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ROLE OF HEPCIDIN IMPAIRMENT IN CHRONIC KIDNEY DISEASE - IMPLICATION FOR NEW THERAPEUTICS OPPORTUNITIES

Artigo de revisão

Área científica de Farmacologia e Terapêutica

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Março – 2013

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List of abbreviations and acronyms

BMP 6 - Bone morphogenetic protein 6

BMP-RI - Bone morphogenetic protein receptors type I

BMP-RII - Bone morphogenetic protein receptors type II

BMP-REs - Bone morphogenetic protein responsive elements

CKD - Chronic kidney disease

CV - Coefficient of variation

CRP - C-reactive protein

ELISA - Enzyme-linked immunosorbent assay

ESA - Erythropoiesis-stimulating agents

EPO - Erythropoietin

eGFR - Estimated glomerular filtration rate

GDF15 - Growth differentiation factor 15

HFE - Hemochromatosis gene

HJV - Hemojuvelin

HFE2 - Hemojuvelin gene

HIF - Hypoxia-inducible factor

IFN-γ - Interferon-gamma

IL-1 - Interleukin-1

IL-6 - Interleukin-6

IV - Intravenous

LPS - Lipopolysaccharide

LM-MS - Liquid Chromatography MS

MS - Mass spectrometry

MALDI - Matrix-assisted laser desorption ionization

RIA - Radioimmunoassay

siRNA - Short interference ribonucleic acid

STAT3-RE - STAT3-responsive element

SELDI- MS - Surface-enhanced laser-desorption ionization mass spectrometry

TOF-MS - Time-of-flight mass spectrometry

TfR1 - Transferrin receptor 1

TfR2 - Transferrin receptor 2

TNF-α - Tumor necrosis factor-alpha

TWSG1 - Twisted gastrulation protein

Resumo

A anemia da doença renal crónica (DRC) é responsável por um aumento significativo da morbilidade e mortalidade entre os pacientes, bem como por uma pior qualidade de vida. A desregulação da homeostasia do ferro é um mecanismo fisiopatológico chave neste processo e a hepcidina é reconhecida como um mediador fundamental. Quando degradada pela hepcidina, a ferroportina é incapaz de mobilizar o ferro para a eritropoiese, causando anemia. São vários os factores que influenciam a expressão e actividade da hepcidina, incluindo os níveis de ferro, um elevado estado inflamatório, a hipóxia e a actividade eritropoética. Os mecanismos moleculares responsáveis pela regulação da síntese e actividade da hepcidina estão a tornar-se mais conhecidos ao longo dos últimos anos, devido a uma intensa investigação experimental e clínica nesta área do conhecimento.

Na DRC, a diminuição da produção de eritropoietina (EPO), já não é reconhecida como o factor exclusivo para o desenvolvimento de anemia, e um estado de elevada inflamação assume um papel primordial, tanto directamente como através do aumento da hepcidina. Deste modo, as terapias actuais, baseadas no uso de ferro intravenoso (IV) e agentes estimuladores da eritropoiese (ESA), embora parcialmente eficazes, não tratam a origem do problema, exigindo frequentemente elevadas doses, com possíveis efeitos adversos e até mesmo o desenvolvimento de resistência à terapêutica com ESA. Tratamentos alternativos que permitam baixar a hepcidina e/ou a inflamação estão a ser investigados, com resultados promissores no tratamento da anemia e na superação da resistência aos ESA. Finalmente, muitos métodos analíticos foram testados para medir os valores de hepcidina, a fim de serem utilizados como ferramentas para o diagnóstico e seguimento do tratamento nestes pacientes, com resultados interessantes. Nesta revisão, sumariamos o papel da hepcidina na homeostasia do ferro, os mecanismos moleculares subjacentes a este processo, o tratamento actual para a doença renal crónica e o papel da hepcidina e da inflamação na fisiopatologia da doença. Revemos também as novas estratégias terapêuticas para diminuir a inflamação e os níveis de hepcidina, bem como a possibilidade de usar estes marcadores para melhorar o diagnóstico e o acompanhamento do tratamento de pacientes com DRC e anemia.

Palavras-chave

- Hepcidina; anemia; doença renal crónica; inflamação; agentes estimuladores da eritropoiese; novas oportunidades terapêuticas; melhorar o diagnóstico.

