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## Laboratory use of hepcidin in renal transplant recipients

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### Abstract

Hepcidin is a small peptide with a critical role in cellular iron homeostasis, as it regulates utilization of stored iron and antimicrobial defense in inflammation (bacterial and fungal). Since it was isolated in 2000, and especially in the last decade, numerous studies aimed to evaluate the clinical use of plasma and urine hepcidin as a marker of anemia, especially anemia of chronic disease and post-transplant anemia (PTA). Hepcidin regulation is delicately tuned by two inflammatory pathways activated by interleukin-6 (IL-6) and bone morphogenic proteins (BMPs) and iron regulated pathway sensitive to circulating transferrin-iron (TR-Fe) complex. BMP-mediated pathway and TR-Fe sensitive pathway seem to be connected by hemojuveline, a BMP co-factor that interacts with transferrin receptor 2 (TRF2) in cases of high TR-Fe circulatory concentration. In addition to these regulatory mechanisms other regulators and signaling pathways are being extensively researched.

Hepcidin has been identified as an important contributor to morbidity and mortality in end stage renal disease (ESRD) but no such association has yet been found in case of PTA. However, there is an association between higher doses of erythropoiesis-stimulating agents (ESA) and mortality in the posttransplant period and the assumption that hepcidin might play a role in ESA resistance in PTA. Thus the review's main goal was to summarize papers published on the association of hepcidin with PTA, give up-to-date information on hepcidin regulation and on potential therapeutics that optimize hepcidin regulation. We also compared the performances of tests for hepcidin determination and reviewed research on immunosuppressants' (IS) effect on hepcidin concentration.

**Key words:** hepcidin; kidney transplantation; post-transplant anemia; iron homeostasis

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### Introduction

We reviewed all studies accessed by literature search of PubMed and SCOPUS from January 2000 to January 2015. The terms used as key words for the search were "hepcidin kidney transplantation" and "hepcidin post-transplant anemia". Additional papers were included after revision of reference lists in the found papers. Acquired data covers hepcidin physiology - regulatory pathways of hepcidin synthesis, isoforms of hepcidin and proteins that bind hepcidin in the circulation, as well as the role of kidneys in hepcidin excretion. Changes in hepcidin concentration after renal transplantation (TX) and their clinical significance could be useful

to nephrologists and other clinicians involved in the management of renal transplant recipients. On the other hand, specialists in laboratory medicine could benefit from a systematic review of all available methods used for hepcidin determination in plasma and urine and data on their precision and analytical recovery that we collected. We also gave possible reasons for different hepcidin reference values reported using different methods. Researchers in pharmaceutical industry could be interested in mechanisms that were reported to lower hepcidin and possibly lead to the development of therapeutics that target hepcidin regula-

tion. Finally, nutritionists could benefit from measurement and monitoring of hepcidin in studies assessing nutritional vs. iron substitution therapy where closer iron absorption is necessary. In this article we tried to address the contribution of high hepcidin concentration to post-transplant anemia (PTA), the possible relationship between hepcidin and other markers of iron status and glomerular filtration rate (GFR) after renal TX, the alteration of hepcidin concentration depending on the type of IS used after renal TX, the association between hepcidin and prohepcidin in the circulation; the differences in performances of tests for hepcidin determination and their current standardization.

### Physiology of hepcidin

#### Biosynthesis and processing of hepcidin

Hepcidin is a 25 amino acid (aa) peptide that regulates iron release from its stores. Krause *et al.* isolated hepcidin from human blood ultrafiltrate in the year 2000 and detected it as a cystein rich peptide with molecular mass of 2789.8 Da on matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) (1). It is predominantly synthesized in the liver. Low levels of hepcidin expression have also been detected in various human organs, such as heart, brain, gastrointestinal tract, bladder, lungs, glands, skeletal muscle and kidneys (Table 1). Immunohistochemical studies of liver biopsy samples using various region specific antibodies enabled the localization of hepcidin on the basolateral membranes of hepatocytes in periportal zones, with their concentration decreasing from portal triads towards central veins. There, hepcidin cDNA is translated to

**TABLE 1.** Expression of the hepcidin gene in different tissues (1).

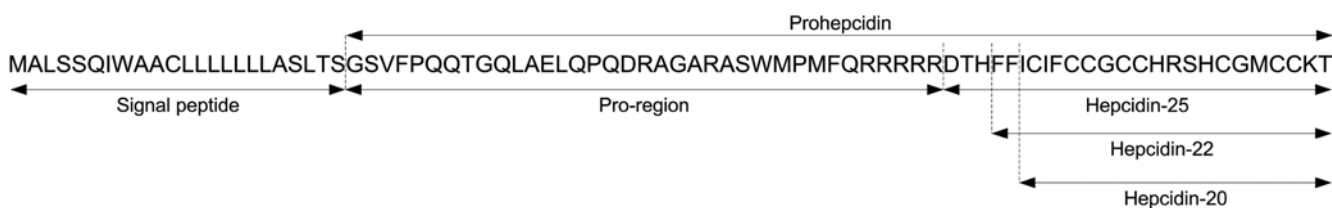
Tissue	Expression of hepcidin gene*
Stomach, duodenum, bladder, thyroid gland, adrenal gland, prostate gland, salivary gland, trachea, tonsils, lungs	1-10-fold
Brain	25-fold
Heart	105-fold
Liver	1550-fold

Gene expression was measured using real-time quantitative polymerase chain reaction (RT-PCR). Hepcidin expression for each tissue was divided by the value of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene and standardized by kidney expression (1-fold expression).

84 aa preprohormone with 24 aa signal peptide at the N terminal. After cleavage of the signal peptide, a 60 aa prohepcidin is formed, which is a prohormone without biological activity. Prohepcidin is converted to active hepcidin in plasma by convertases in blood or liver capillaries wall cells. It is possible that hepcidin-25 undergoes some N-terminal cleavage that results in the production of two smaller isoforms (hepcidin-20 and -22), or that these isoforms are products of prohepcidin cleavage by convertase. All isoforms of hepcidin are excreted in urine. Peptide sequence of human hepcidin is shown in Figure 1 (2).

#### Metabolic role of hepcidin

Like hepcidin-25, hepcidin-22 is an antimicrobial agent (*in vitro*), but the biological significance of hepcidin-20 is unknown. Active hepcidin has an immune and iron-regulatory role. Immunological-



**FIGURE 1.** Amino acid sequence of hepcidin precursors and 20-, 22- and 25-hepcidin peptides (2). Vertical dashed lines denote cleavage sites resulting in different peptide forms.

ly, hepcidin is consistent with type II acute phase proteins and three inflammatory mediators are needed for its induction in hepatocytes – IL-6, BMP6 and activin B. Immunological activation of hepcidin gene (*HAMP* – hepcidin antimicrobial peptide) transcription was first discovered in hepatocytes as a reaction to pro-inflammatory IL-6, but similar activation was detected in monocytes. In monocytes incubation with IL-6 and interferon- $\gamma$  (INF $\gamma$ ) increased hepcidin mRNA in a dose-dependent manner, 10-fold higher than in control hepatocyte cultures (HepG2 cells), as well as faster (peak induction of hepcidin mRNA in monocyte was at 1 hour of incubation and in HepG2 cells at 3 - 6 hours) (3). Higher hepcidin plasma concentrations in pro-inflammatory *milieu* are needed for protective reasons. In bacterial infection hepcidin “hides” iron in its stores from bacteria that have high-affinity iron-binding siderophores for retrieving iron from hosts’ transferrin and using it for their optimal growth and metabolism. It also kills bacteria on contact, like other defensin proteins (4). It is possible that in the same way elevated hepcidin makes it harder for cancer cells to get the iron they need for their growth (5).

