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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.

Therefore, 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.

Results showed that sTfR highly associates 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.

In conclusion, 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.

Keywords: Hepcidin; Iron deficiency; sTfR; Thalassemia; Iron

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]. Fig. 1A 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

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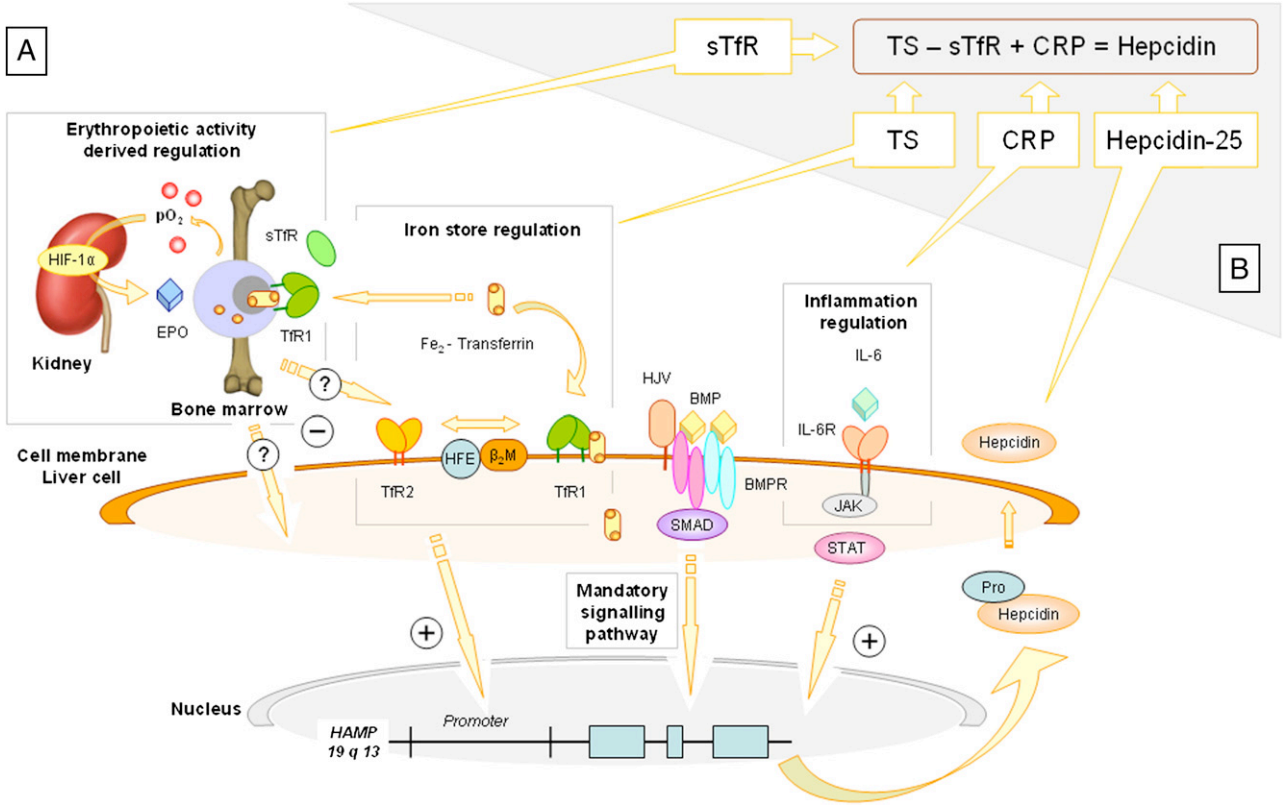


Fig. 1. Model of pathways involved in hepcidin regulation as basis for a predictive algorithm. (A) A regulation model constructed from the combined results of recent publications [6–11], focused on three relevant sites involved in hepcidin regulation: kidney, bone marrow, and liver cells. It comprises 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.

