation. In all other samples, urine hepcidin values were within the normal range. In HFE-C282Y homozygotes, we observed substantially lower urinary hepcidin levels compared to those in HFE-C282Y heterozygotes (*P*=.01) (Figure 3).

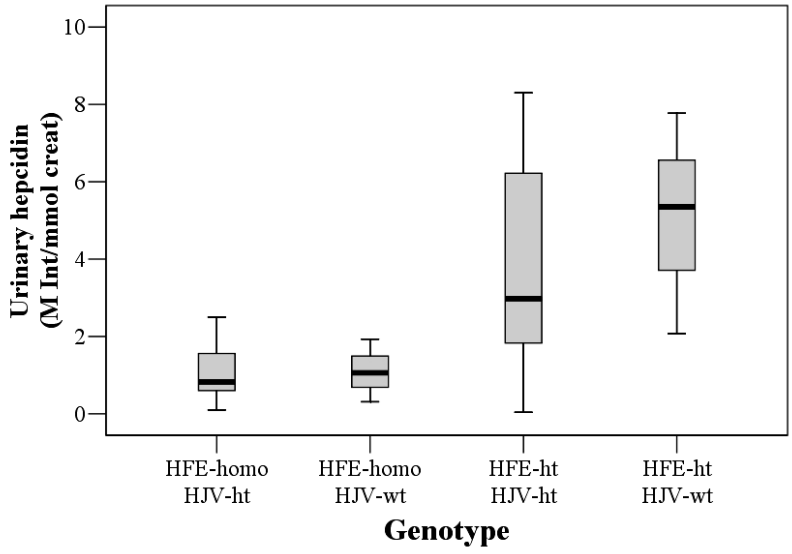

Figure 3. Boxplot showing urinary hepcidin-25 levels (minimum, p25, median, p75, and maximum) for different genotypes. HFE-homo: homozygous for the C282Y mutation in the *HFE* gene; HFE-hetero: heterozygous for the C282Y mutation in the *HFE* gene (compound heterozygotes are excluded); *HJV*-ht: heterozygous for the L165X mutation in the *HJV* gene; *HJV*-wt: wildtype for the L165X mutation in the *HJV* mutation.

HJV-L165X as a modifier of HFE-related HH

Iron indices, iron removed/age and urinary hepcidin levels were similar for HJV heterozygotes (L165X) and the HJV-wildtypes, also when stratified by *HFE* genotype (Figure 3). In this large family the presence of the HJV-L165X mutation does not aggravate the phenotypic appearance of *HFE*-related hemochromatosis, nor does the co-existence of the heterozygous HJV-L165X and the heterozygous HFE-C282Y mutation lead to iron accumulation.

Discussion

A novel truncating mutation (HJV-L165X) was found to be responsible for the non-HLA linked hemochromatosis described 30 years ago in a Dutch family [26,28,29]. The family now appears to harbor various combinations of the HFE-C282Y and the HJV-L165X alterations.

The early stop codon created by the HJV-L165X mutation may very well lead to nonsense-mediated decay of the corresponding messenger RNA. If the aberrant message is translated, however, it codes for a protein that lacks the GPI anchor signal, such that the potential of HJV to associate with the membrane will be completely absent. In both cases, it can be anticipated that the regulation of HJV release by muscle cells, and thus regulation of body iron homeostasis, is severely impaired in these patients. Thus far, about 25 different mutations have been detected in the *HJV* gene clustered in exons 3 and 4. Several of these mutations result in a premature truncation [35,36].

The identification of HFE-related modifiers will contribute to the debate on whether population screening for HH should be undertaken or whether alternative strategies should be implemented to improve early detection [5]. One popular approach is the screening of relatives of a clinically overt HFE-C282Y-homozygous proband [37]. This family (cascade) screening may become even more cost-effective if a dominant modifying factor can be identified. So far, however, the long sought genetic modifier that explains the highly variable clinical expression of HH in subjects homozygous for the C282Y-mutation has not been found. Nevertheless, some support for genetic modifiers comes from several cases in which the interaction of the homozygous HFE-C282Y with heterozygous mutations in hepcidin resulted in more severe iron overload than in HFE-C282Y homozygous patients matched for age and gender [25]. Another recent and illustrative example of digenic inheritance is the juvenile phenotype that results from compound HFE-C282Y/H63D heterozygosity in combination with homozygous TfR2 missense mutations [38]. These findings appear to fit to the experimental evidence that HFE and TfR2 pathways share parts in common [39].

Earlier papers suggest that *HJV*-mutations may also modify the penetrance of HFE-C282Y homozygotes [24,25]: in a cohort study of 310 HFE-C282Y homozygous patients, nine patients were found with an additional *HJV* missense mutation in the

heterozygous state (L101P, S105L, E302K, G320V, N372D or R335Q). The iron indices of eight patients appeared to be more severe than those observed in HFE-C282Y homozygous patients of identical sex and similar age ranges [25]. In another study, 136 HFE-C282Y homozygous, 43 heterozygous, 42 *HFE*-C282Y/H63D compound heterozygotes, and 62 control subjects were scanned for mutations in the *HJV* gene. In one compound heterozygous patient with a severe phenotypic presentation, a novel heterozygous HJV-N196K mutation was found [24]. In yet another study, no modifying effect was observed. In the latter, two patients presented with a coinheritance of a homozygous HJV-G320V mutation, and a heterozygous HFE mutation. Five of eleven family members carried at least one of the three most common HFE mutations (C282Y, H63D or S65C) and were heterozygous for the HJV-G320V mutation. None of these 5 individuals had any clear evidence of iron loading [40].

Our data indicate that the HJV-L165X mutation is no apparent modifier of C282Y mutation in the *HFE*-gene, based on serum parameters. We should emphasize however, that as most family members had been adequately phlebotomized, serum iron parameters were not appropriate as a measure of the iron burden. We found an alternative in the retrospective assessment of the quantity of iron removed by phlebotomy throughout the years (iron removed/age) [31]. Although this approach may have some shortcomings in that iron intestinal uptake may increase upon enhanced iron intake and by the phlebotomy itself, it offers a rough and second best estimate of the severity of the hemochromatosis. Also, results on TS-measurement and the DFO-test performed in the early 70s before treatment confirm the absence of a clinically relevant modifying effect of the HJV-L165X mutation. Finally, in addition to this conventional assessment of iron burden, we also analyzed the hepcidin-25 levels in urine with our novel MS assay [9]. Through both experimental and clinical studies hepcidin has been proposed as the unifying pathogenetic factor in HH, and its concentration to be inversely correlated to the severity of the phenotype[1]. Indeed, we found statistically significant lower urinary hepcidin levels in HFE-C282Y homozygotes in comparison to HFE-C282Y heterozygotes. On the other hand, and in line with the observed conventional iron parameters, the similarity in hepcidin levels between HFE-C282Y mutated subjects with or without the newly identified heterozygous *HJV* mutation, provides up to date evidence that the truncated hemojuvelin has no impact on the penetrance of the HFE-mutation. Last but not least, it should be noted that against a background of multiple small variations, environmental variation and unknown genes, we cannot exclude a minor effect of the HJV-L165X mutation on the iron homeostasis. But if present, this effect is expected to be of low clinical relevance.

The homozygous HJV-L165X truncating mutation predisposes to an aggressive form of hemochromatosis, which presents at a young age. Early recognition of this rare form of hemochromatosis is of prime importance because timely treatment can prevent organ damage and can preserve fertility, as illustrated by our index case,

who was followed for 30 years. We assessed both conventional iron parameters (TS and ferritin), as well as results from a DFO-test, iron removed/age and hepcidin levels, partly before and sometimes long after normalization of iron load by phlebotomy in an extended family in which the HFE-C282Y and the HJV-L165X were shown to co-exist. We found that the presence of the heterozygous HJV-L165X mutation does not clearly aggravate the phenotypic appearance of HFE-related hemochromatosis. This indicates that one wildtype copy of HJV is sufficient for HJV-mediated regulation of iron metabolism and that one mutated or absent copy of the hemojuvelin protein is not a clinically relevant modifying factor for the penetrance of the homozygous HFE-C282Y mutation. These findings are in agreement with the view recently proposed by Anderson and Frazer [41] in which HFE and HJV each are, at least in part, participants in distinct regulatory pathways.