Abstract

Anemia of chronic kidney disease (CKD) is responsible for a significant increase in morbidity and mortality among patients, as well for a poor life quality. Impairment of iron homeostasis is a key pathophysiological mechanism in this process and hepcidin is being recognized as a pivotal player. Ferroportin is an iron exporter present in the surface of cells that permits the absorption and mobilization of iron trough cells. When degraded by hepcidin, ferroportin is unable to mobilize iron for erythropoiesis, causing anemia. Many factors influence hepcidin expression and activity, including the iron status, a high inflammatory state, hypoxia and the erythropoietic activity. The molecular mechanisms behind the regulation of hepcidin synthesis and activity are becoming clearer during the last years due to a deep experimental and clinical investigation on this area of knowledge.

In CKD, erythropoietin (EPO) impairment isn't, any more, recognized as the solely factor for anemia development, and the high inflammatory state is assuming a primordial role, both directly, as well as through augmentation of hepcidin levels. Thus, the current therapies based on intravenous (IV) iron and erythropoiesis-stimulating agents (ESA), although partially effective, don't address the root of the problem, often requiring high doses, with possible adverse effects and even development of resistance to ESA therapy. Alternative therapies that lower hepcidin and/or inflammation are being investigated with promising results in the treatment of anemia and in overcoming resistance/hyporresponsiveness to ESA. Finally, many analytical methods have been tested to measure hepcidin levels, in order to be used as tools for diagnosis and treatment follow-up in these patients, with interesting results. In this review, we summarize the role of hepcidin in iron homeostasis, the molecular mechanisms behind that process, the current treatment for CKD and the role of hepcidin and inflammation in the physiopathology of the disease. We also review the new therapeutic strategies to lower inflammation and hepcidin, as well as the possibility to use this markers to improve diagnosis and treatment follow-up of CKD patients with anemia.

Keywords

Hepcidin; anemia; chronic kidney disease; inflammation; erythropoiesis-stimulating agents; new therapeutic opportunities; improve diagnosis.

I. Introduction

The anemia that accompanies the chronic renal disease (CKD) is associated with an elevation in precocious mortality and morbidity rates, as well as with a decrease in life quality of patients [1-3]. The existing treatments include hemodialysis, together with the administration of iron and erythropoiesis-stimulating agents (ESA) [4]. However, these treatments are only partially effective and often require high doses, with the consequent adverse effects [5, 6]. Furthermore, approximately 10% of patients develop resistance to therapy [7], complicating the treatment and the prognosis.

It is established that inflammation plays a central role in this type of anemia, as it limits erythropoiesis [8], suppresses the production of erythropoietin (EPO) [9] and, mainly, changes iron homeostasis. Hepcidin, a protein produced in hepatocytes and stimulated by inflammation, especially interleukin-6 (IL-6), plays a central role in this process since it promotes retention of iron in tissues [10]. Hepcidin degrades ferroportin, which leads to the accumulation of iron in macrophages and hepatocytes, and to a reduced flow from duodenal enterocytes to the circulation [11-13]. Thus, although there are adequate deposits of iron (thereafter normal or elevated ferritin), the circulating and available amount for hemoglobin synthesis is reduced (low serum iron, transferrin saturation and reticulocyte counts). This condition is defined as "functional anemia". In recent years, some authors have suggested that changes in the expression and/or activity of hepcidin may be related to resistance to ESA treatment, a process which appears to be mainly caused by disturbances in iron homeostasis and by a pro-inflammatory state [14, 15].

Through these findings, hepcidin has been identified as a possible marker for diagnosis, prognosis and/or as a therapeutic target for this anemia and/or resistance to ESA therapy. In fact, it is believed that the measurement of hepcidin may provide data, more

precise and correct, than those given by the current markers (serum iron, transferrin and ferritin). In parallel, treatments that attempt to lower the levels of hepcidin or its activity (inhibitors of synthesis, antagonists of their action, or stabilizers of ferroportin or soluble hemojuveline [4, 14]) or which inhibit inflammation (IL-6 antagonists, statins and others[4, 14, 15]) are becoming very promising options to manage and/or treat the "functional anemia" and the resistance to ESA therapy.

II. Aims and methodology of the revision

The aim of this article is to review the current scientific knowledge regarding the role of hepcidin imbalances in this functional inflammatory anemia and resistance to ESA therapy, and analyze the diagnostic, prognostic and/or therapy proposals that can be open through its modulation and measurement.