Hepcidin’s iron-regulatory function consists in disabling the passage of stored iron from enterocytes into the duodenum; and from hepatocytes and macrophages into the circulation. This is achieved by hepcidin’s binding to the iron channel ferroportin. Ferroportin is the only iron exporter in these iron storage cells and it is rapidly degraded after hepcidin’s binding (6). After ferroportin degradation, the retained iron is “trapped” in cytoplasmic ferritin form and unavailable to be used for erythropoiesis.

Use of radiolabeled  $^{125}\text{I}$ -hepcidin revealed that about 90% of hepcidin circulates bound to  $\alpha_2$ -macroglobulin (specific binding) and the rest is bound to albumin (nonspecific binding with non-saturable kinetics) or unbound. Hepcidin bound to  $\alpha_2$ -macroglobulin seems to be active – it was found to decrease ferroportin expression in cell culture (7). The recent study by Huang *et al.* showed that serum iron levels are reduced to a significantly greater extent in mice treated with  $\alpha_2$ -macroglobulin-hepcidin complex than in those

treated with unbound hepcidin. The researchers suggested that this binding delays hepcidin excretion by the kidney and increases its circulatory half-life and efficacy compared to free hepcidin (unbound  $^{125}\text{I}$ -hepcidin was significantly increased in the kidney and urine relative to  $^{125}\text{I}$ -hepcidin complexed to  $\alpha_2$ -macroglobulin) (8).

### Regulation of hepcidin activity

Although hepcidin peptide was isolated in 2000, *HAMP* gene was not discovered until two years later, in 2002 by Nicolas *et al.*, on chromosome 19 (NCBI Gene ID 57817) (9). An inflammatory triggered pathway includes IL-6 that binds to IL-6 receptor (IL-6-R) and activates Janus kinase 2/signal transducer and activator of transcription 3 (JAK2/STAT3) signaling to up-regulate *HAMP*. This activation was also observed in cultures of primary human hepatocytes from micro-inflamed ESRD (10). Very intensively studied inflammatory pathways include members of transforming growth factor- $\beta$  (TGF- $\beta$ ) superfamily (BMPs 2, 4, 5, 6, 7 and 9) that were found to induce BMP signaling by phosphorylation of Sma and Mad related proteins (SMADs). Most of the data was collected on BMP6, which together with activin B binds to bone morphogenic protein receptor (BMP-R) and phosphorylates cytoplasmic SMAD1, 5 and 8 proteins that, together with SMAD4, translocate to the nucleus and stimulate hepcidin expression by interaction with BMP responsive elements on *HAMP* promoter (11,12). In *Bmp6* disrupted mice hepcidin expression was so low that it led to iron overload which could not be compensated by other BMPs. Membrane hemojuvelin (mHJV) was identified as a BMP co-receptor in this pathway. Homozygotes or combined heterozygotes mutations of glycosylphosphatidylinositol-(GPI-) anchored mHJV in humans markedly reduce hepcidin expression and cause juvenile hemochromatosis. This mHJV is an important factor in the iron regulatory pathway of hepcidin expression and represents its connection to BMP-SMAD pathway. When TR-Fe complex concentrations increase in the circulation, like in hemochromatosis, it binds to transferrin receptor 1 (TRF1) and displaces hereditary hemochromatosis protein (HFE) and signals *HAMP* induction, possibly through in-

teraction with TFR2. Hepcidin regulation by HFE, which interacts with TFR2 and mHJV to form a "supercomplex" and leads to up-regulation of hepcidin expression *via* BMP-SMAD signaling, is still not entirely clarified. Use of tagged proteins showed "supercomplex" formation in cell cultures, but their interaction has not been shown *in vivo* (in mice and humans) and the precise molecular mechanism of HFE and TRF2 role in hepcidin regulation is still under investigation (13). Matriptase-2 (MT-2) is a membrane protease in hepatocytes that acts as a hepcidin suppressor and inhibits the "supercomplex" formation. MT-2 was tested in human and animal models and it was found that MT-2 mutations cause high hepcidin expression and iron deficiency anemia refractory to therapy. MT-2 cleaves mHJV in hepatocytes and mHJV cannot act as a BMP co-receptor. Neogenin is a ubiquitous cell surface protein that forms a tertiary complex with MT-2, BMP receptor and HJV thus facilitating MT-2 mediated HJV cleavage. It was found to be essential for HJV function in mice, but this is still to be confirmed in humans (14). Another hepcidin up-regulating mechanism appears to be activated by endoplasmic reticulum stress, which activates hepcidin promoter gene through the cyclic adenosine monophosphate (cAMP) response element-binding protein H (CREBH), a liver-specific transcription factor (15). It physiologically prevents iron utilization and iron overload; its down-regulation was first documented in iron overload disorders - most forms of hereditary hemochromatosis (16-19) and later in cases of thalassemia (20). Except hereditary causes for hepcidin down-regulation, such as HFE mutations in hereditary hemochromatosis and mHJV mutation in juvenile hemochromatosis, the most specific evidence on hepcidin suppression were found for MT-2. Other studies identified hypoxia-inducible factors (HIF), HIF-1 $\alpha$  and HIF-2 $\alpha$ , which presumably have a role in down-regulation of hepcidin in hypoxia. They bind to enhancer element of erythropoietin (*EPO*) gene and activate its transcription (21). A study on hepatocyte cultures showed that proteolytic cleavage of prohepcidin to hepcidin is not regulated by TR-Fe complex or the HIF pathway (22). Two other research groups found that hepcidin expres-

sion was not directly regulated by HIFs (23,24). Thus the effect of HIFs on hepcidin down-regulation is still controversial. One study found that hepatocyte mHJV was elevated only in chronic kidney disease (CKD) patients undergoing hemodialysis (HD), not in renal transplant recipients or in CKD patients who weren't treated with HD. In this study each of the 3 patient's groups had only 31 patients (25). Molecular mechanisms responsible for the increase in hepcidin concentrations in hypoxia are yet to be clarified. Several studies showed that the induction of erythropoiesis causes down-regulation of hepcidin, and not hypoxia or anemia. In cases of bone marrow suppression by cytotoxic agents or irradiation, the down-regulation of hepcidin does not occur (26,27). Furthermore, when EPO was subcutaneously administered to healthy humans, hepcidin was profoundly suppressed in the next 24 hours (and gradually recovered during the following two weeks), while soluble transferrin receptor and growth differentiation factor-15 (GDF15) remained constant (28). Ashby *et al.* examined few candidates for bone marrow-derived mediators of hepcidin suppression. EPO was considered to lead to hepcidin suppression by reducing transferrin saturation, but hepcidin decrease in serum precedes the reduction in transferrin saturation (29). A study performed on freshly isolated mice hepatocytes and human hepatocytes (cell line HepG2) showed that hepcidin mRNA was down-regulated by EPO, administered in supraphysiological, therapeutic doses, in a dose-dependent manner. This effect was blocked by anti-EPO receptor (anti-EPO-R) antibody, along with a down-regulation of CCAAT/enhancer-binding protein  $\alpha$  gene (*CEBPA* gene) mRNA that is responsible for expression of signaling protein C/EBP $\alpha$  (CCAAT/enhancer-binding protein  $\alpha$ ). Protein C/EBP $\alpha$  binds to *HAMP* promoter and directly reduces hepcidin expression as a downstream result of EPO binding to EPO-R on hepatocyte surface (30,31) (Table 2). Schematic representation of hepcidin regulation pathways can be found in Figure 2.