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 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 (Fig. 1B) 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 h 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 h 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

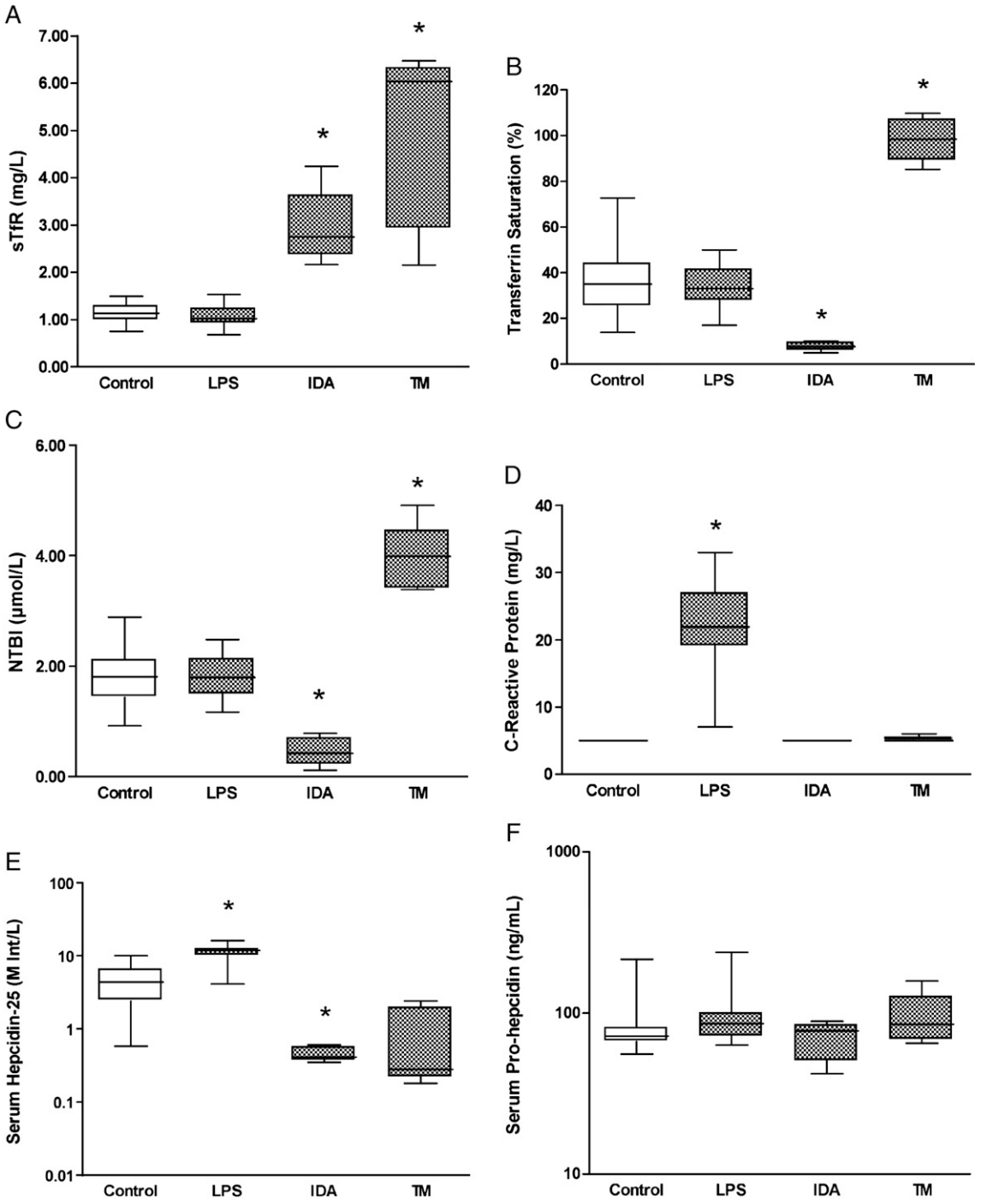


Fig. 2. Laboratory results of biochemical parameters representing regulatory pathways of hepcidin. Box plots show 25 and 75 percentile with median, and error bars represent minimum and maximum values. *Indicates significant difference from control group ($P<0.05$; non-parametric one-way ANOVA with Dunn's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