Acknowledgements

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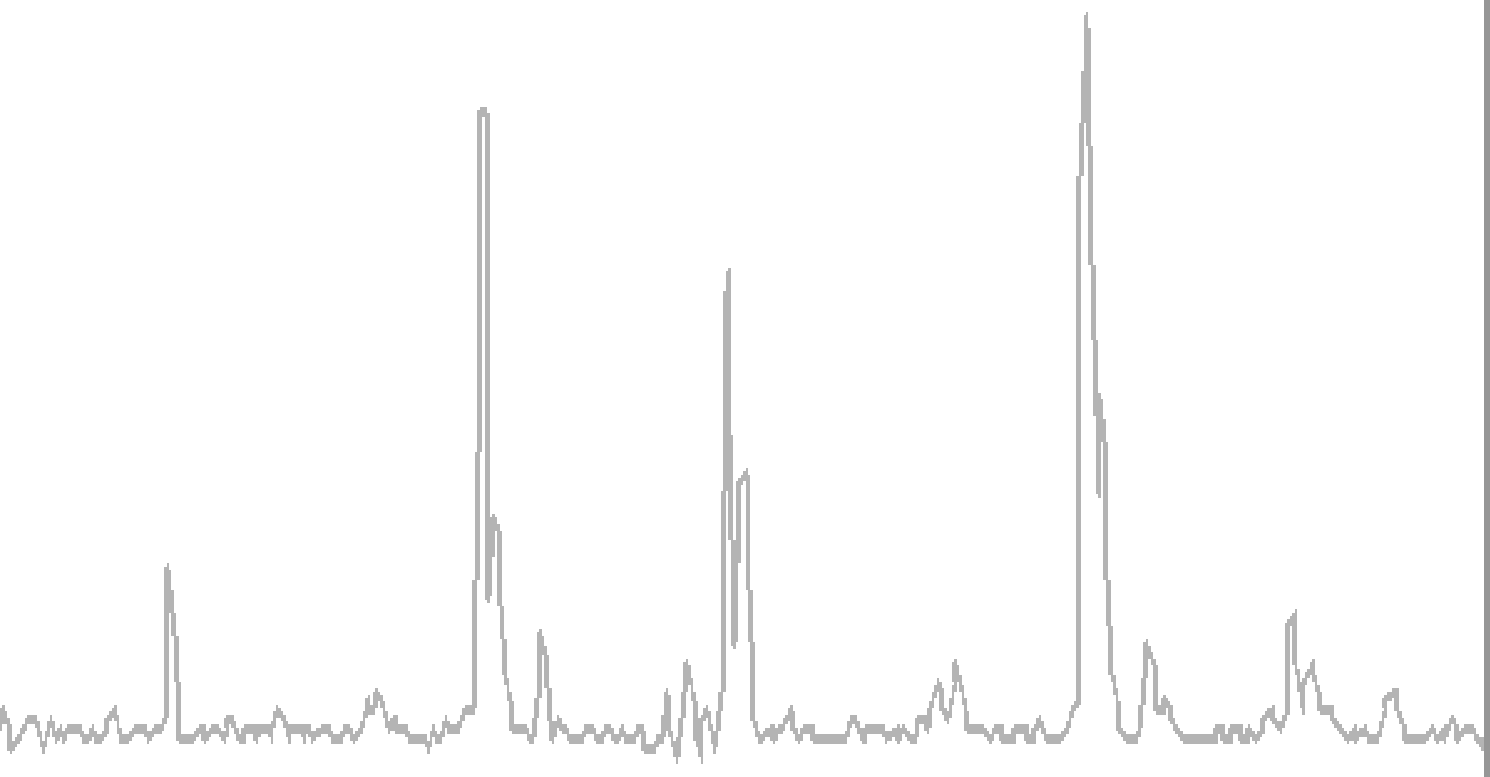
Summary, General discussion & future perspectives



Summary, conclusions and perspectives

Although iron is essential for life, its reactive properties require tight regulation in order to prevent pathological side effects. Hepcidin, a liver produced peptide hormone, is thought to be the central regulator of body iron metabolism. Its production is foremost under control of erythropoietic activity of the bone-marrow, the amount of circulating and stored body iron, and inflammation. Hepcidin exerts its regulatory function on iron metabolism by binding to the transmembrane iron exporter ferroportin present on macrophages, the basolateral site of enterocytes, and hepatocytes. As a result, internalization and degradation of ferroportin prevents iron release into the circulation. Although hepcidin was first discovered in human urine and serum, most evidence on hepcidin regulation and mode of action comes from *in vitro* work and mice studies that often use hepcidin mRNA expression as a read out. Human studies were largely impeded because suitable hepcidin assays were not available except for the one documented specific immuno-dot assay to measure hepcidin in urine. The major aim of this thesis was the development of a high through-put assay for hepcidin in order to study the regulation of this peptide, and to gain knowledge on the role of hepcidin in iron metabolism.

Chapter 2 reports an alternative approach for quantification of hepcidin in urine by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS). By this technique we circumvented the difficulties immunochemical-based methods encountered due to technical problems of antigen- and antibody production. The MS-based assay showed to be capable of detecting the 3 isoforms of hepcidin normally found in urine from which the identity of the peptide peak equivalent to the 25 amino acid peptide (hepcidin-25) was confirmed with a commercially available synthetic human hepcidin-25 peptide. Validation of the method by analyses of samples with various hepcidin levels showed a strong correlation with the immuno-dot assay. We concluded that this new urinary hepcidin assay might become an important tool in future studies.

Further improvements of the assay protocol and the use of a different ProteinChip array type made it also possible to measure hepcidin in serum as described in Chapter 3. This time, state of the art proteomic techniques confirmed that next to the three hepcidin isoforms in urine, hepcidin-25 in serum had the same amino-acid sequence as the 25 amino acid peptide found in urine. The analytical performances were acceptable although further improvements were warranted such as the use of an internal standard in order to eliminate matrix differences and to allow quantification of hepcidin levels. Preliminary validation of both serum and urine application showed to be capable of differentiation of various disorders of iron metabolism, this despite

influence of pre-analytical and biological variations. Urine hepcidin turned out to be more affected by multiple freeze-thaw cycles and storage conditions, but less influenced by diurnal variation, than is serum hepcidin. Standardized sampling for serum hepcidin analyses was therefore advised when used in future clinical studies.

Chapter 4 describes that the knowledge of hepcidin regulation gained by *in vitro* studies was translatable to the human *in vivo* situation. Therefore we measured soluble transferrin receptor (sTfR) and transferrin saturation (TS) as biochemical markers reflecting erythropoietic activity and the iron store regulatory pathways of hepcidin, respectively, and C-reactive protein (CRP) as reflector of inflammation related regulation in patients with iron metabolism disorders and controls. In parallel serum hepcidin-25 and prohepcidin were also assessed. These regulatory pathway reflecting parameters were also combined in an algorithm that turned out to predict accurately the actual measured hepcidin levels. We concluded that despite the small setting of the study and the simplified approach of the algorithm, the complex regulation of hepcidin can be determined mainly by these three regulatory pathways. In future studies the robustness and suitability of this algorithm in combination with actual measured serum hepcidin levels in clinical differentiation has to be validated, as potential tool to gain insight in the balance between the regulators and the production of hepcidin in specific iron metabolism disorders.
The role of sTfR as communication factor between the bone marrow and the liver was suggested but not proven by the HepG2 expression results which showed an inverse association between hepcidin and sTfR. Because of the use of patient serum in these *in vitro* experiments, influence of other components on hepcidin expression in the cells could not be excluded. The use of purified sTfR in the same experimental setting might clarify the regulatory effect of this plasma protein on hepcidin expression.

Chapter 5 describes the effects of lipopolysaccharide (LPS) as an upstream inflammation activator on urinary hepcidin excretion levels in a 24-hour time course analysis with the use of an *in vivo* human endotoxemia model. Previous studies had shown that interleukin-6 (IL-6) mediated hepcidin increase and consequent hypoferremia during inflammation. The temporal associations between plasma cytokines, hepcidin levels, and serum iron parameters in healthy individuals after LPS injection displayed a dramatically induction of IL-6 within 3 hours after injection. Consequently, urinary hepcidin levels peaked within 6 hours, followed by a significant decrease in serum iron. This study confirmed the importance of the IL-6-hepcidin axis in development of hypoferremia in inflammation and highlighted the rapid responsiveness of this iron regulatory system.

Next to the putative role of hepcidin in the redistribution of iron between the intra- en extra-cellular body compartments during inflammation, *in vitro* studies suggested also

involvement of nitric oxide (NO). Chapter 6 describes a study in which the course of iron parameters, cytokine profiles and urine hepcidin levels were compared in a human *in vivo* endotoxemia model in the absence and presence of the selective inducible NO synthase (iNOS) inhibitor aminoguanidine. It was shown that CRP, Tumor necrosis factor-α, and IL-10, urinary hepcidin excretion levels and circulating iron levels showed no significant differences between both experimental settings. Therefore we concluded that, compared to hepcidin, NO plays no role of significance in body iron homeostasis during systemic inflammation *in vivo*.