Lakhal *et al.* showed that infusion of iron chelator deferoxamine increases plasma GDF15 *in vivo*, but the finding was limited by small number of partici-

**TABLE 2.** Molecular regulation of hepcidin transcription (15,31).

Regulator	Mechanism of regulation	Result
<b>BMP</b>	Binds to BMP receptors on hepatocytes	SMAD-mediated induction of <i>HAMP</i> transcription
<b>mHJV</b>	Acts as a BMP co-receptor on hepatocytes	Increases signal for SMAD-mediated <i>HAMP</i> transcription
<b>sHJV</b>	Competes with HJV for BMP	Decreases signal for SMAD-mediated <i>HAMP</i> transcription
<b>MT-2</b>	Cleavage of HJV on hepatocytes	Inhibits BMP and SMAD-mediated <i>HAMP</i> transcription
<b>IL-6</b>	Binds to IL-6 receptor on hepatocytes	Activates JAK2/STAT3-dependent signal pathway to promote <i>HAMP</i> transcription
<b>TR-Fe</b>	Binds to transferrin receptor 1 or 2 on hepatocytes	Activates ERK 1/2-mediated <i>HAMP</i> transcription
<b>HFE</b>	Binds to transferrin receptor 1 and 2 on hepatocytes (competes with TR-Fe)	Inhibits activation of ERK 1/2-mediated <i>HAMP</i> transcription
<b>Endoplasmatic reticulum stress</b>	Activates <i>CREBH</i> in hepatocytes	<i>CREBH</i> binds and activates <i>HAMP</i> promoter gene
<b>EPO</b>	Binds to EPO-R on hepatocytes	Deactivates <i>CEBPA</i> transcription and decreases C/EBP $\alpha$ -mediated activation of <i>HAMP</i> promoter
<b>HIF</b>	Binds to enhancer elements of <i>EPO</i> and <i>HAMP</i> genes	Activates transcription of <i>EPO</i> and deactivates transcription of <i>HAMP</i> . Reduces hepcidin (?)
<b>Activin B</b>	Activates SMAD signaling pathway in hepatoma-derived cell cultures	Contributes to inflammatory SMAD-mediated <i>HAMP</i> transcription <i>in vivo</i> (?)
<b>GDF15</b>	Induces changes in SMAD (?)	Decreases SMAD-mediated <i>HAMP</i> transcription (?)
<b>TWSG1</b>	Blocks BMP signaling pathway	Decreases SMAD-mediated <i>HAMP</i> transcription (?)

BMP – bone morphogenic protein; *HAMP* – hepcidin gene; mHJV – membrane hemojuvelin; sHJV – soluble hemojuvelin; MT-2 – matriptase-2; IL-6 – interleukin 6; JAK2/STAT3 – Janus kinase 2/signal transducer and activator of transcription 3; TR-Fe - transferrin-iron complex; ERK 1/2 – extracellular-signal-regulated kinase 1/2; HFE - hereditary hemochromatosis protein; *CREBH* - cAMP response element-binding protein H; EPO-R – erythropoietin receptor; *CEBPA* – CCAAT/enhancer-binding protein  $\alpha$  (C/EBP $\alpha$ ) gene; HIF – hypoxia inducible factor; GDF15 – growth differentiation factor 15; TWSG1 – twisted gastrulation 1.

pants (8 healthy volunteers) and the increase was modest compared to *in vitro* responses in human cell lines. They investigated GDF15 further and in a study with slightly larger number of subjects (21 iron-replete and 22 iron-deficient) found that GDF15 was higher in iron-deficient subjects. GDF15 is a BMP antagonist, but was not elevated in healthy subjects, iron deficiency or reduced hepcidin expression by blood loss (32).

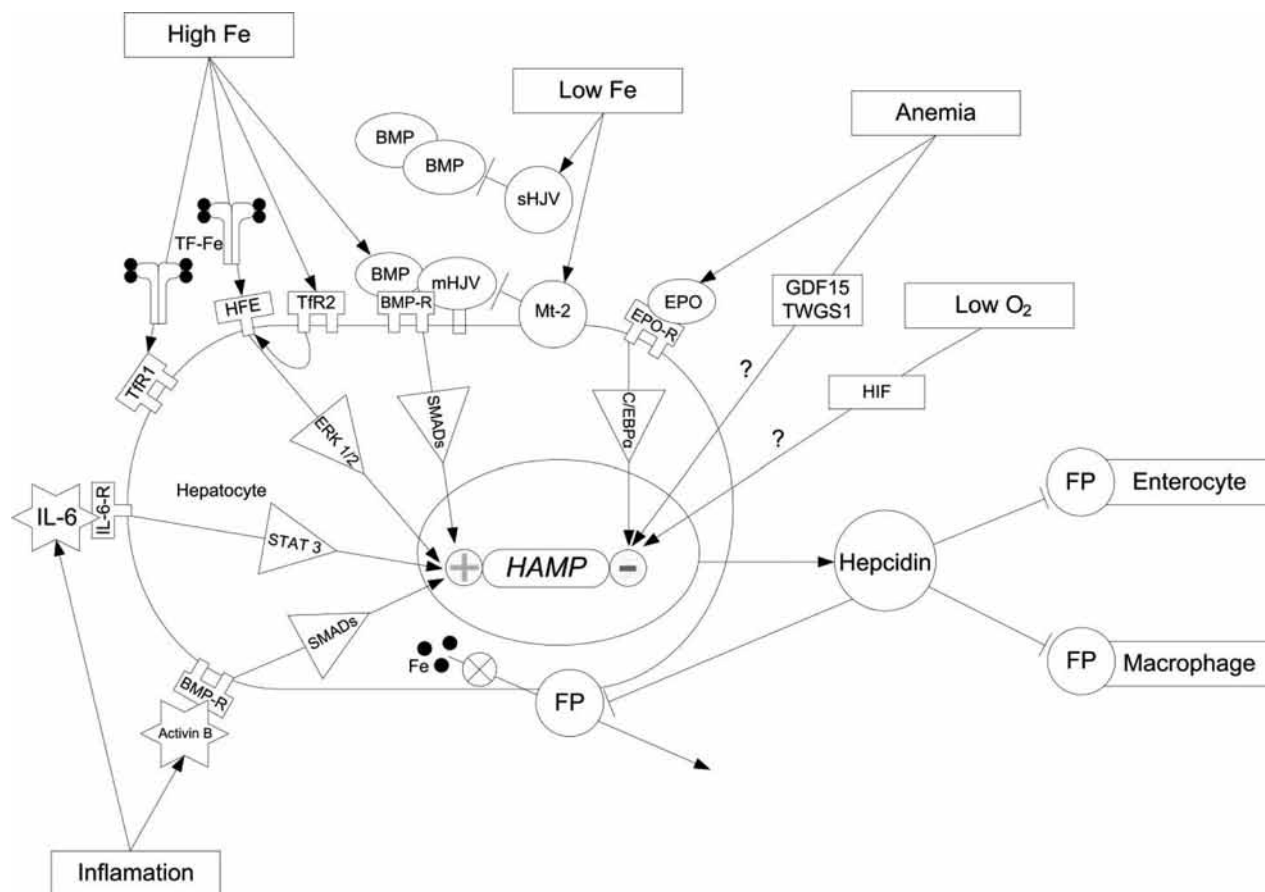
## Hepcidin in renal transplant recipients

### The role of hepcidin in pathogenesis of anemia and its significance as biomarker of anemia

The European TRESAM Survey published in 2003 reported anemia prevalence of 38.6%, and very similar prevalence of 42% was reported in 10 Euro-

pean centers in 2010 (33,34). This makes anemia more prevalent in kidney transplant patients than in GFR-matched CKD-patients. PTA shows two peaks. The early peak (within 3 months after TX) occurs because of low pre-transplant hemoglobin (Hb), blood loss or infection during surgery, aggressive hydration or frequent blood sampling after surgery, delayed graft production of EPO and IS therapy influence (35). The late peak is associated with a decline in kidney function (36-38).