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 (Fig. 1), 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:

$$(\mathrm{TS} - \mathrm{sTfR}) + \mathrm{CRP} = \mathrm{Hepcidin} \tag{1}$$

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 Eqs. (2), (3), and (4) before importation in the algorithm (Eq. (1)):

$$\mathrm{TS} = \frac{(\mathrm{TS}_i - \mathrm{TS}_{\mathrm{ME}_{\mathrm{control}}})}{\mathrm{TS}_{\mathrm{ME}_{\mathrm{control}}}} \tag{2}$$

$$\mathrm{sTfR} = \frac{(\mathrm{sTfR}_i - \mathrm{sTfR}_{\mathrm{ME}_{\mathrm{control}}})}{\mathrm{sTfR}_{\mathrm{ME}_{\mathrm{control}}}} \tag{3}$$

$$\mathrm{CRP} = \frac{(\mathrm{CRP}_i - \mathrm{CRP}_{\mathrm{ME}_{\mathrm{control}}})}{\mathrm{CRP}_{\mathrm{ME}_{\mathrm{control}}}} \tag{4}$$

where i is the individual value and $\mathrm{ME}_{\mathrm{control}}$ is the 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.

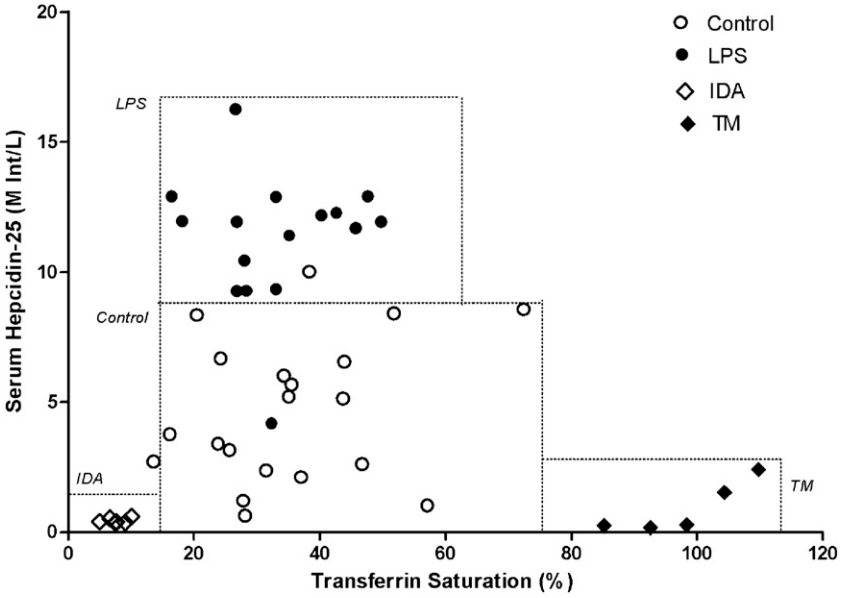


Fig. 3. Serum hepcidin-25 levels in relation to transferrin saturation levels in various iron metabolism disorders. Bracket lines give a rough indication of clusters of disorders of iron metabolism of similar etiology. Compared to the control population, TM patients show a small inter-individual variation 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.

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 h, 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 min at 95 °C, 40 two-step cycles were performed (30 s, 95 °C; 1 min, 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 one-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.

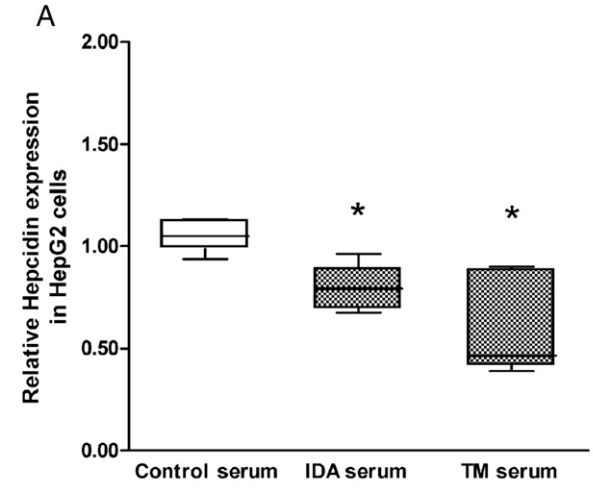
Results

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. We found that sTfR concentrations were strongly increased in both iron-deficient and thalassemia major patients (Fig. 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 (Fig. 2B). As shown in Fig. 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 ($R=0.958$; $P<0.001$, data not shown).