The method used for the preparation of this paper consisted in a review of medical literature on the subject. The study was mainly supported by search medical/scientific articles in the PubMed database, using the following combination of terms in the title:

- Search 1: (hepcidin) AND ("chronic kidney disease" OR CKD OR "renal failure")
- Search 2: (hepcidin) AND (erythropoietin)
- Search 3: (hepcidin) AND (therapeutic OR therapeutics)

This research resulted in 22, 29 and 9 articles, respectively. In addition, the revision of the topic and references list of these relevant articles was also considered. Other scientific databases were searched, including Medline, Biomednet, Scopus, Science Citation Index expanded and Web of Science.

III. Role of hepcidin in iron homeostasis

a) Iron homeostasis: brief review

Iron, an essential element for life, is involved in the transport of oxygen, as a component of heme (in hemoglobin and myoglobin) and is present in numerous enzymes essential for cellular respiration (e.g. redox mechanisms) [16], survival and growth [4]. In fact, iron is necessary for a normal hemoglobin synthesis and erythropoiesis. However, free iron is toxic [17], in a way that organisms had to develop intricate mechanisms to transport iron attached to molecules, deliver it where is strictly needed and store it in proteins when in excess. But, in the opposite way, there's also a low bioavailability of iron in human diet; so, iron is recycled when possible and losses are reduced to the minimum (they represent <0.1% of the 3–4 g of total iron and have to be replaced by diet) [16]. This is most probably the reason why there isn't a proper pathway to excrete iron, even in the presence of iron abundance, which can lead to iron overload.

Iron is absorbed by enterocytes (approximately 1 to 2 mg per day [18]) of duodenum and exported [19] by ferroportin to the circulation, where it binds to transferrin, that delivers most of the iron to bone narrow for erythropoiesis. Hemoglobin of the red cells contains most of the iron of our body (2.5 g of iron). Iron is also recycled in the spleen, where reticuloendothelial macrophages phagocytize senescent erythrocytes and degrade hemoglobin [16]. The excess is stored as ferritin (responsible for one-third of the body's iron stores) in erythrocytes, liver and reticuloendothelial macrophages. Every time iron is needed, it is exported from iron reserves trough ferroportin channels to circulation until it reaches its destination [10, 13].

b) Hepcidin as a key player in iron homeostasis

Hepcidin is an acute phase protein that regulates iron absorption and its distribution (Fig. 1) [10]. It is produced mainly in the liver and is encoded as a prepropeptide, cleaved to a propeptide of 60 amino acids, and it's only secreted in its mature form, a 25 amino acid protein (convertases, like furin, PACE4, PC5/6 and PC7/LPC process hepcidin *in vitro*) [20]. Its peptide resembles a bent hairpin held together by four disulfide bonds [20]. Its production occurs in response to a number of stimuli, like inflammation and high iron blood levels. It is excreted by the kidneys, although only 5% of the hepcidin from plasma appears intact in urine [21]. Maybe it isn't feely filtered or it is reabsorbed and degraded by proximal tubular cells. It acts by binding to ferroportin and degrading it [22]. Ferroportin is a membrane iron export protein present in cells that export iron, such as, duodenal enterocytes, splenic and hepatic macrophages and hepatocytes [23], which has a fundamental role in the iron metabolism. Animal models without ferroportin expression die in the embryonic stage because they can't mobilize iron [23]. Patients with thiol residue Cys326 mutations in ferrportin (probably the binding local to hepcidin) have a form of early-onset iron overload disorder [24]. Nevertheless, hepcidin seems not to be the only ferroportin regulator, as cellular iron can, independently, regulate ferroportin expression [25].

When attached to hepcidin, the proper degradation mechanism involves ferroportin conformational changes and endocytosis, and the ferroportin-hepcidin complex is degraded in lysosomes (this is another way, apart from the renal one, to clear hepcidin) [16, 22]. By doing so, hepcidin inhibits iron absorption from enterocytes and iron deliver from its reserves (e.g. liver, reticuloendothelial macrophages) to erythrocytes (Fig. 1) [11, 13, 22]. In fact, an injection of hepcidin in mice caused a prolonged (more than 48 hours) hypoferremia, although hepcidin was cleared in a few hours by kidneys [26]. This may be due to the fact that organism takes a long time to re-synthetize degraded ferroportin [16]. Thus, the clinical

effects will be normocytic and normochromic anemia characterized by low serum iron, low reticulocyte counts, low transferrin saturation and, paradoxically, high levels of ferritin (in contrast to iron deficiency anemia where it's low) [27, 28].