Hepcidin is predominantly produced in liver upon iron overload, hypoxia and inflammation, but kidneys are also involved in the synthesis of hepcidin, as well as in its elimination. Thus hepcidin concentrations are influenced by kidney graft function and post-transplant iron status. Hepcidin synthesis is up-regulated in uremia as in other inflammatory



**FIGURE 2.** Regulation of hepcidin antimicrobial peptide (*HAMP*) expression.

Iron abundance (high Fe) induces bone morphogenic proteins (BMPs) binding to their receptor (BMP-R) and SMAD-mediated induction of *HAMP*. Membrane hemojuvelin (mHJV) is a BMP co-receptor. Matriptase-2 (MT-2) is active in iron deficiency and hypoxia, inhibits BMP signal transmission. Soluble hemojuvelin (sHJV) is cleaved from HJV by furine convertase or type II transmembrane serin protease TMPRSS6. It competes with sHJV in BMP and has a negative effect on BMP signal transmission. High iron concentration causes binding of transferrin-iron complex (TR-Fe) to transferrin receptor 1 (TfR1). This binding displaces hereditary hemochromatosis protein (HFE) and signals *HAMP* induction, through interaction with transferrin receptor 2 (TfR2) and extracellular-signal-regulated kinases 1/2 (ERK1/2) signal pathway.

Inflammation stimulates hepcidin production through two mediators, interleukin-6 (IL-6) and activin B. IL-6 binds to IL-6 receptor (IL-6-R) on hepatocyte and activates Janus kinase 2/signal transducer and activator of transcription 3 (JAK2/STAT 3) signal pathway. Activin B binds to BMP-R and activates SMADs. Both inflammatory mediators have a positive effect on *HAMP* expression. Anemia causes an increase in production of erythropoietin that binds to erythropoietin receptor (EPO-R) and down-regulates expression of signaling protein CCAAT/enhancer-binding protein α (C/EBPα) and hepcidin. The mechanisms of hypoxia inducible factor (HIF), growth differentiation factor 15 (GDF15) and twisted gastrulation (TWGS) as *HAMP* modulators is still controversial.

states. Przybyłowski *et al.* conducted a well designed study including 170 renal allograft recipients and 168 patients after orthotopic heart TX, with very similar IS therapy used and no signs of inflammation or rejection. The prevalence of PTA was 37% among kidney transplant patients and 34% among heart transplant patients. Anemic kid-

ney transplant patients, as well as anemic heart transplant recipients, exhibited significantly higher hepcidin when compared to their non-anemic counterparts. Based on their results it seems that the observed PTA and impaired kidney function (also present after heart TX), and not inflammation, were the leading causes of hepcidin elevated

concentrations since eGFR and ferritin, but not hsCRP were found to be predictive of hepcidin concentrations (39). Interestingly, the authors concluded that it was plausible that hepcidin is not a pathogenic factor for PTA among heart or kidney transplant recipients. The role of hepcidin in pathogenesis of anemia of chronic disease is very well documented, especially in ESRD, since it is an important contributor to morbidity and mortality in ESRD.

### **The role of hepcidin in resistance to erythropoiesis-stimulating agents (ESA) after renal transplantation (TX)**

High plasma hepcidin concentrations decrease iron availability to bacteria and cancer cells leading to anemia of chronic disease, despite the therapy with ESA (e.g. cancer, autoimmunity or chronic infection) (40). A functional iron deficiency can be often observed in renal transplant recipients – iron stores are adequately filled (normal or high ferritin levels and transferrin saturation at 20% or less), but iron is unable to be released from its stores, and subsequently erythropoiesis is inadequate. Since clinicians treat PTA with ESA, hepcidin could be used to clarify the often found disbalance in iron metabolism and resistance to ESA in the post-transplant period. Therefore, hepcidin could become an important tool to predict ESA responsiveness and to guide treatment with ESA and intravenous iron. The prediction of ESA resistance would be an important step forward in treating anemia after renal TX. Two cohort studies have reported an association between higher doses of ESA and mortality (41,42), as well as the results of randomized controlled trials that reported an increased mortality or morbidity in patients who were targeted to high Hb concentrations (43,44).

### **Hepcidin in relation to other biomarkers of anemia**

Many studies showed an association between hepcidin and ferritin, either by correlation of the two parameters or ability of ferritin to predict hepcidin by multiple regression analysis. Indeed, hepcidin and ferritin have some common characteris-

tics – their plasma concentration is decreased in iron deficiency and increased in inflammation (but not specifically, as they are both elevated in various cancers), and can be used in treating anemia after renal TX. In this sense, hepcidin shows some advantages over ferritin – it reflects iron availability and not just iron stores, and better reflects the status of iron homeostasis than single parameters such as transferrin saturation, soluble transferrin receptors and CRP (45). A study was done by Kato *et al.* to evaluate hepcidin and prohepcidin as potential predictors of ESA responsiveness in a small number of CKD patients on HD. Neither hepcidin nor prohepcidin were found to be good predictors of ESA responsiveness (46). Besides the small number of subjects (only 75), the limitations of this study were that hepcidin was measured only semiquantitatively (peak intensity of serum hepcidin-25 by SELDI-TOF MS). Multiple regression analysis did not show statistically significant association between prohepcidin and hematopoietic parameters, but prohepcidin had a weak relationship with ESA dosage. The biggest disadvantage of using prohepcidin is that it is not an active form of hepcidin, and there is no clear relationship between prohepcidin and hepcidin concentration in circulation, since available part of hepcidin degrades to iron-regulatory inactive isoforms (hepcidin-20 and -22). Malyszko *et al.* conducted a study that enrolled 70 subjects after renal TX and correlated hepcidin to other renal markers. Hepcidin was associated with total protein, creatinine, ferritin, eGFR (calculated by MDRD equation) and time after TX. Multiple regression analysis showed that only eGFR and ferritin could be considered as predictors of hepcidin. Since in healthy subjects hepcidin showed no association with creatinine and eGFR, it could be assumed that hepcidin is accumulated in renal insufficiency CKD and could be used as a marker of it (47). The limitation of hepcidin determination in this study was the determination by ELISA, which is not completely specific for hepcidin-25 (hepcidin-20 and -22 can also be measured). When compared to other markers of iron status, such as iron, ferritin and transferrin saturation, hepcidin 25 seems to be the most sensitive indicator of iron absorption. This is an indica-



tion that in the future hepcidin could substitute conventional assays for measuring iron status. The only drawback is that hepcidin is limited to non-inflammatory conditions, but this concerns also other tests for monitoring iron concentrations.

### Elimination of hepcidin by kidneys

Since hepcidin is a very small peptide, it is filtered freely in the glomeruli and almost completely reabsorbed in the tubules (fraction extraction of hepcidin estimated in patients with hemochromatosis and thalassemia was found to be less than 3%) (48,49). Thus, it is clear that hepcidin is eliminated by the kidneys, but it is still unclear which process is determining the rate of hepcidin elimination - filtration, reabsorption, local production and/or degradation. A study with limited number of participants has provided evidence that tubular reabsorption (*via* megalin-mediated endocytosis) and local production in distal kidney tubules may contribute to urine hepcidin concentrations. This production is stimulated by monocytes in inflammatory conditions, but monocytes are also capable of producing hepcidin, at least in cell cultures, when stimulated with pro-inflammatory cytokine. Furthermore, an increase of urine hepcidin-25 was considered a potential marker for tubular degradation in patients with CKD since it seems to accumulate in plasma and, according to Peters *et al.*, it is due to inadequate tubular reabsorption in patients with biopsy proven tubular disease (similar observation was found earlier for prohepcidin by Taes *et al.*) (50,51). But the effect of uremia and how residual renal function determines hepcidin clearance is yet to be determined. Studies on a larger number of human subjects are necessary to confirm if inflammation after renal TX causes distal tubules and/or monocytes in kidney to produce hepcidin, determine the extent of this production and its clinical value. If local hepcidin production would prove to be valuable in monitoring the graft function, that would justify the determination of hepcidin concentrations in urine samples as markers of kidney inflammation and possibly predictors of post-transplant tubular degradation and renal rejection.