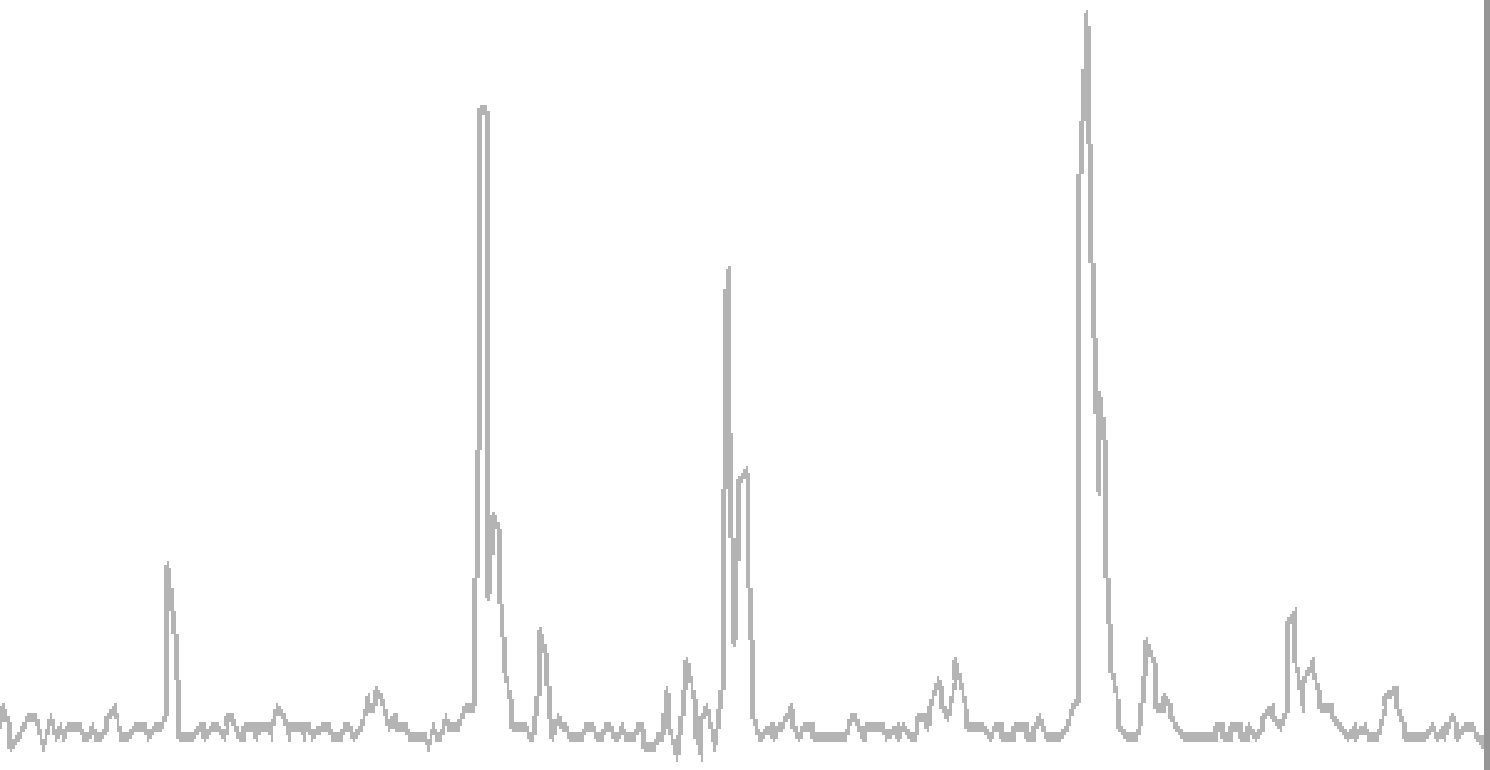
Chapter 7 describes the use of urinary hepcidin analysis as tool to support the search for genetic mutations in a patient suffering of non-HFE hereditary hemochromatosis (HH). The very low hepcidin levels found in the proband suggested sequencing of the *hepcidin* (*HAMP*) and *hemojuvelin* (*HJV*) genes. As a result, a new homozygous truncating mutation, L165X, of the *HJV* gene was found. These data exemplify that hepcidin analysis might have a role as a diagnostic test for HH, provided that abnormalities in liver functions, inflammation and a short interval between sample collection and phlebotomy are excluded. Therefore, assessment of hepcidin values might reduce the workload and costs of the cumbersome procedures of screening for sequence variations in the multiple genes responsible for hemochromatosis.

In addition, Chapter 1 suggests several differential diagnostic and therapeutic implications of hepcidin analysis. Recognition of iron deficiency anemia (IDA) in the context of anemia of chronic disease (ACD) is currently performed with routine biochemical parameters which all have their own disadvantages. In contrast to increased levels of hepcidin in ACD, classic IDA in man is associated with low hepcidin levels which make hepcidin a potential marker for detection of IDA in ACD. However, studies in anemic patients suffering from diseases such as rheumatoid arthritis, inflammatory bowel diseases, cancer, and end stage renal disease are needed to validate the potency of hepcidin measurements under these conditions.
In iron loading anemias such as thalassemia, studies have suggested hepcidin or hepcidin/ferritin index values at the lower end of reference range as a result of suppressed hepcidin production due to high and less effective erythropoietic activity. These findings may be relevant in the search for non-invasive measures of iron burden and improved therapeutic interventions for these often congenital diseases.
To date, the forecast how patients will react on erythropoietin (EPO) treatment is complicated by the co-existence of several factors that contribute to anemia, such as inflammatory activity and liver toxic therapy. Determination of hepcidin levels might turn out to be of value in the prediction of a response as well as in the monitoring of EPO treatment in these patients.
Additional investigations are warranted to determine hepcidin levels in chronic kidney disease, end stage renal disease, upon hemodialysis and EPO and/or iron treatment in relation to iron and inflammatory status and blood counts. These studies will pave

the way for novel diagnostic and more optimized therapeutic strategies in patients treated with EPO.

In conclusion, improved hepcidin assays are expected to increase insight in circulating hepcidin levels in various conditions and its kinetics. This knowledge aids in the development of hepcidin agonists and antagonists or the targeting of other proteins of the hepcidin regulatory circuitry pathways that will then be of value in the treatment of these iron related disorders.

Samenvatting in het Nederlands



Samenvatting, conclusies en beschouwingen

Het element ijzer is onlosmakelijk verbonden met de levende cel door de essentiële reacties die het vervult in die cel. Dezelfde chemische reactiviteit van het ijzer vraagt echter om een zorgvuldige regulatie van dit element om te voorkomen dat eventuele toxische neven reacties tot schade in die zelfde cel kunnen leiden. In hoofdstuk 1 wordt in een overzicht beschreven dat het, door de lever geproduceerde, eiwit hormoon hepcidine een centrale rol vervult in de regulatie van het totale lichaamsijzer. De productie van hepcidine staat op zijn beurt voornamelijk onder controle van de rode bloedcel aanmaak in het beenmerg, de hoeveelheid circulerend en opgeslagen lichaamsijzer en ontstekingsactiviteit in het lichaam. Hepcidine reguleert de opname en afgifte van ijzer door binding aan ijzer exporterende eiwit ferroportin dat zich in de celmembraan van darm epitheelcellen, levercellen en macrofagen bevindt. Als gevolg van deze binding verdwijnt de ijzertransporter van de celmembraan waardoor er geen ijzer meer afgegeven kan worden aan het bloed. Ondanks het feit dat hepcidine voor het eerst ontdenkt is in het serum en urine van de mens, is de meeste kennis over de regulatie en werking van dit eiwit verkregen uit laboratoriumstudies met muizen en celkweken waarbij DNA of RNA uit (lever)cellen wordt gebruikt om een indruk te krijgen van de hepcidine productie. Studies in de mens worden beperkt door deze arbeidsintensieve en belastende manier van monsterafname. Daarnaast was er tot voor kort maar één immunochemische test beschreven waarbij met behulp van antilichamen gericht tegen het hepcidine de concentratie van dit eiwit in urine gemeten kon worden. Het belangrijkste doel van dit proefschrift is dan ook het ontwikkelen van een hepcidine bepaling in serum of urine waardoor studies naar regulatie van dit eiwit en inzicht naar de rol binnen de ijzerstofwisseling in de mens vergroot kunnen worden.

Hoofdstuk 2 beschrijft een testmethode om hepcidine in urine te meten met behulp van surface-enhanced laser desorption/ionization time-of-flight massaspectrometrie (SELDI-TOF MS) na voorscheiding op een analyse chip met specifieke bindingseigenschappen. Door het gebruik van massaspectrometrie (MS) wordt het gebruik van antilichamen tegen hepcidine, en dus de technische problemen om ze te maken omzeild. De nieuwe MS-methode blijkt in staat drie verschillende vormen van het hepcidine eiwit te detecteren in urine waarbij het eiwit met een lengte van 25 aminozuren (hepcidine-25) dat betrokken is bij de ijzerregulatie geïdentificeerd is aan de hand van de massa die overeen komt met een commercieel verkrijgbaar synthetisch product van humaan hepcidine-25.
De hepcidine concentraties gemeten met de MS-methode komen sterk overeen met de resultaten verkregen met de eerder genoemde immunochemische test. Op basis

van deze resultaten is geconcludeerd dat deze nieuwe MS-methode voor metingen van hepcidine in urine goed bruikbaar is voor toekomstige studies.

Zoals beschreven is in hoofdstuk 3, maakt verdere verbeteringen van het meetprotocol en het gebruik van een analyse chip met een ander type bindingsoppervlak het uiteindelijk mogelijk ook hepcidine te detecteren in serum. Door middel van hoogwaardige massa spectrofotometrische scheidingstechnieken is aangetoond dat het in serum detecteerbare hepcidine-25 dezelfde aminozuurvolgorde heeft als het hepcidine dat in urine gemeten wordt. De analytische prestaties van deze nieuwe testmethode zijn acceptabel. Echter, het gebruik van een interne standaard zou de testmethode verder kunnen verbeteren omdat daardoor de invloed van de verschillen in samenstelling tussen serum en urine worden geëlimineerd en kwantificering mogelijk wordt. De eerste studies met zowel serum als urine metingen laten zien dat onderscheid tussen verschillende ijzergerelateerde aandoeningen mogelijk is ondanks invloed van opslag en biologische dagvariaties op de uitslag. Hepcidine in urine blijkt namelijk meer beïnvloed te worden door veelvuldig invriezen en ontdooien en de temperatuur waarbij het bewaard wordt, terwijl hepcidine in serum juist onder invloed staat van het dag/nacht ritme. Er wordt dan ook voor klinische studies met serum aanbevolen op vaste tijden monsters af te nemen, om vergelijking van uitslagen tussen personen mogelijk te maken.