This type of anemia (with high levels of hepcidin) occurs in many situations, such as chronic infections, cancer, rheumatoid arthritis and chronic kidney disease, and is called "anemia of inflammation" or "functional anemia" [29]. It's hypothesized that our body developed this iron sequester mechanism to protect us against invading pathogens, many of which require iron to growth [30, 31]. In fact, patients with hereditary hemochromatosis, a disease where hepcidin levels are low, have susceptibility to be infected by unusual microorganisms like *Vibrio*, *Yersinia* and *Listeria* [20].

Moreover, experiments in mices with hepcidin overexpression showed that they had the classical features of functional anemia [32]. A report from Weinstein *et al*. (2002) showed that an hepatic adenoma resection, secreting high hepcidin levels, in a patient with a severe anemia, resolved the patient condition [33]. In contrast, patients with hepcidin deficiency, due to hereditary or acquired causes, have a great propensity to develop hemochromatosis (iron overload disorder) [16].

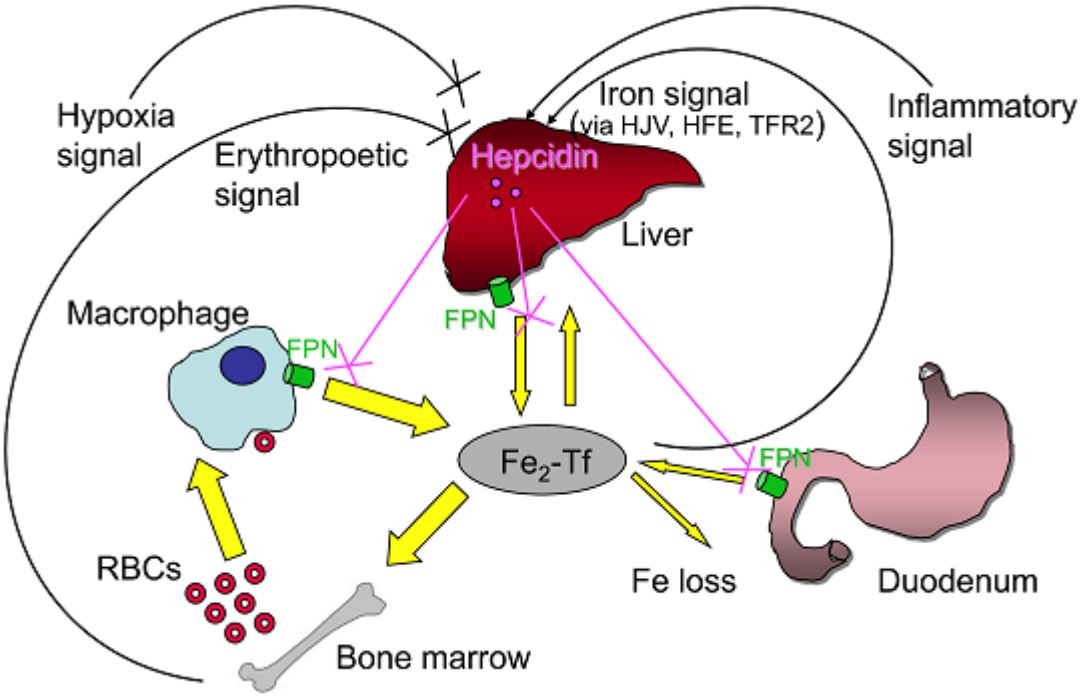

Figure 1 – Hepcidin role in iron homeostasis and the factors that influence its production. RBC (red blood cells), FPN (ferroportin), HFE (hemochromatosis proteins), TFR2 (transferrin receptor 2), and HJV (hemojuvelin). From Babitt *et al.* (2010).