### Hepcidin and kidney disorders

As an acute phase protein, hepcidin is induced in inflammation. The subclinical inflammation often found in patients with CKD limits its use as a marker of renal function. Since study by Peters *et al.* associated serum hepcidin with tubular degradation in CKD (hepcidin inversely correlated to eGFR), serum and urine hepcidin was investigated as a marker of acute kidney injury (AKI). Urinary hepcidin was found to be a protective marker for AKI after cardiopulmonary bypass surgery. Interestingly, a smaller rise in hepcidin's urine concentrations implied a greater renal injury (resulting in lesser hepcidin reabsorption from primary filtrate) and was associated with higher risk of AKI (52). Two papers published by Ho *et al.* found that increased urine hepcidin concentrations indicated lower risk of developing AKI after cardiac surgery, and this was confirmed in a larger study with 100 subjects. The increased local production of hepcidin in kidney is attributed to the prevention of oxidative damage induced by free iron in AKI (hepcidin binds  $Fe^{2+}$ ) (53,54). It was found that in patients with lupus nephritis changes in urine hepcidin-25 and -20 predict renal flares. Hepcidin-25 serum concentration decreased in a renal flare and hepcidin-20 increased 4 months before a renal flare (this urine proteomics study was limited to 25 samples) (55). Lack of evidence regarding clinical significance of urine hepcidin concentration after renal TX renders urine hepcidin use in monitoring controversial. CKD is associated with increased serum hepcidin and it contributes to anemia and ESA resistance. If ESA resistance could be predicted or even abolished by hepcidin monitoring or reduction, it would mean better management of CKD patients, and need for lower doses of ESA to correct anemia, since higher doses carry a risk of dose-related side effects. It seems that hepcidin reflects status of chronic (in CKD) and acute inflammation (in AKI), but by different mechanisms. In CKD hepcidin is up-regulated in the liver and in AKI presumably in the kidney, and therefore it is necessary for future research to measure hepcidin in serum and urine. Since hepcidin is an inflammatory inducible peptide, it can only be used as an

indicator of renal function changes in cases of CKD without acute inflammation. In case of AKI, hepcidin rise in serum is caused by increase of TR-Fe and IL-6 that induce hepcidin expression, thus it can act in preventing iron oxidative damage. But why a post-surgically better functioning kidney tubules (not prone to subsequent AKI) do not completely reabsorb hepcidin and degrade it is still unknown.

### The influence of immunosuppressive therapy

Immunosuppressive therapy (IS) after renal TX contributes to the development of anemia through different mechanisms. Antimetabolites, such as azathioprine, mycophenolic acid (MPA) and mycophenolate mofetil (MMF), have a direct effect on bone marrow cells and their suppression may aggravate anemia (39,56). In a cohort study with 878 renal transplant recipients it was reported that 58% of them on MMF were anemic and presented with lower GFR than patients not given MMF. A possible confounding factor was that all patients on treatment with ESA were classified as anemic, irrespective of hemoglobin concentrations. Perhaps those patients would be better classified by hemoglobin concentration. The effect of this potential classification error is not large, since only 68 adults were stimulated by recombinant EPO and 75% stayed anemic despite therapy (57). In a smaller study on 170 renal transplant recipients, those treated with MMF or MPA showed lower hemoglobin compared to patients not treated with MMF or MPA. Similar observations were found in patients treated with mTOR inhibitors sirolimus or everolimus, which can cause myelosuppression and anemia particularly when administered together with MMF. Maiorano *et al.* conducted a study that included 42 biopsy-proven chronic allograft nephropathy patients randomized to receive cyclosporine or sirolimus. Based exclusively on hepcidin results, the authors suggested that sirolimus interferes with iron metabolism and that in this case PTA does not present the features of inflammation-related anemia, since serum hepcidin was similar in the two groups after therapy rand-

omization. However, their observation is based only on hepcidin, ferritin and transferrin concentrations, not on other inflammatory markers, and the study in general is of limited power due to a small number of patients and the fact that it measured prohepcidin and not the active hepcidin-25 (58). Thauinat *et al.* investigated CRP, IL-6 and IL-10, and reported an opinion opposite to Maiorano: sirolimus induces a defective IL-10-dependent autoregulation of inflammation, thereby inducing anemia, in cases of late introduction of sirolimus (59). A retrospective study performed on 93 allograft recipients with sirolimus-induced anemia and microcytosis showed no impact of MMF on the occurrence or severity of PTA (60). In assessing the influence of IS therapy on hepcidin anemia after renal TX first thing that should be made clear in future studies is which IS (if any) can be associated with subclinical inflammation that could cause a rise in hepcidin (and lead to functional iron deficiency and impaired gastrointestinal absorption of iron). Hepcidin measurement in such anemic patients might help prove IS role in PTA pathogenesis. This mechanism seems more likely for mTOR inhibitors, at least for now. As far as MMF/MPA therapy is concerned, it seems that anemia is caused by IS effect on bone marrow, rather than the inflammatory effect that could cause a hepcidin rise.

### Hepcidin determination

The small size of hepcidin and its different isoforms made it a challenge to produce a specific immunoassay and therefore hepcidin was considered a hormone very difficult to measure. Radioimmunoassay (RIA) is very sensitive and specific immunoassay method that was first considered for hepcidin measurement. It uses a competitive principle - radioactive  $^{125}\text{I}$  bound to tyrosine or histidine of synthetic hepcidin-25 (in known amount) is mixed with serum from patient containing an unknown quantity of hepcidin. The radioactive and non-radioactive hepcidin molecules compete for polyclonal rabbit anti-hepcidin-25 antibody. Immunocomplexes are separated using a secondary antibody-PEG assisted separation and quantified by radioactivity measurement. Since this

method has been suppressed by non-radioactive immunoassays (ELISA), the only commercially available RIA test is "Hepcidin-25 RIA kit" (Bachem, UK). Manufacturer has declared measurement range of 0.1-13 ng/mL, using the hepcidin-25 as standard. Ashby *et al.* evaluated this assay and found it to be linear up to 200 ng/mL, with a detection limit of 0.6 ng/mL, intra-assay precision 7.2% (at 3 ng/mL) and 5.8% (at 35 ng/mL), and inter-assay precision 7.6% (at 3 ng/mL) and 6.7% (at 35 ng/mL). Analytical recovery was 98% (5 ng/mL) and 97% (40 ng/mL). Cross-reactivity with synthetic hepcidin-20 was observed, and it was 9.6% (200 ng/mL). There was no cross-reactivity with prohepcidin (25). A 10-20% cross-reactivity with hepcidin-20 and -22 was found (61).