Hoofdstuk 4 laat zien dat de kennis met betrekking tot hepcidine regulatie verkregen uit laboratorium studies met muizen en celkweken ook toepasbaar is op de regulatie in de mens. Hiervoor zijn circulerend serum transferrine receptor (sTfR) en transferrine saturatie (TS) gemeten als biochemische merkers voor respectievelijk beenmerg activiteit en lichaamsijzer voorraad en het acute fase eiwit C-reactief proteine (CRP) als merker voor ontstekingsactiviteit in het lichaam. Elk van deze merkers weerspiegelen een proces dat de hepcidine productie beïnvloedt. Gelijktijdig zijn tevens hepcidine-25 en het voorlopereiwit prohepcidine in serum gemeten van gezonde vrijwilligers en verschillende groepen patiënten met aandoeningen van het ijzermetabolisme. Vervolgens zijn deze drie merkereiwitten in een formule samengevoegd waarbij na invulling van de in serum gemeten relatieve concentraties van elke merker een hepcidine waarde kan worden berekend die nauwkeurig overeen komt met de werkelijk gemeten serum hepcidine-25 concentratie. Uit deze data is geconcludeerd dat ondanks het beperkte aantal gemeten monsters en de eenvoud van de formule, de concentratie van het circulerende serum hepcidine voornamelijk bepaald wordt door de drie onderzochte regulatie processen. In toekomstige studies zullen de robuustheid en bruikbaarheid van de formule in klinische differentiatie gevalideerd moet worden om zo meer inzicht te krijgen in de balans tussen de verschillende regulatoren en de werkelijke hoeveelheid hepcidine die geproduceerd wordt in ijzermetabolisme gerelateerde aandoeningen. Tevens

wordt in dit hoofdstuk een hepcidine expressiestudie in levercellen beschreven waarbij de rol van sTfR als communicatie eiwit tussen het beenmerg en de lever is bestudeerd. De resultaten laten een sterke associatie zien tussen de sTfR concentratie in serum en de hoeveelheid hepcidine die door de levercellen tot expressie worden gebracht. Echter, een direct oorzakelijk verband wordt niet bewezen door deze resultaten. Nieuwe experimenten met bijvoorbeeld gezuiverd sTfR eiwit kunnen waarschijnlijk meer duidelijkheid geven over de potentie van dit eiwit in de regulatie van hepcidine.

Hoofdstuk 5 beschrijft het effect van bacterieel endotoxine op de uitscheiding van hepcidine in urine gedurende 24 uur, waneer deze stof ingespoten wordt in gezonde vrijwilligers. Eerdere studies hadden al aangetoond dat interleukine 6 (IL-6), een hormoon geproduceerd door witte bloedcellen tijdens ontstekingsreacties, in staat is de productie van hepcidine te verhogen. Als gevolg hiervan werd een afname van de ijzerconcentratie in het bloed gezien. In onze studie wordt 3 uur na injectie van endotoxine een sterke toename van IL-6 gemeten, waarop vervolgens een toename van hepcidine uitscheiding in urine gezien wordt tot 6 uur na injectie, gevolgd door een afname van ijzer in het bloed. Deze data bevestigen dat de IL-6-hepcidine as een belangrijke rol speelt bij het ontstaan van ijzergebrek tijdens inflammatie in de mens. Tevens illustreert deze studie de hoge snelheid waarmee dit proces plaatsvindt.

Naast het effect dat hepcidine op het circulerende ijzer in het bloed heeft tijdens infecties, zijn uit diverse laboratoriumexperimenten met celkweken aanwijzingen verkregen dat stikstofoxide (NO) hier ook een rol speelt. Dit NO wordt tijdens inflammatie geproduceerd door een enzym genaamd “induceerbaar NO synthase (iNOS)”. In hoofdstuk 6 wordt een studie beschreven waarbij wederom gezonde vrijwilligers met endotoxine zijn ingespoten, nu echter in combinatie met of zonder de specifieke iNOS remmer aminoguanidine. Diverse infectie parameters in het bloed en urine hepcidine concentraties zijn ook hier gedurende 24 uur vervolgd maar lieten geen verschillen zien tussen aan- of afwezigheid van aminoguanidine. Geconcludeerd is dat NO minder invloed uitoefent op de ijzerregulatie in het lichaam tijdens systemische ontstekingsreacties in de mens, in vergelijking tot hepcidine.

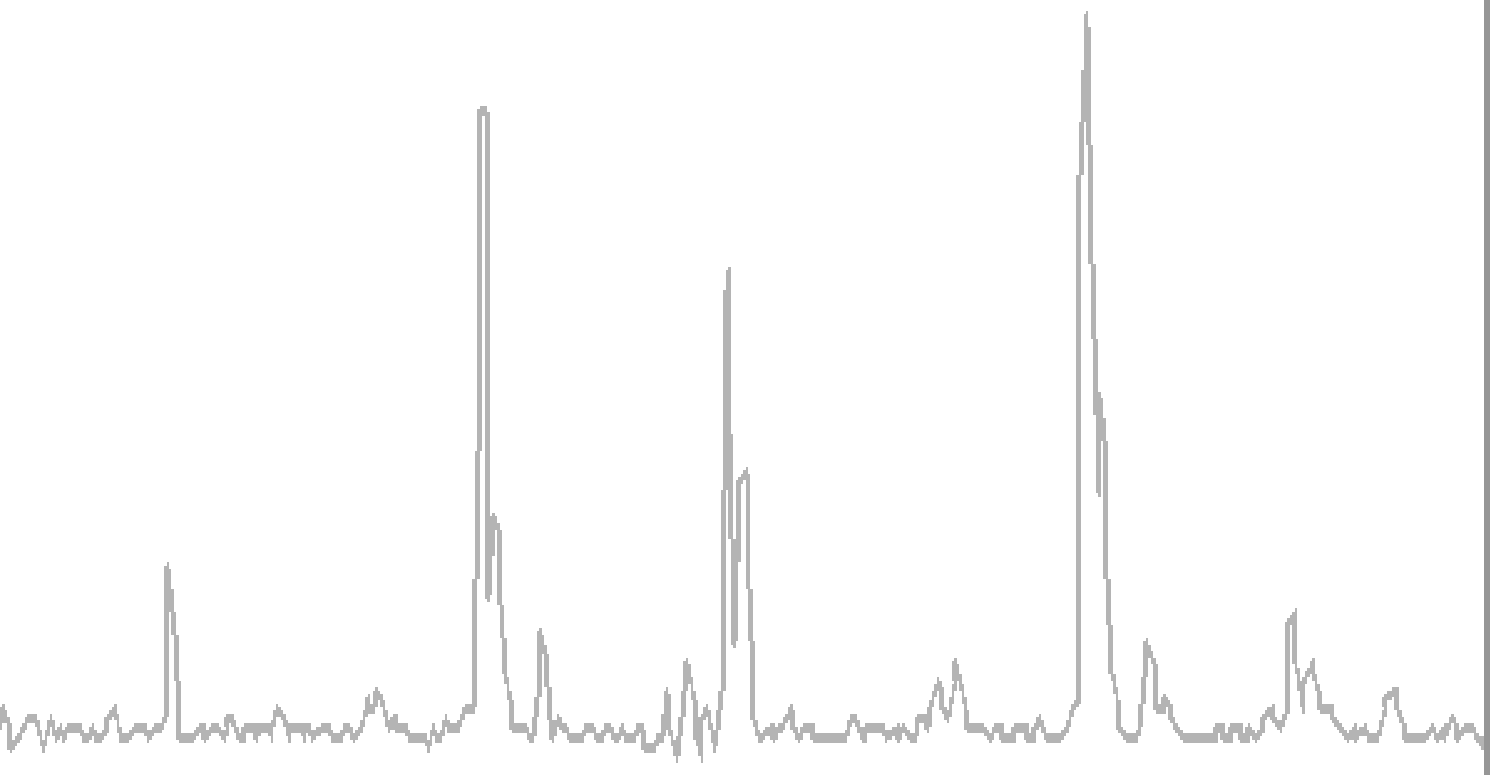
Uit hoofdstuk 7 blijkt dat het meten van de concentratie hepcidine in urine een goed hulpmiddel is in de zoektocht naar genen verantwoordelijk voor ijzerstapeling. Dit is geïllustreerd aan de hand van een patiënt lijdend aan ijzerstapeling die niet wordt veroorzaakt door een genetisch defect van het HFE eiwit. De zeer lage hepcidine concentratie gemeten in deze patiënt, wijzen op een mutatie in het *hepcidine* (*HAMP*) of *hemojuveline* (*HJV*) gen. Verder onderzoek leverde dan ook een nieuwe mutatie in het *HJV* gen op, aangeduid als L165X. Deze data laten zien dat de hepcidine meting een waardevolle rol kan vervullen als screentest bij genetische ijzerstapeling op

voorwaarde dat leverafwijkingen en ontstekingsprocessen zijn uitgesloten en de afname van het analysemonster voldoende ruim voor of na een aderlating heeft plaatsgevonden. Het meten van hepcidine zou daarmee een gunstig effect kunnen hebben op de werklast en onkosten die een uitgebreide genetische screening van hemochromatose kandidaat genen met zich mee brengt.