Many factors can influence hepcidin production, like iron status, inflammation, hypoxia, erythropoietic activity, EPO and anemia (Fig. 1) [4, 16, 18]. Iron affects hepcidin production in a feedback loop. When in excess, it can enhance its production and, when in deficiency, it can suppress it. Patients with various types of hemochromatosis have low levels of hepcidin [18]. The molecular mechanisms behind this are becoming better understood. Inflammation is a great promoter of hepcidin production. It was already tested in humans that injection of lipopolysaccharide, which increases IL-6 levels, leads to an elevation in hepcidin levels, followed by the consequent hypoferremia [34]. Moreover, in monkeys with collagen-induced arthritis, anti-IL-6 receptor antibodies caused a diminution in hepcidin and C-reactive protein (CRP) levels in a week, with improvement of anemia parameters in 4 weeks

[35]. Anemia with preserved erythropoietin production and enhanced erythropoietic activity are potent suppressors of hepcidin production, through the formation of hepcidin inhibitor proteins by erythroid precursors [36, 37]. This is logical, because increased erythrocyte formation requires great iron consumption and iron availability. These situations are pretty evident in ineffective erythropoiesis, where there is an erythroid hyperplasia, although the reticulocyte counts are low because of apoptosis in later stages of erythropoiesis. A perfect example is β-thalassemia, where levels of hepcidin are very low, even in the presence of high iron serum levels and iron overload [38, 39]. Hypoxia also suppresses hepcidin expression (Fig. 1) [14, 15, 18].

c) Pathway for the regulation of hepcidin expression

Great advances in understanding molecular mechanisms of hepcidin have been made by studying the iron overloaded disorder called hereditary hemochromatosis [40]. These patients, consistently, have mutations in hemochromatosis protein gene (HFE), transferrin receptor 2 gene (TFR2), and hemojuvelin gene (HFE2) and subsequent low hepcidin levels, despite high iron blood and ferritin levels [4, 18]. Very rare mutations in hepcidin itself cause a severe early-onset form of hemochromatosis [41].

Hepcidin is encoded by the HAMP gene [18] and it is known that its transcription is enhanced by iron "sufficiency" and inflammation and suppressed by hypoxia, anemia and iron deficiency.