The first ELISA for serum hepcidin was developed and evaluated by Ganz *et al.* It was a competitive assay with biotinylated hepcidin-25 (Intrinsic LifeSciences, La Jolla, CA, USA) as a tracer and a synthetic hepcidin was used for standard curve measurement. No accuracy or recovery data was reported, but intra-assay precision of 5-19% and inter-day precision of 12% was found. Measurement range was declared at 5.6 - 4000 ng/mL, but precision data indicate that the detection limit may be closer to 50 ng/mL. The extent of cross-reactivity with hepcidin-20 and -22 has not been stated for this assay, and should be examined in the future. Their evaluation study enrolled 114 healthy volunteers and showed a diurnal increase of serum hepcidin at noon and 8 p.m. compared with 8 a.m., and a transient rise as a response to iron ingestion. Using this immunoassay they found normal values of hepcidin to be 29 - 254 ng/mL in men and 17 - 286 ng/mL in women (48). The same ELISA test was later reported to have median hepcidin concentrations of 73 ng/mL for healthy subjects and 270 ng/mL for CKD patients. Researchers concluded that their findings are in agreement with those reported by Ashby *et al.* for hepcidin levels in CKD patients using radioimmunoassay (62), which determined normal range for healthy subjects at 2 - 56 ng/mL with median hepcidin concentrations of 11 ng/mL. In CKD patients, hepcidin concentrations ranged from 3 - 153 ng/mL with a median 26.5 ng/mL (25). Considering the very wide range, the

method would probably be better suited for hepcidin measurement in renal transplant recipients and CKD patients rather than healthy subjects, since they are expected to have higher hepcidin. Precision must be taken into account, considering that precision data indicates that the detection limit of competitive ELISA could be much higher than manufacturer states (50 ng/mL, and not 5.6 ng/mL), which is in range with hepcidin concentrations expected in healthy subjects. Besides the difference in calibration, antibody affinity for different isoforms, binding to  $\alpha_2$ -macroglobulin and albumin, circadian rhythm and lack of standardization in blood collection may also contribute to variations in hepcidin concentration in healthy population.

Commercially available kit "Hepcidin-25 (bioactive) ELISA" (DRG International Inc., Springfield, NJ, USA) uses a sandwich principle with monoclonal antibodies against hepcidin-25 in serum/plasma, with no detectable cross-reactivity to prohepcidin and no reports on cross-reactivity to hepcidin-20 and -22. This presents a problem when measuring hepcidin in patients with ESRD since they have higher hepcidin-20 and -22 concentrations in serum (63). The manufacturer declares an assay range of 0.35 - 80 ng/mL, with possible dilution up to 100-fold for hepcidin concentrations higher than dynamic range. The mean intra-assay coefficient of variation (CV) stated is 5.1% and inter-assay CV 12.7%. DRG also offers a "Pro-hepcidin ELISA kit" for prohepcidin measurement in serum, plasma and homogenised tissue. Its declared detection range is 3.95 - 1000 ng/mL, and the same intra- and inter-assay CV < 10% (<http://www.drg-international.com>). These assays have not yet been evaluated in the routine clinical setting.

Kulaksiz *et al.* have also developed an ELISA method for serum prohepcidin quantification. It uses an anti-hepcidin rabbit antibody for prohepcidin and N-terminally biotinylated prohepcidin as a competitor and streptavidin-peroxidase to facilitate detection. This assay is reported to be very reproducible and sensitive, but its most important disadvantages are the detection of the pro-peptide region of hepcidin, the lack of clear correlation with active hepcidin-25 and no correlation with

iron status markers (e.g. iron, ferritin, transferrin) (64-66).

Mass spectrometry is useful for quantifying small molecules and peptides. The molecule undergoes ionization and fragmentation and the generated charged fragments are recognized and measured by their mass-to-charge ratios. For hepcidin measurement several technological approaches have been used – surface enhanced laser desorption-time of flight mass spectrometry (SELDI-TOF MS), MALDI-TOF MS and liquid chromatography tandem mass spectrometry (LC MS/MS). The main advantages of these methods are the separation of hepcidin isoforms and quantification of only hepcidin-25 without interferences of hepcidin-20 and -22, and quantification can be done in serum and urine samples. Using SELDI-TOF MS with desAsp-hepcidin (hepcidin-24) as internal standard, Swinkels *et al.* showed good precision (< 10% CV), but a difference in desAsp-hepcidin and hepcidin-25 binding properties that may indicate that this is not the best choice of internal standard (49). Others used isotope-labeled hepcidin as internal standard in SELDI-TOF MS with good intra-day (< 10%) and inter-day (< 20%) precision, as well as limit of detection of 10 ng/mL (67). Those studies had no accuracy and recovery data presented. Kemna *et al.* evaluated SELDI-TOF MS for quantification of hepcidin isoforms in serum and urine in 73 subjects with different iron status, from iron deficiency to hereditary hemochromatosis. They used synthetic human hepcidin-25 for calibration. Interestingly, they found all three isoforms in urine, but no hepcidin-22 in serum, which supports the evidence that hepcidin-20 and -25 are produced in hepatocytes and released in blood stream, while hepcidin-22 is a urinary degradation product of hepcidin-25 (68). MALDI-TOF MS with isotope-labeled hepcidin as internal standard, but only for urine samples, because serum samples displayed poor peak resolution. This method shows limit of detection 1 - 300 nmol/L (2.8 - 836.9 ng/mL) with intra-assay CV 12.1% and inter-assay precision 13.2% (66).

LC MS/MS was first used in hepcidin quantification by Murphy *et al.*, after reverse solid phase extraction. Calcitonine gene-related peptide was used as internal standard. The method showed good ac-

curacy and precision and a limit of quantification of 1.0 ng/mL (69). Recent methods that used synthetic stable isotope-labeled hepcidin as internal standard were in concordance, with limits of quantification from 1.1 - 2.5 ng/mL and upper limits between 159 and 500 ng/mL. They showed good inter-day precision (CV < 10%) (70,71). Li *et al.* used 60 control samples and 50 CKD patients, and found hepcidin means for them to be 10 ng/mL and 99 ng/mL, respectively. This method was validated to FDA criteria for bioanalytical assays (70). Study that used standardized serum and urine hepcidin sampling showed reference values in healthy subjects to be 0.77 to 200 nmol/L for urine, when measured by LC-MS/MS, with a coefficient of renal excretion ranging from 0.1 to 16.4%. The serum reference values were 0.48 - 100 nmol/L, in agreement with that obtained by Li *et al.* (72). A short overview of hepcidin measurement methods and their characteristics is presented in Table 3.

Studies evaluating hepcidin concentrations in CKD found elevated concentrations in HD patients compared with healthy volunteers, but absolute values of serum hepcidin varied up to 10-fold depending on the assay (25,48,62,66,73,74). On the other hand, a few found hepcidin concentrations to be lower after HD (63,75). These contradictory results must be researched in the future and the effect of HD should also be taken into account. Also, an ELISA method should be further improved to detect only free hepcidin-25. In fact, the lack of standardization in hepcidin testing, as well as the lack of specificity for free, total or bound hepcidin (i.e. to  $\alpha_2$ -macroglobulin), have led Kroot *et al.* to initiate a round robin on hepcidin serum and urinary quantification. They sent 12 replicates of samples, 8 urine samples and 7 plasma pool samples with a wide range of hepcidin from healthy subjects and added synthetic hepcidin-25, to be measured in various laboratories by 8 different methods (competitive RIA, MALDI-TOF MS, two SELDI-TOF MS methods, two LC-MS/MS and two competitive ELISA methods). ELISA methods showed higher hepcidin concentrations compared to LC-MS/MS method (in urine hepcidin means up to 11-fold and SD up to 27-fold and in serum hepcidin means up to 8-fold and SD up to 30-fold). An-