Aanvullend worden er in hoofdstuk 1 diverse suggesties gedaan voor diagnostische en therapeutische toepassingen van de hepcidine bepaling. In de eerste plaats zou de hepcidine meting een rol kunnen gaan spelen in het herkennen van een ijzergebreksanemie tijdens inflammatie. Op dit moment wordt dit gedaan met een combinatie van biochemische testen die elk hun eigen tekortkomingen hebben. In tegenstelling tot de hoge hepcidine concentraties tijdens inflammatie, worden bij ijzergebrek lage hepcidine waarden gemeten, waarmee hepcidine een potentiële merker kan zijn bij een combinatie van beide processen. Door meting van monstermateriaal van patiënten waarbij zowel ijzergebrek als inflammatie de oorzaak kunnen zijn van bloedarmoede zoals in reumatoïde artritis, ontstekingen in het maagdarmkanaal, kanker, of ernstige nierziekten, kan inzicht verkregen worden in de diagnostische waarde van de hepcidine meting onder deze condities.
Hepcidine of de hepcidine/ferritine ratio hebben daarnaast in diverse studies laten zien dat het goede klinische merkers zouden kunnen zijn voor het schatten van de ijzerbelasting bij aangeboren vormen van anemie die gepaard gaan met ijzerstapeling. Lage waarden wijzen op een onderdrukking van de hepcidine productie veroorzaakt door een sterke, maar inefficiënte, rode bloedcel aanmaak in het beenmerg van deze patiënten. Deze bevindingen kunnen relevant zijn voor het verkrijgen van een goede maat voor de hoeveelheid ijzer opgeslagen in het lichaam zonder belastende ingrepen en voor het verbeteren van behandelingsmethoden voor deze vaak aangeboren aandoeningen.
Als laatste toepassingsmogelijkheid wordt de voorspelling van de reactie op het toedienen van erytropoietine (EPO) of het vervolgen van deze therapie genoemd. Deze toepassingsmogelijkheden worden momenteel bemoeilijkt door de verschillende factoren die bijdragen tot het ontstaan van een anemie bij EPO behandelde patiënten zoals ontstekingen en de toediening van lever toxische medicijnen. Toekomstig onderzoek zal moeten uitwijzen welke hepcidine waarden gevonden worden in patiënten met kanker of chronische nierziekten die behandeld worden met EPO en/of ijzerpreparaten in vergelijking tot ijzerwaarden in het bloed, infectiestatus en het aantal bloedcellen. Deze studies zullen naar verwachting mogelijkheden creëren voor nieuwe diagnostiek en betere therapieën in patiënten die behandeld worden met EPO.
Concluderend kan gesteld worden dat door verbeterde hepcidine analysemethoden het inzicht in de regulatie van het eiwit en informatie over de te verwachten concentraties onder verschillende condities vergroot zal worden. Deze kennis zal bijdragen tot het ontwikkelen van betere diagnostische bepalingen, en de

ontwikkeling van medicijnen die de werking van het hepcidine eiwit of ander eiwitten betrokken bij de regulatie van hepcidine kunnen beïnvloeden als nieuwe therapie in de behandeling van ijzer gerelateerde aandoeningen.

Dankwoord



Dankwoord

Tja, en dan nu het dankwoord. Misschien wel het moeilijkste deel van het hele proefschrift. Niet dat bedanken iets lastigs is, maar je wilt vooral niemand vergeten. Hoe klein het aandeel van iemand ook geweest is, en ook al word *ik* uiteindelijk aangekeken op de inhoud, dit proefschrift is voor mij het gezamenlijke resultaat van al die mensen die op de één of andere manier de afgelopen 3 jaar betrokken zijn geweest bij mijn promotie onderzoek. Daarvoor mijn hartelijke dank.

Uiteraard ontkom ik er niet aan toch een aantal mensen in het bijzonder te noemen.

Allereerst wil ik mijn promotor Hans Willems bedanken voor het mogelijk maken van dit onderzoek op de afdeling klinische chemie (AKC) van het UMC St Radboud.
Tijdens mijn hoofdvak stage op het AKC ben ik waarschijnlijk voldoende betrouwbaar overgekomen dat je het zelfs aandurfde me een combinatie traject aan te bieden. Het eerste deel, het promotie onderzoek, zit er op, nu nog de interne opleiding tot klinisch chemicus verder afmaken. Voorlopig blijf ik dus nog even.

Dorine, geweldig. Ik weet niet hoe je het doet maar onder jouw directe begeleiding is onderzoek inspirerend. Ik wist niet dat ik het in me had maar jij hebt blijkbaar de onderzoeker in me naar boven kunnen halen. De drive waarmee jij onderzoek bedrijft is aanstekelijk, stimulerend en voor mij van cruciaal belang geweest om dit project af te kunnen ronden. Ik wil je hiervoor heel erg bedanken.
Daarnaast wil ik je ook bedanken voor alle gezelligheid gedurende de congressen in het buitenland, de vele discussies en je luisterende oor op die momenten dat het nodig was.

Natuurlijk wil ik ook jou bedanken, Harold. In de rol van co-promotor heb je menig artikel voorbij zien komen voor correctie. Als man van weinig woorden wist je feilloos de zwakke punten aan te geven waardoor het uiteindelijke resultaat vele malen beter werd. Jouw kennis over eiwitten is van grote waarde gebleken gedurende het onderzoekstraject. Ik heb veel van je geleerd, dank hiervoor.

Coby, zonder jou was het niet gelukt. Onze samenwerking en vele discussies hebben er voor gezorgd dat de hepcidine analyse is wat ze is. Jouw kennis en ervaring over hoe je eiwitten in urine moet benaderen heeft het project een solide basis gegeven. Het stond voor mij dan ook snel vast dat jij één van mijn paranimfen *moest* worden. Ik kijk terug op een leuke en succesvolle samenwerking en hoop dat Joyce hetzelfde mag ervaren gedurende haar promotieonderzoek.

Verder bedank ik natuurlijk de research analisten van het eerste uur: Rian en Erwin. Het was erg gezellig samen met de vissen en de droppot. De eerste stappen binnen het hepcidine werk waren al door jou gedaan, Rian. Blotten met slechte commerciële antilichamen bleek uiteindelijk toch niet de weg te zijn om hepcidine aan te tonen maar heeft ons veel geleerd over het gedrag van het peptide onder testomstandigheden. Later bleek jouw kennis en vaardigheden met de prohepcidine ELISA goed bruikbaar. Ik wil je hiervoor nogmaals bedanken. Erwin, naast het vele PCR werk van de verschillende ijzer-genen is jouw rol binnen het eiwit werk vooral het laatste jaar uitgebreid door de vele externe hepcidine-samenwerkingsprojecten. Ik dank je voor je inzet op alle analyses.

Joyce, now it's up to you. Je hebt het stokje van me overgenomen als junior onderzoeker en zult nu ook op zoek moeten naar de krenten in de onderzoekspap. Voorlopig zullen we nog veel samen kunnen optrekken, met dezelfde mensen om ons heen, in dezelfde stimulerende omgeving. Ik wens je heel veel succes.

Renée. Ik heb jou gevraagd of je naast Coby ook paranimf wilde zijn tijdens mijn promotie. Ik heb ervaren dat het goed is een maatje te hebben op de werkvloer, en dat ben je. Jouw sociale karakter en de liters koffie die daar bij horen zijn vaak onmisbaar gebleken tijdens de eindfase van dit project. Dank hier voor.

April; how unorganized can you be? You improved my English; I didn't improve your Dutch. It was a privilege having you as a colleague, especially at the iron meetings in Barcelona, Kyoto and London. Thanks for our intensive and noisy discussions.
Boukje, dat de epidemiologie zo snel hepcidine zou kunnen gebruiken was voor mij een prettige verrassing. Deze toepassing heeft toch maar mooi een publicatie opgeleverd: I owe you one.

Jacqueline en Edmée, bedankt voor jullie interesse in mijn project en de tips om het tot een goed einde te brengen. Verder alvast bedankt voor de inspirerende gesprekken over "het vak". Ik ben er zeker van dat er nog vele zullen volgen.
Verder wil ik Annemarie en Siem niet vergeten voor hun getoonde betrokkenheid en gezelligheid tijdens de koffie en lunch pauzes – gaan we buiten zitten?
Alle collega´s van het AKC, ook jullie bedankt voor je soms "persoonlijke bijdrage" aan het onderzoek, maar vooral ook voor de interesse naar wat er zich in het kenniscentrum afspeelt.