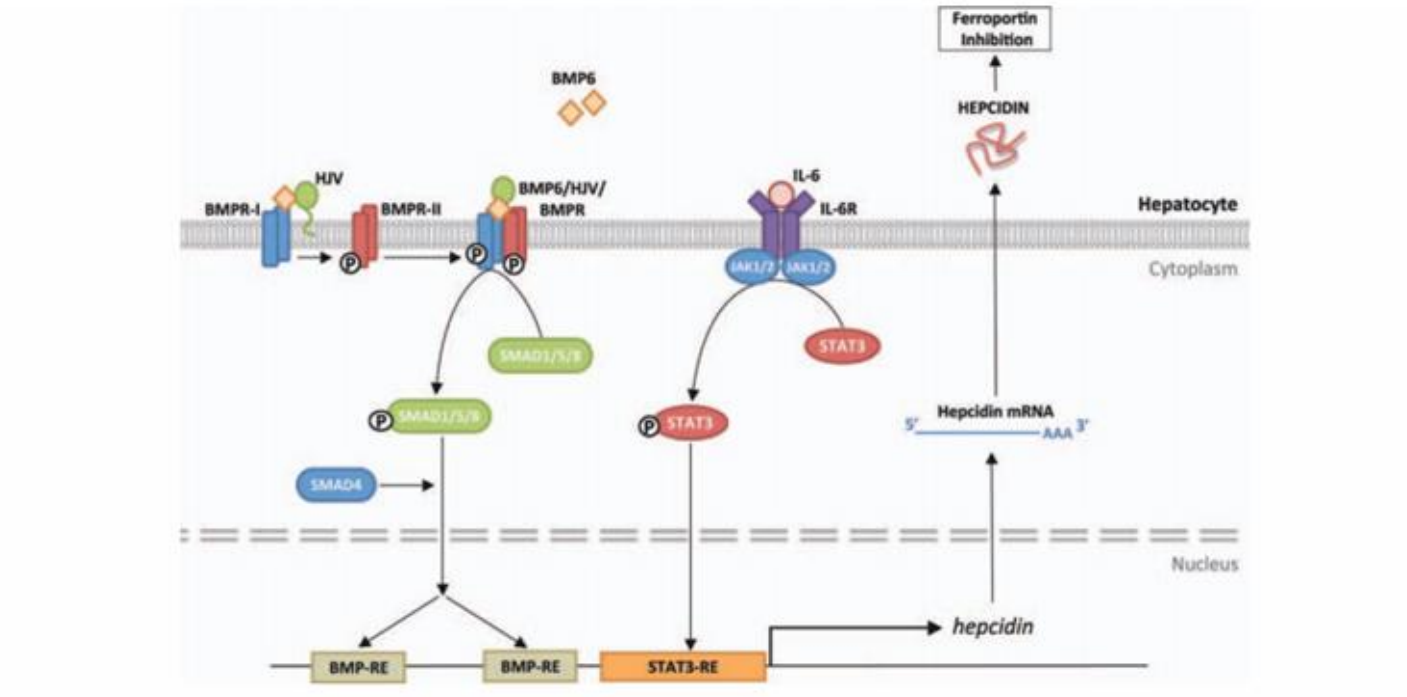

Figure 2 - Hepcidin regulation by BMP6–HJV–SMAD and IL-6–STAT3 signaling pathways. BMP 6 (bone morphogenetic protein 6), BMP receptors type I (BMP-RI) and type II (BMP-RII), hemojuvelin (HJV), BMP-responsive elements (BMP-REs), STAT3-responsive element (STAT3-RE). From Sun *et al. (*2012).

Iron "sufficiency": With sufficient blood iron, transferrin-bound iron binds to transferrin receptor 1 (TfR1) and displace hemochromatosis gene (HFE) from there, which then binds to transferrin receptor 2 (TfR2) [20]. HFE and TfR2 induce hepcidin expression through the morphogenic protein receptor complex BPM/SMAD signaling pathway [42] (Fig.

2). The morphogenic protein receptor complex consists of BPM-6 (it's though that its expression is regulated by iron [43], BMP receptor (BMP type I and type II serine threonine kinase receptors [4]) and hemojuvelin (HJV) [42], which, all together, activates the SMAD signaling cascade. HJV enhances the SMAD signaling and thus, stimulates hepcidin expression, mainly with low BMP ligand levels [42]. This pathway leads to sequential proteins activation that translocate to the the nucleus where hepcidin expression is induced trough BMP responsive elements (BMP-RE) located on the promoter region of hepcidin gene (Fig. 2) [4, 18]. Deletion in any of the genes that express BPM/SMAD signaling pathway molecules result in hepcidin deficiency [4, 18].

Inflammation: Inflammation also plays a central role in hepcidin activation. The proinflammatory cytokine IL-6 activates the JAK/STAT3 signaling pathway (binding to IL-6 receptor) and promotes hepcidin expression trough STAT3 responsive element (STAT3- RE) on the promoter region of hepcidin gene [44-46]. Other proinflammatory cytokines, such as interleukin-1 (IL-1) may play a similar role [47], as shown by the elevated expression of hepcidin mRNA independently of IL-6 in mouse hepatocytes and in IL-6 knockout mice with chronic inflammation [20]. Zhang *et al.* (2006) and Vecchi *et al.* (2009) refer reported another possible pathway to induce hepcidin expression in inflammation. Proinflammatory cytokines and bacterial lipopolysaccharide (LPS) are thought to cause endoplasmic reticulum (ER) stress and, thus, activate CREBH (cyclic AMP response element-binding protein H) which, in turn, activates numerous acute phase genes and induces the hepcidin production, by binding to the promoter gene [48, 49]. The available data suggests there's an interaction between JAK/STAT3 and BPM/SMAD signaling pathway, as they cooperate to promote hepcidin expression. Inhibition or abolishment of BMP/SMAD pathway resulted in blunted responses to hepcidin transcription by the IL-6 pathway [44].

Iron deficiency: In contrast, iron deficiency, produces low hepcidin levels because it is though that activates TMPRSS6 or matriptase-2, which is a hepcidin suppressor [50]. TMPRSS6 is a liver transmembrane serine protease that clivates membrane-bound HJV into soluble HJV. The mechanism may be due to the inhibition of hepcidin induction by HJV in the BMP complex or by the action of soluble HJV that, presumably, binds competitively to the BMP receptor complex inhibiting the signaling pathway and impairing hepcidin expression [50-52].