**TABLE 3.** Comparison of methods for hepcidin determination.

Method	Sensitivity (ng/mL)	Specificity	Main feature	Limitation
<b>RIA (Hepcidin-25 RIA Kit, Bachem) (61)</b>	0.1	10 - 20% cross reactivity with hepcidin-20 and -22	High sensitivity; Low intra- and inter-assay CV (< 8%)	Radioactive marker
<b>Competitive ELISA (Ganz <i>et al.</i>) (48)</b>	5.6	Cross reactivity with hepcidin-20 and -22 not evaluated	Wide measurement range (up to 4 µg/mL)	High imprecision at < 50 ng/mL (not suitable for healthy subjects)
<b>Hepcidin-25 (bioactive) ELISA (DRG) (<a href="http://www.drg-international.com">http://www.drg-international.com</a>)</b>	0.35	No detectable cross reactivity with prohepcidin (0.04%); No data on cross reactivity with hepcidin-20 and -22	Low intra-assay CV (5.1%) and inter-assay CV (12.7%)	Serum/plasma determination only
<b>SELDI-TOF MS (67)</b>	10	Specific for hepcidin-25	In agreement with competitive ELISA (Ganz <i>et al.</i> ) for mean hepcidin concentrations in healthy subjects	No standardization; no internal standard
<b>MALDI-TOF MS (66)</b>	2.8	Specific for hepcidin-25	High accuracy (90–110% recovery); Small sample volume (5 µL of urine)	Urine determination only
<b>LC-MS/MS (70)</b>	2.5	Specific for hepcidin-25	Good inter-assay precision (CV < 10%); Validated to FDA criteria	Matrix of standards does not mimic human serum (rabbit serum used)

RIA – radioimmunoassay; CV – coefficient of variation; ELISA – enzyme-linked immunosorbent assay SELDI-TOF MS – surface enhanced laser desorption-time of flight mass spectrometry; MALDI-TOF MS – matrix assisted laser desorption ionization-time of flight mass spectrometry; LC MS/MS – liquid chromatography tandem mass spectrometry.

analytical variations for these methods were found to be generally low and similar for all methods (76). This huge difference in ELISA and LC-MS/MS hepcidin measurement could be due to the fact that ELISA lacks the sensitivity to distinguish hepcidin-25 from hepcidin-20 and -22. Therefore, in case of hepcidin determination introduction in routine laboratory practice, patients should be monitored exclusively using the same, possibly standardized, method. Before commercial hepcidin ELISA can be implemented in routine laboratory practice, cross reactivity for hepcidin-20 and -22 should be evaluated by an independent party. If MS based methods will become routinely used, despite their expensive and time-consuming nature, a few criteria should be agreed upon: a standard internal standard, commutable calibrator that mimics human matrix and adequate quality con-

trol. In light of hepcidin measurement standardization we strongly recommend this be addressed in future studies.

### Potential therapeutic use of hepcidin

Hepcidin agonists and antagonists have been designed in order to modulate iron metabolism. Hepcidin agonists are designed to treat iron overload in hereditary hemochromatosis and thalassemia in order to avoid currently used treatment with phlebotomies and their side effects. Treatments like injection of BMP6, oligonucleotids or small interfering RNA against *Tmprss6* mRNA and minihepcidin (PR65) showed results in decreasing hepcidin concentrations and iron overload in mouse models of hereditary hemochromatosis and/or thalassemia. Minihepcidin is a lipophilic

peptide that contains 9 aa of the hepcidin's N-terminus which are important for its activity (77). MT-2, as the strongest inhibitor of hepcidin, has been targeted by antisense oligonucleotides against its mRNA (*Tmprss6* mRNA) or small interfering RNA (siRNA) against *Tmprss* mRNA. Injection of sHJV and HJV antisense oligonucleotides selectively inhibited BMP induction of hepcidin expression (78).

The search for optimal treatment for anemia of chronic disease gave even more candidates that showed successful in decreasing hepcidin. Heparin, and his subsequently developed non-anticoagulant forms that bind to BMPs, showed promise in blocking hepcidin transcription and decreasing hepcidin plasma concentration after intravenous injection in mice. Similar approaches have been tried with injections of dorsomorphin and its derivative LDN-193189, which bind to BMP-R and act as inhibitors of BMP-mediated hepcidin transcription pathway (78,79). Injections of BMP and IL-6 had showed the same effect, but to a different extent - injection of BMP-2, -4, -5, -6, -7 and -9 showed a hepcidin mRNA increase up to 1000-fold, while IL-6 initiated an increase of only 3.3-fold (80). Anti-hepcidin was used for hepcidin neutralization and hepcidin mRNA suppression in a mouse model of disease. This treatment was able to increase iron and hemoglobin and overcome anemia of chronic disease (81). Treatment with antibodies such as anti-IL-6 receptor antibody (tocilizumab) was the first potential therapeutic tested on humans and it improved anemia of inflammation in multicentric Castleman disease (82,95). Spiegelmer NOX-H94, non-immunoreactive L-oligoribonucleotide that binds human hepcidin with high affinity (83), and anticalins that bind human hepcidin were used to block hepcidins target ferroportin or hepcidin itself. Anti-ferroportin antibodies and anticalin PRS-080 are in phase I of clinical trials (84,85). Lowering *HAMP* expression by these or similar mechanisms in humans could increase iron release from stores and help with recovery of anemia patients (Table 4).

## Final considerations

This review is the first one that focuses on hepcidin in renal TX recipients. Specific topics regarding

management of renal transplant recipients have been addressed, such as IS therapy influence on PTA and hepcidin, and the role of hepcidin in resistance to ESA. We addressed hepcidin at a molecular, biochemical and clinical level. In inflammation hepcidin gene is induced by IL-6 (via JAK2/STAT3 signal pathway), as well as BMP6 (via pSMAD1/5/8 and SMAD4 pathway) (10). The resulting effect is the unavailability of iron supplies to microorganisms with high-affinity iron-binding siderophores that make them able to use iron from the host's transferrin. mHJV was identified as a BMP co-receptor in hepcidin's expression signaling pathway. MT2 is a hepcidin suppressor, as it binds mHJV on hepatocytes and stops its BMP co-receptor activity. Homozygotes or combination heterozygotes mutations of mHJV in humans markedly reduce hepcidin expression and cause juvenile hemochromatosis type 2A, whereas mutations in HAMP cause juvenile hemochromatosis type 2B (87). Besides hemochromatosis and thalassemia, the hereditary causes for hepcidin down-regulation, later studies identified hypoxia-inducible factors - HIF-1 $\alpha$  and HIF-2 $\alpha$  as a possible down-regulators of hepcidin in hypoxia. Some researchers found that HIF-1 $\alpha$  subunit binds to enhancer element of *EPO* gene activating its transcription, but also seems to bind to enhancer element of *HAMP* gene and down-regulates it (21). Others claim that HIF-2 $\alpha$  exclusively regulates *EPO* gene, based on expression studies on hepatocytes and immunohistochemical studies that localized Hif-2 $\alpha$  but not Hif-1 $\alpha$  to *EPO*-producing renal cells (88). This down-regulation mechanism is still controversial, since there have been some studies claiming that only erythropoietic activity suppresses hepcidin expression, rather than it being regulated by HIFs in hypoxia (26,27). Molecular mechanism for hepcidin plasma increase in hypoxia or anemia should be further investigated. Other potential down-regulators as GDF15 and TWSG1 also seem promising in further clarifying hepcidin regulation. They are released by erythroid precursors in some iron-loading anemias. TWSG1 expression is increased in thalassemic mice during early erythroblast maturation (suggested mechanism is inactivation of BMP/SMAD activation of *HAMP*). High