Zonder SELDI geen analyse, vandaar dat ik hierbij zeker de SELDI-faciliteit wil noemen. Arnoud, Mark en Waander, bedankt voor alle ondersteuning tot nu toe. Het is ons toch maar mooi gelukt de SELDI een plek te geven binnen het diagnostische veld. Dat er nog veel toepassingen mogen volgen.

Als ik SELDI zeg, bedoel ik natuurlijk ook Davy. Ik heb zeer goede herinneringen aan onze samenwerking in “Het Ciphergen tijdperk”. Jouw kennis en bijdrage zijn tot op de dag van vandaag nog voelbaar.

Verder wil ik hierbij alle artsen bedanken die betrokken zijn geweest bij het verzamelen van patiënten materiaal voor de verschillende studies. In het bijzonder wil ik Peter Pickkers noemen. Het humane endotoxine model wat jij begeleide bleek een lot uit de loterij en een bron van materiaal voor vele studies. Onze samenwerking heb ik als geweldig inspirerend ervaren, dank hier voor. Ik hoop dat de toekomst nog veel mag gaan brengen.

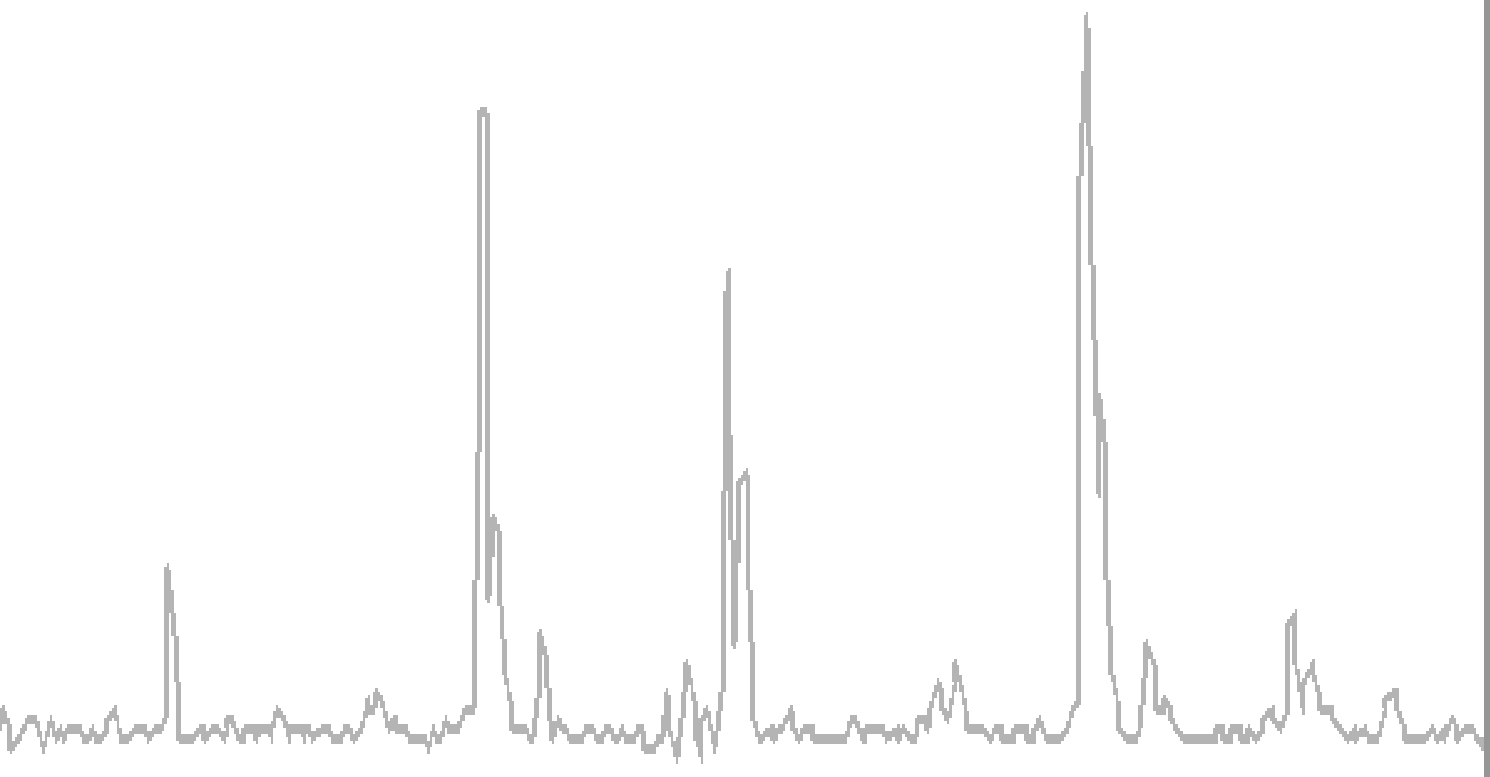
I also like to thank our collaborators from abroad. Specifically I’d like to mention Tomas Ganz and Ella Nemeth from UCLA. Working together with the pioneers on hepcidin analysis was a privilege. Your contribution to my first papers and the donation of peptides and antibodies turned out to be a success. Many thanks for your cooperation!

Uiteraard mag ik ook niet de “Nederlandse IJzer Congres Familie” vergeten: Jo Marx, Cees van Deursen, Herman Kreeftenberg en Esther Jacobs. Met erg veel pezier denk ik terug aan onze congressen. Hopelijk was ik niet te vermoeiend. Volgend jaar European Iron Club in Zwitserland?

Ten slotte wil ik familie en vrienden heel hartelijk bedanken voor hun belangstelling en steun gedurende dit project. Ik beloof dat mijn gespreksstof in de toekomst weer wat meer omvat dan enkel ijzer en hepcidine.

Lieve Ria, we zijn er bijna. Mede door jou is het mogelijk geweest dit traject in te gaan waarvan het promotie onderzoek maar een onderdeel is. Ondanks alles ben je blijven geloven in dit project en heb je me ondersteund waar mogelijk. Dank je wel, ik ben trots op je en heb ongelofelijk veel zin in de toekomst die voor ons ligt.