CRP levels were only elevated in the endotoxin-treated group (Fig. 2D), which showed the highest serum hepcidin values (Fig. 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 (Fig. 2E). In contrast to hepcidin, serum prohepcidin levels displayed no significant difference between all



	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)

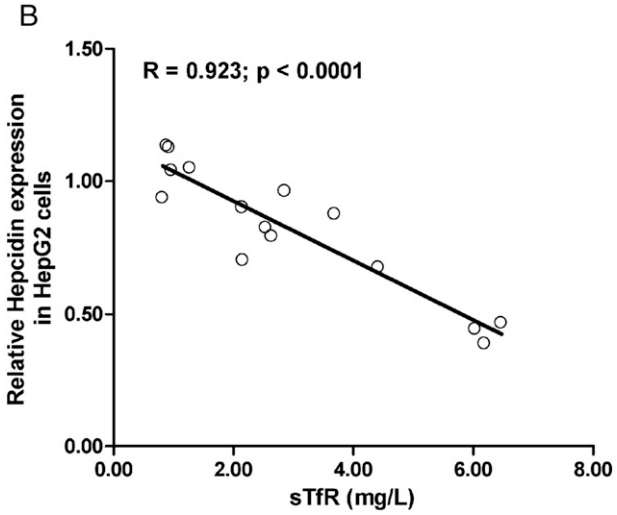


Fig. 4. Hepcidin mRNA expression in HepG2 cells in relation to sTfR concentrations of human sera. Panel A: Relative hepcidin expression measured in HepG2 cells after 72 h incubation with 10% serum of 5 healthy controls, 5 iron-deficient patients (IDA), and 5 thalassemia major patients (TM). Laboratory 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 (*$P<0.05$). Panel B shows a strong association between the expressed hepcidin levels and the sTfR levels in the sera added.

the groups tested (Fig. 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).

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 (Fig. 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.

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 (Fig. 4A). The relative hepcidin expression values strongly correlated to the measured sTfR values ($R=0.923$; $P<0.0001$; Fig. 4B).