Anemia with preserved EPO production: Another situation where hepcidin production is inhibited is in the presence of anemia with preservation of EPO production [36, 37]. The proteins mainly involved in these mechanism are growth differentiation factor 15 (GDF15) and twisted gastrulation protein (TWSG1), produced by erythroid precursors [36, 37]. The intact erythropoiesis activity (with normal EPO levels) is essential for hepcidin suppression, because some *in vivo* experiments, where cytotoxic agents or irradiation suppressed erythropoiesis, led to normal hepcidin levels, even in the presence of anemia [53]. GDF15 interferes with the BPM/SMAD signaling pathway, by an unknown mechanism, thus inhibiting hepcidin expression. Curiously, GDF15 knockout mice didn't have iron homeostasis impairment, suggesting that its action may be limited to anemias (like β-thalassemia) with ineffective erythropoiesis, where there is GDF15 overproduction [20, 36]. TWSG1, per se, is thought to interfere with BMP protein, disrupting BPM/SMAD signaling pathway [18, 20].

Hypoxia: Hypoxia is a potent suppressor of hepcidin. The complete physiological regulation is still incomplete but it seems to be related with the hypoxia-inducible factor (HIF) pathway [54]. In normoxic conditions, HIF pathway proteins are degraded by oxygen hydroxylase and von Hippel–Lindau protein oxygenases, but, when in hypoxia, those enzymes are inactivated and HIF accumulates. Evidence suggests that activated HIF pathway

proteins bind to hepcidin promoters impairing their expression [54]. In fact, mice with deletion of von Hippel–Lindau protein (thus, simulating hypoxia conditions) had, consistently, low levels of hepcidin, but when added a new deletion to a gene encoding HIF, hepcidin levels where restored to normal (the inhibitory effect of HIF ended) [55]. Interestingly, furin (which cleaves HJV) and TFR1 are encoded by HIF target genes [56, 57], which suggests that HIF could indirectly lower hepcidin trough inhibition of BPM/SMAD signaling pathway [56, 57]. Finally, hypoxia stimulates EPO production, which, as seen above, inhibits hepcidin, and that may act as a bias factor in understanding HIF-induced hepcidin suppression.

IV. The "inflammatory anemia" of CKD and the central role of hepcidin

Functional anemia is very common among patients with inflammatory conditions (such as CKD, cancer, chronic infection and autoimmune diseases) [29] and is a predictor of poor prognosis (longer hospitalization, cognitive impairment, heart failure, and increased morbidity) [1-3]. In fact, patients with CKD have an increase prevalence of anemia as they progress through the different stages of the disease: 10% in CKD stages 1 and 2, 20–40% in stage 3, 50–60% in stage 4 and more than 70% in end-stage renal disease (stage 5) patients [58, 59].

There are many important factors contributing to the anemia of CKD: a) renal lesion leads to a poor production of EPO [60]; b) the accumulated uremic toxins have an important inhibitory effect on erythropoiesis as well as erythrocytes life span [29, 60]; c) hemodialysis patients loose red blood cells (and, thus iron) due to blood trapping in the dialysis apparatus and repeated phlebotomy [60]; d) the administration of ESA to elevate hemoglobin levels leads to a rapid iron depletion [60]; e) iron mal-absorption in CKD patients is due, not only to hepcidin, but also to other factors [61]. However, inflammation and hepcidin are being increasingly recognized as pivotal players in the pathogenesis of anemia in CKD causing the denominated "functional anemia".

Patients with CKD have high serum levels of inflammatory markers [62, 63], such as CRP, IL-1, IL-6 (mentioned above as essential in the molecular pathway for hepcidin expression), interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) and it's recognized that inflammation plays a central role in the development of anemia by many ways. First of all, it enhances the production of hepcidin, as above explained; but it also directly inhibits erythroid colony formation, bone narrow response to EPO [8], EPO production [64] and red blood cells life span.

High hepcidin values in dialysis patients might be due, not only to inflammation, but also to the fact that hepcidin is cleared by the kidneys. Although there is still controversial data regarding this matter, some studies have demonstrated that glomerular filtration rate in non-dialysis patients correlates inversely with hepcidin levels [65, 66], and patients with CKD disease stage 2-4 have hepcidin levels between levels found in control subjects and dialysis patients [67, 68]. The role of dialysis in reducing hepcidin levels is still unclear. Malyszko *et al.* (2007) attribute elevated hepcidin levels (in patients with CKD on hemodialysis and kidney allograft recipients) to patient inflammation state and impaired renal function [60].