Curriculum Vitae

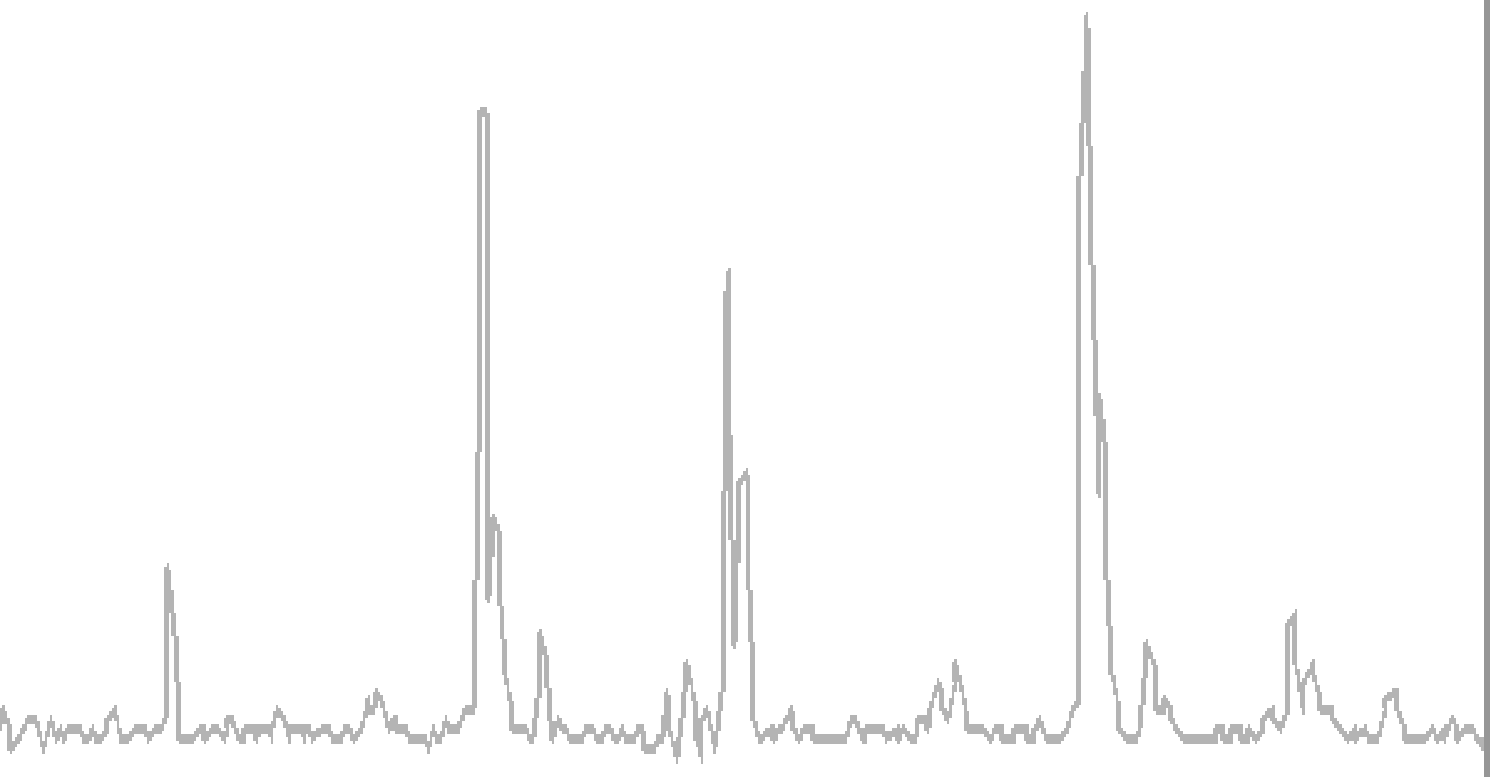


Curriculum Vitae

Erwin Hendrikus Johannes Maria Kemna werd geboren op 12 juli 1967 te Almelo. In 1985 behaalde hij zijn HAVO diploma aan het St. Canisius College te Almelo. In 1985 begon hij met het Hogere Laboratorium Onderwijs (HLO), medische richting, in Hengelo. De stage en afstudeerperiode werden doorlopen op het klinisch chemisch laboratorium van het Wezenlanden Ziekenhuis te Zwolle (Dr. K. Miedema en Drs. E. van Voost tot voorst). Het HLO diploma werd verkregen in 1989. Na het vervullen van de dienstplicht als sergeant-analist in het Militair Hospitaal Dr. A. Mathijssen in Utrecht, begon hij in 1990 als klinisch-chemisch analist in het Deventer Ziekenhuis. In 1992 trouwde hij met Ria Schilder. In 2001 nam hij ontslag in Deventer en begon de studie Biomedische wetenschappen aan de Katholieke Universiteit in Nijmegen (KUN). Het doctoraal examen met hoofdvakstage Pathobiologie (Prof. Dr. J.L. Willems) werd behaald in april 2004. Van mei 2004 tot februari 2007 was hij werkzaam als junior onderzoeker op de Afdeling Klinische Chemie (AKC) in het Universitair Medisch Centrum St. Radboud te Nijmegen (Prof. Dr. J.L. Willems, Dr. D.W. Swinkels en Dr. H. Tjalsma). Tijdens deze periode werd het onderzoek verricht dat is beschreven in dit proefschrift. Sinds februari 2007 is hij bij dezelfde vakgroep in opleiding tot Klinisch Chemicus.

Erwin Hendrikus Johannes Maria Kemna was born on July 12th 1967 in Almelo, The Netherlands. After he finished junior high school in 1985, he started a study to become a laboratory technician and graduated in 1989 as Bachelor of Science. During military service he worked as laboratory technician at the Central Military Hospital in Utrecht, The Netherlands. In 1990 he moved to Deventer, The Netherlands, to start at the Clinical Chemistry Laboratory of the local hospital. In 1992 he married Ria Schilder. In 2001 he ended his job in Deventer and started his study biomedical science at the University of Nijmegen, The Netherlands. He was awarded the degree of Master of Science in April 2004 on a project on folic acid in serum and erythrocytes, supervised by Prof. Dr. J.L. Willems. In May of that same year he started his PhD project on hepcidin at the department of Clinical Chemistry at Radboud University Nijmegen Medical Centre, The Netherlands, until February 2007, under supervision of Dr. D.W. Swinkels, Dr. H. Tjalsma and Prof. Dr. J.L. Willems. The results from this project are presented in this thesis. In February 2007 he started his internships at the same department in order to become a Clinical Chemist.

List of Publications



List of publications

Kemna EHJM, Tjalsma H, Wetzels JF, and Swinkels DW. Measuring serum hepcidin concentrations. Nat Clin Pract Gastroenterol Hepatol 2005 [10.1038/ncpgasthep0121]

Kemna E, Pickkers P, Nemeth E, van der Hoeven H, and Swinkels D. Time-course analysis of hepcidin, serum iron, and plasma cytokine levels in humans injected with LPS. Blood. 2005;106:1864-1866.

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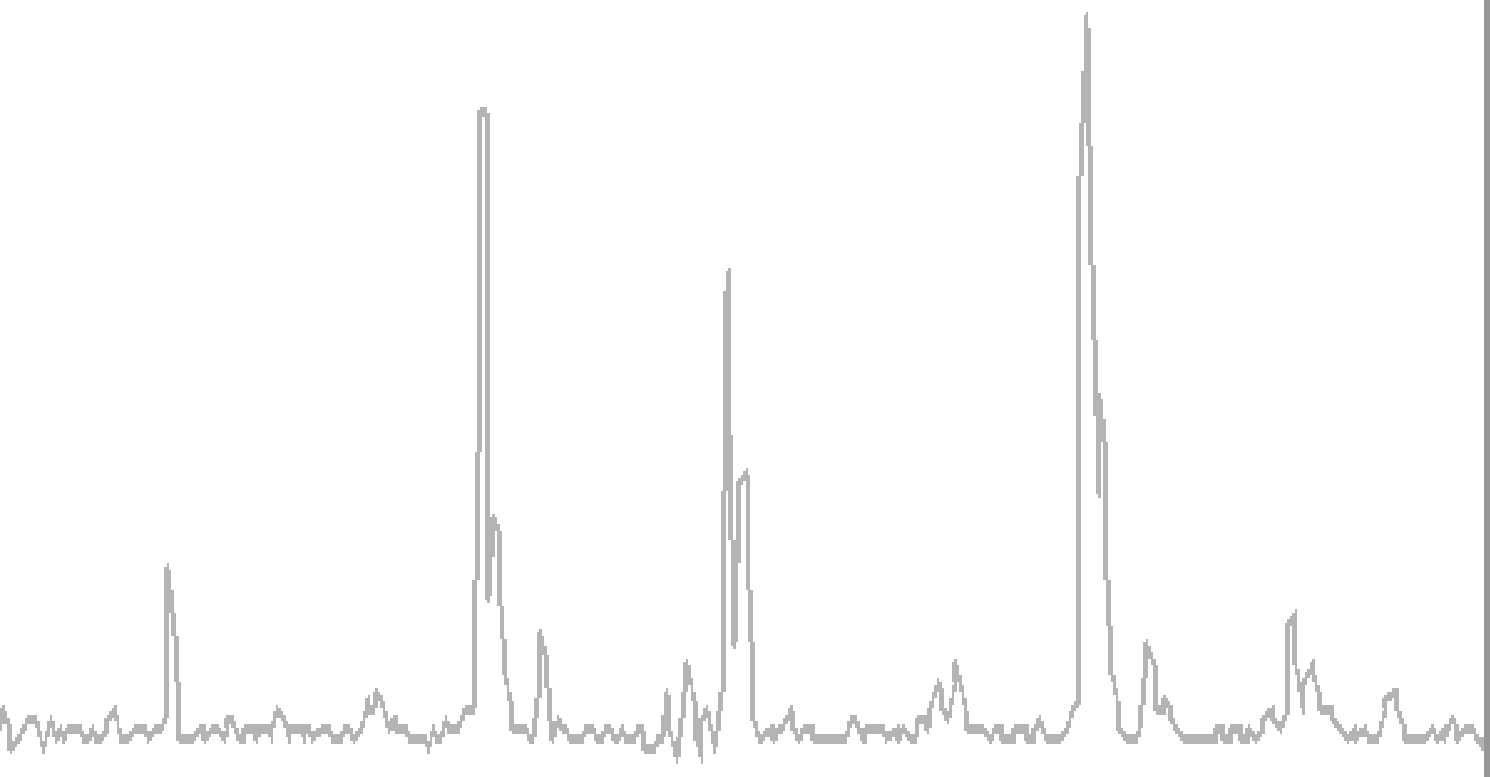
Color Figures