**TABLE 4.** Potential therapeutics for hepcidin regulation and their effect (15, 28, 77-86).

Target molecule	Potential therapeutic substance*	Effect
<b>BMP6</b>	Anti-BMP6	Decrease in hepcidin
	Injection of BMP6	Increase in hepcidin
	Non-anticoagulant heparins	Decrease in hepcidin
<b>BMP-R</b>	Dorsomorphin	Decrease in hepcidin
	Injection of LDN193189	Decrease in hepcidin
<b>HJV</b>	Injection of sHJV	Decrease in hepcidin
	HJV antisense oligonucleotides	Decrease in hepcidin
<b>MT-2 mRNA (<i>Tmprss6</i> mRNA)</b>	<i>Tmprss6</i> mRNA antisense nucleotides	Increase in hepcidin
	siRNA against <i>Tmprss6</i> mRNA	Increase in hepcidin
<b>STAT3</b>	Phosphorylation inhibitor AG490	Decrease in hepcidin
<b>IL-6-R</b>	Anti-IL-6-R	Decrease in hepcidin
	Injection of IL-6	Increase in hepcidin
<b>Ferroportin</b>	Minihepcidin (PR65)	Increase in hepcidin
	Anti-ferroportin antibodies	Decrease in hepcidin
<b>Hepcidin</b>	Anti-hepcidin speigelder NOX-H94	Decrease in hepcidin
	Anticalin PRS-080	Decrease in hepcidin
<b>EPO</b>	Injection of EPO	Decrease in hepcidin

\*The substances listed above were tested only on animal models (murine, cynomolgus monkey and zebrafish), hepatoma cell cultures (HepG2 cells) or are in the beginning of clinical trials. Only anti-IL-6 was tested on humans in a multicentric study. BMP6 – bone morphogenic protein 6; anti-BMP6 – antibody against BMP6; BMP-R – bone morphogenic protein receptor; sHJV – soluble hemojuvelin; MT-2 – matriptase 2; *Tmprss6* – gene for MT-2; siRNA – small interfering RNA; STAT3 – signal transducer and activator of transcription 3; IL-6-R – interleukin 6 receptor; anti-IL-6-R – antibody against interleukin 6 receptor.

GDF15 concentrations can suppress *HAMP* in cell models, but the underlying molecular mechanism has not yet been characterized (89). A potential up-regulator activin B has been found to have effect on SMAD-mediated regulation of *HAMP* transcription in hepatoma-derived cell cultures but that is yet to be confirmed *in vivo* (90).

Up to 90% of circulating hepcidin is bound to  $\alpha_2$ -macroglobulin, and this complex was shown to reduce iron concentrations to a significantly greater extent than free hepcidin. It is argued that the reason is slower hepcidin urine excretion, but this remains to be confirmed in humans (8). In renal transplant recipients this could be of great significance, since their renal excretion is limited by graft function. In case of degrading graft function plasma hepcidin elevation could theoretically contribute to PTA and resistance to ESA treatment, as it was shown in CKD. EPO has been identified as a hepcidin suppressor, but it is unclear if it is ineffec-

tive in hepcidin suppression in ESA-resistant PTA or his suppression is simply not adequate enough compared to other active hepcidin up-regulating mechanisms. In renal recipients with worsening graft function hepcidin is expected to be up-regulated because of uremia and/or subclinical inflammation (by IL-6 activation of JAK2/STAT3-mediated *HAMP* transcription). Another reason for plasma hepcidin increase is iron substitution therapy after renal TX that has a positive effect on *HAMP* transcription by BMPs. It also increases TR-Fe displacement of HFE from TRF1 and formation of HFE-TRF2-mHJV supercomplex that induces *HAMP*. Could that lead to higher hepcidin serum concentration and cause PTA? Could it be in that extent that it could become a marker of kidney function or predictor of PTA? At this point of research and with different measurements challenges hepcidin faces it is still too early to say.

Hepcidin has been shown to be a good marker of iron status after renal TX and it is superior to current markers of iron homeostasis (iron, transferrin saturation and soluble transferrin receptor) (45). Many studies have reported considerable hepcidin and ferritin association in anemia (39,47,58,91), but with one important advantage of hepcidin – instead of reflecting how much iron is circulating or stored, it is the most sensitive indicator of iron absorption and reflects how much iron is accessible under current conditions (inflammation, iron concentration, anemia and hypoxia). Because hepcidin serum concentration is regulated by all of these processes, hepcidin gives a better insight into complex iron homeostasis. Since hepcidin acts in preventing iron oxidative damage in inflammation (as seen in AKI), its' use should be limited to non-inflammatory states (similar to other acute phase proteins), at least when one is assessing renal TX recipients for possible chronic rejection (recipients without subclinical inflammation can be selected by determining high sensitive CRP). On the other hand, researchers that studied hepcidin after cardiac surgery found increased urine hepcidin protective for AKI (52). This justifies further research on inflammation induced local production of hepcidin in kidneys and possibly designing a study that assesses diagnostic abilities of urine hepcidin in predicting AKI in renal TX recipients.

In addition to its correlation with ferritin, hepcidin correlates with creatinine, total protein and eGFR (calculated by MDRD) in patients after renal TX, but only ferritin and eGFR could be used as predictors of hepcidin (47). It is clear that high hepcidin concentration contributes to PTA, but additional research is needed to determine if hepcidin can be used to predict ESA resistance in renal TX recipients. Since iron and recombinant EPO are often used for PTA treatment, future studies will also have to assess the effect of each of these therapeutics on hepcidin regulation, since high iron induces HAMP expression and EPO decreases it.

Research on effect of IS therapy on hepcidin is limited to a single study by Maiorano *et al.* that randomized patients to receive sirolimus and cyclosporine and showed no difference in serum hepci-

din concentrations in patient groups (58). This is confounding because sirolimus has been argued to induce PTA. Possible reason for contradictory results in these studies is the difference in immunological risk for transplant recipients and subsequent difference in dose adjustment in each study. Inadequate level of immunological suppression achieved by sirolimus could result in inadequate T and B-cell suppression and larger than necessary production of IL-6 in B-cells. This IL-6 could theoretically induce *HAMP* transcription and hepcidin. Other IS that have been associated with a higher incidence of anemia after renal TX are azathioprine, MPA and MMF (92,93), and their effect on hepcidin concentration should be investigated in future research.

Measurement of hepcidin should be standardized, since immunochemical and MS methods show non comparable hepcidin results (up to 10-fold higher in healthy subjects measured by immunochemical methods) (25,48,62,66,73,74). MS methods show higher precision and specificity to hepcidin-25, but lack of universal internal standard. In order to be able to compare hepcidin results from MS methods a universal internal standard is needed. Calibrators used in MS methods should mimic human serum and urine. Cross reactivity between hepcidin-25 and hepcidin-20 and -22 should be reduced using more specific antibodies in immunochemical tests. Most researchers agree that measurement of prohepcidin cannot be a substitute for determination of hepcidin because there is no clear relationship or correlation between serum concentrations of prohepcidin and hepcidin (6,94).

Various innovative potential therapeutics, that decrease or increase hepcidin concentration, are currently being tested. Anti-ferroportin antibodies and anticain PRS-080 are in phase I of clinical trials (84,85), whereas anti-IL-6 receptor antibody (tocilizumab) was shown to improve anemia of inflammation in multicentric Castleman disease (82,95). If these substances prove safe for human use and useful in decreasing plasma hepcidin the next step should be testing them in a multicentric randomized trial on patients with PTA to determine if they are able to reduce ESA-resistance after renal TX.



## Conclusion

Hepcidin is a central regulatory protein of iron homeostasis and is greatly influenced by immunity. High hepcidin concentrations reflect the status of chronic (in CKD) as well as acute inflammation (in AKI), but the specific mechanisms involved in hepcidin increase are different. CKD is associated with accumulation of hepcidin in serum which contrib-

utes to anemia and ESA. Hepcidin is the most sensitive marker of iron absorption and the only marker that reflects iron availability. Because of those characteristics it is extensively researched as a therapeutic target, both in anemia and iron overload disease.

## Potential conflict of interest

None declared.

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