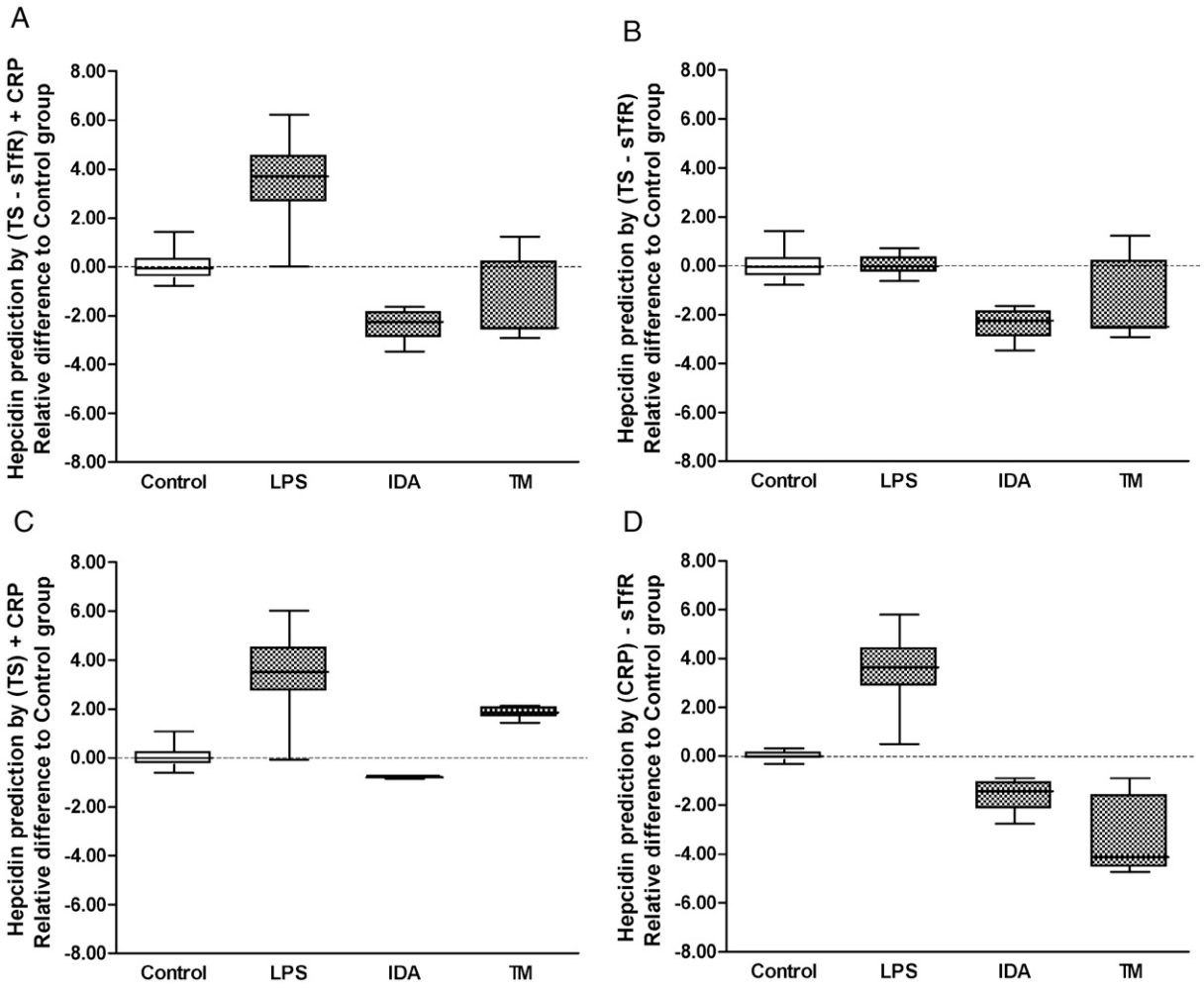


Fig. 5. Hepcidin prediction by an algorithm based on measured TS, sTfR, and CRP levels. Levels display the relative difference to the control group. Box plots show 25 and 75 percentile with median, and error bars represent minimum and maximum values. Dotted line indicates relative level of control population. 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. Fig. 5A shows that the predicted hepcidin levels closely resemble the actual measured levels in the studied groups as depicted in Fig. 2E. Linear regression analysis resulted in a strong significant correlation between the calculated and measured serum hepcidin levels ($R=0.756$, $P<0.001$; 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 outcome level of hepcidin under different conditions (Figs. 5B–D). When CRP was left out of the equation, this only influenced the hepcidin levels in the endotoxin-treated group (Fig. 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 (Fig. 5D).

Discussion

We defined an algorithm based on serum TS (iron store), sTfR (erythropoietic activity), and CRP levels (inflammation) to delineate the regulatory processes of hepcidin regulation in humans with various diseases of iron metabolism. 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 ($R=0.978$; $P<0.001$; 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". The algorithm might be of diagnostic use in the search for the genetic defect in patients with distorted iron homeostasis by revealing a "regulation gap" between measured and calculated hepcidin levels. In current clinical practice, similar tools are employed in the differentiation between various specific disorders. 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 (Fig. 2A) suggest individual differences in erythropoietic drive. Combined with differences in iron burden, this might explain the variation in serum hepcidin levels as displayed in Fig. 2E [21]. 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 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, are 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 provide insight in the contribution of each of the regulatory processes to the circulating hepcidin levels.

Acknowledgments

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