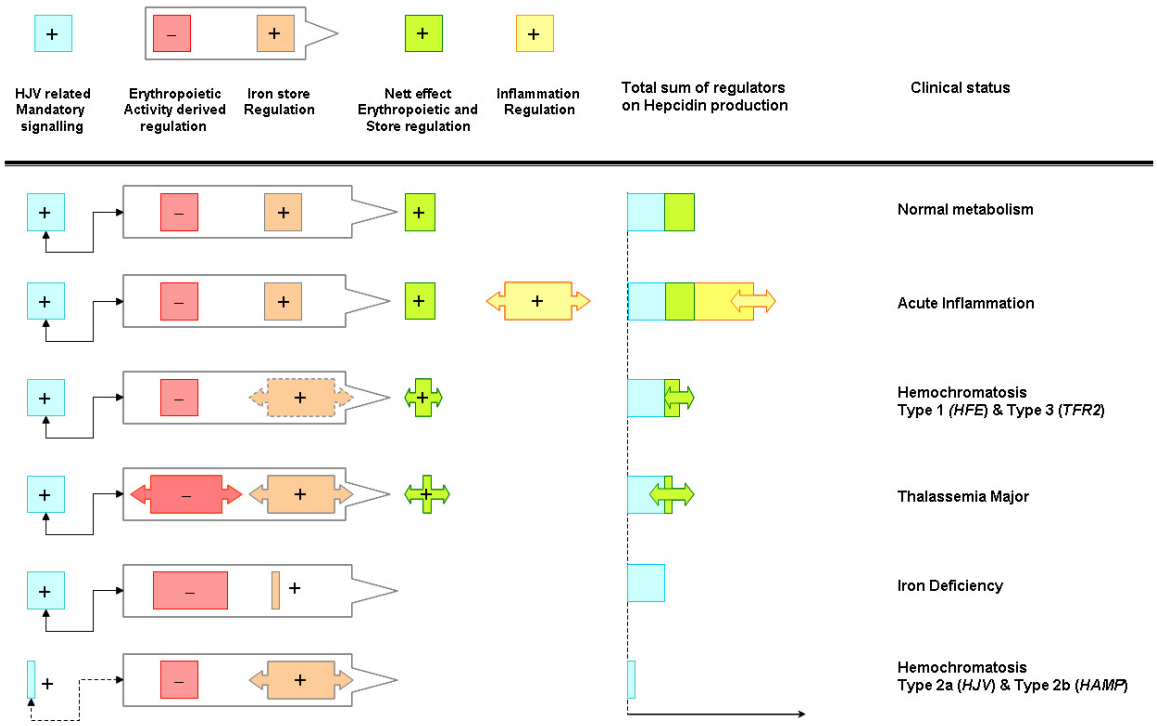


Figure 4, chapter 1, Page 22. An additive hypothetical model of hepcidin regulation in different disorders of iron metabolism. The model is based on the evidence provided by studies that are referred to in Table 1 (page 24) and that are at least partially described throughout the text of the paper. Different conditions of iron metabolism disorders are shown on the right in relation to the expected circulating hepcidin levels (second column from the right). The size of every colored box indicates the relative contribution of the specific regulator and should be interpreted in relation to the box size under normal conditions. In case the regulation pathway is affected by a gene mutation, a dotted line is drawn. The sum of all regulators indicates the expected hepcidin level. The erythropoietic regulator has a negative effect on hepcidin production and if strong enough is capable to suppress or overrule the inductive action of the iron store regulator. This might result in a nett decrease of hepcidin production, as occurs in the iron loading anemias, such as thalassemia major. Gene mutations in the iron store regulation pathway, such as in *HFE* or *TfR2*, reduce the effect of this store regulating pathway. This results in a lower hepcidin response than predicted from the elevated body iron stores, e.g. inappropriately low hepcidin levels Both, store and erythropoietic activity regulation depend on mandatory signaling by HJV/SMAD pathway. In case of disruption of this pathway, e.g. in the presence of HJV mutations induction of hepcidin by elevated iron stores is crippled. Finally, in this model inflammation acts as an additive regulator and is assumed to increase hepcidin levels regardless the activity of the other regulators.

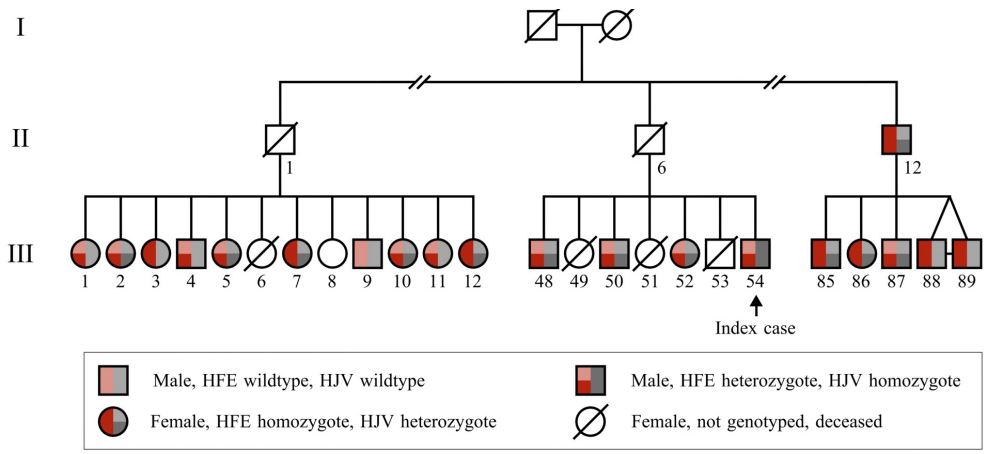


Figure 2, chapter 7, page 98. Family tree showing *HFE* and *HJV* gene mutations.

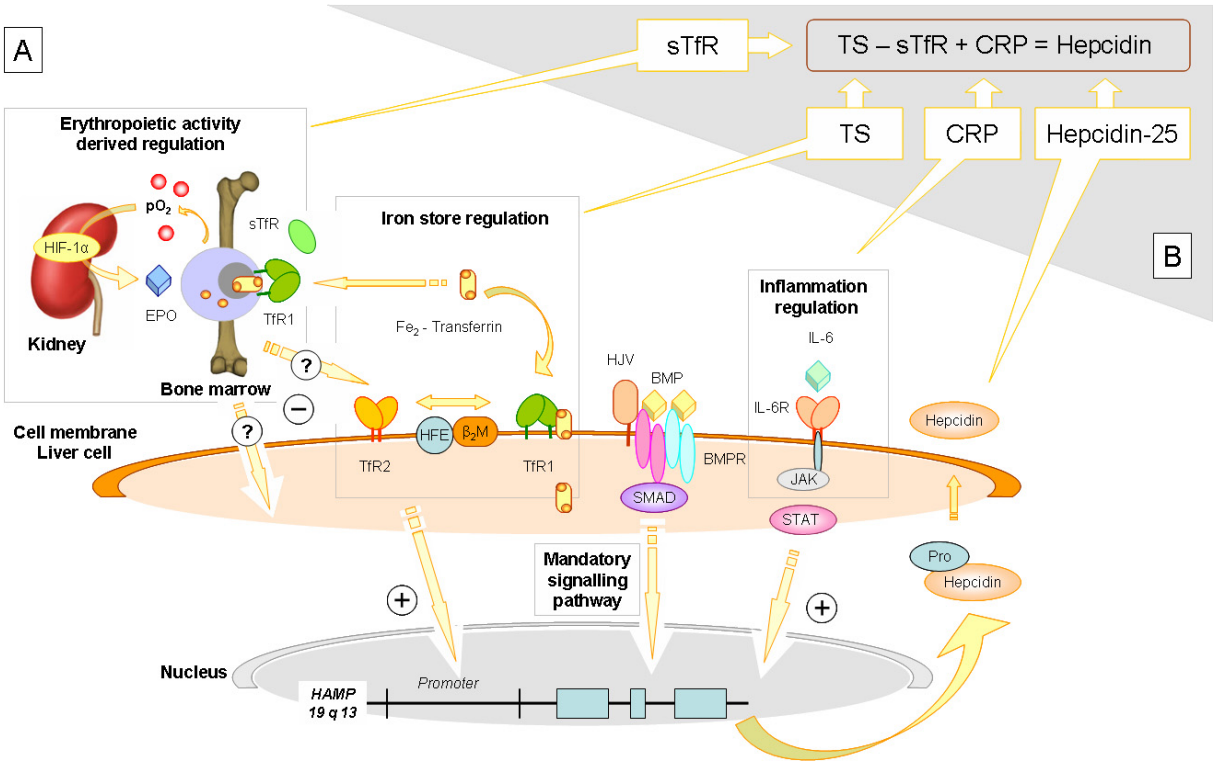


Figure 1, chapter 4, page 64/65. Model of pathways involved in hepcidin regulation as basis for a predictive algorithm. A) A regulation model constructed from the combined results of recent publications[6-11], focused on three relevant sites involved in hepcidin regulation: kidney, bone marrow and liver cells. It comprises three active regulation pathways and a mandatory signaling pathway that together orchestrate the (pro-)hepcidin production by interacting with the hepcidin anti microbial peptide (*HAMP)* gene on chromosome 19q13. In case of hypoxia or anemia, the **erythropoietic activity derived regulation** responds upon low oxygen pressure (pO_2) levels that induce hypoxia inducible factor (HIF)-1α stabilization in kidney cells, which results in erythropoietin (EPO) production of the kidney. EPO increases the erythropoietic activity and thus the need for iron of the bone marrow. Therefore, erythropoietic progenitor cells increase transferrin receptor (TfR)1 up-regulation. Communication between bone marrow and the liver is performed by a, thus fare, unknown factor, in order to release stored iron and increase absorption by the duodenal enterocytes. This factor might negatively interfere in the formation of a hepcidin inducing sensing complex of the **store regulation** pathway which acts upon the liver cells via circulating iron bound to transferrin, or might act by a still unknown direct inhibitory pathway. Glycosylphosphatidylinositol (GPI)-linked cell associated hemojuvelin (HJV) has been suggested to maintain a **mandatory regulation pathway** by bone morphogenetic protein (BMP)/SMAD signaling. Disruption of this pathway cripples the functionality of the erythropoietic activity derived and store regulation. The **inflammatory regulation** pathway, induced by interleukin (IL)-6 followed by Janus kinase (JAK)/signal transducer and activator of transcription (STAT)-3 signaling, is suggested to act more dominant regardless the status of the store and erythropoietic activity regulation pathways, and the HJV/SMAD pathway.
B) Algorithm based on measured TS, sTfR and CRP levels as reflecting parameters for iron store, erythropoietic activity, and inflammation respectively, by which relative hepcidin production can be estimated.