V. Hepcidin as a therapeutic target for the treatment of functional anemia and/or to overcome resistance to ESA therapy

a) Current treatments: focus on ESA therapy

Current treatment of functional anemia is based on IV iron, erythropoiesis-stimulating agents and (in severe cases) on blood transfusions. It has been demonstrated to improve patient's quality of life [4].

As referred above, in CKD there's both a poor response to EPO therapy, and a reduced EPO production which, at least partially, leads to anemia, which might be due to high hepcidin levels and inflammation. Hepcidin is tough to be responsible for the inhibition of erythropoiesis [69], EPO production and, above all, bone narrow response to EPO, thus causing an resistance or hyporesponsiveness to ESA therapy [14]. It's though that inflammation has similar effects as hepcidin in this processes [8, 15, 64]. Thus, ESA therapies represented a great advance in the treatment of this type of anemia [70]. It allowed to rebuild the levels of EPO and (by using high doses of ESA) to overcome resistance to ESA therapy. Even so, Kankay *et al.* (2010) showed that 10 % of the patients maintained anemia with ESA treatment [7] and the use of high doses of ESA isn't deprived of adverse effects. Trials showed that patients with CKD receiving ESA doses in a goal of obtaining >13 g/dL of hemoglobin had a higher incidence of cardiovascular events, stroke, progression of cancer and death [5, 6].

Intravenous (IV) iron is another central piece in this treatment. Studies demonstrated that IV iron partially corrected functional anemia [32, 71], thus diminishing the required doses of ESA [72, 73]. A study revealed that IV iron in combination with ESA improved patients hemoglobin levels compared with those receiving ESA alone [74]. That makes sense,

in part, because ESA can strongly stimulate erythropoiesis, and high-stimulated erythropoiesis needs great iron availability. Further, patients treated with exogenous erythropoietin alone, rapidly develop absolute iron deficiency [60]. Oral iron doesn't have the same effect, as its absorption is blocked by hepcidin [75, 76]. Even IV iron therapy it's controversial, as it is a potent stimulator of hepcidin [65], and can therefore worsen the anemia if given in supra-physiological doses. Additionally, chronic therapy with IV iron, in an iron blockade anemia, can faster the progress to hemochromatosis and it may promote infections and oxidative damage [14, 77, 78].

Although these treatments improved patient life quality by diminishing anemia, the root of the problem might not be properly addressed as high hepcidin and inflammation require high doses of ESA (with the possible adverse effects) and in many patients only partially corrects anemia. Researching new therapeutics, targeting hepcidin and inflammation, are the goal for the future treatment of functional anemia and may prove to be very effective.

b) Correlation between hepcidin, inflammation and ESA

Hepcidin, inflammation and EPO influence each other production and each other effects on human biology (Fig. 3). The augmentation of one can lead to the diminution of other and *vice-versa* [15]. Many studies suggest that inflammation and hepcidin are a major cause for EPO and ESA resistance or hyporesponsiveness [7, 8, 15, 79].

Inflammation reduces EPO production mainly through IL-1 and TNF-α, as shown by Faquin *et al.* (1992) in the Hep3b cell line [9]. It also inhibits growth of cells in the erythropoiesis process and their response to EPO [8]. Goicoechea *et al.* (1998) showed that the use of IL-1 and IFN-γ caused a hypoproliferative anemia through the inhibition of erythroid progenitor cells [80] and that antibodies directed against this cytokines reversed that effect [8]. Some

studies suggested that this cytokines stimulated the growth of that early progenitors although inhibiting, as well, growth at later stages of the process [81]. The study from Allen *et al.* (1999) also says that requirement for higher ESA doses may be the result of greater 'inflammatory activity' and cytokine propagation [8]. Furthermore, evidence shows that patients with high levels of IL-6 and TNF-α where poorly responsive to ESA [82] and that hemodialysis patients, with high levels of CRP (more than 20 mg/l), required 80% higher doses of ESA than control ones (with lower CRP levels) [79].

However, this isn't a one way direction, as ESA is thought to ameliorate the patient's inflammatory state (Fig. 3). The study of Toba *et al.* (2010) showed that ESA therapy normalized endothelial function, vascular inflammation and oxidative stress [83]. Other proved that ESA lowered IL-6, IL-8 and TNF-α levels [65, 84, 85].

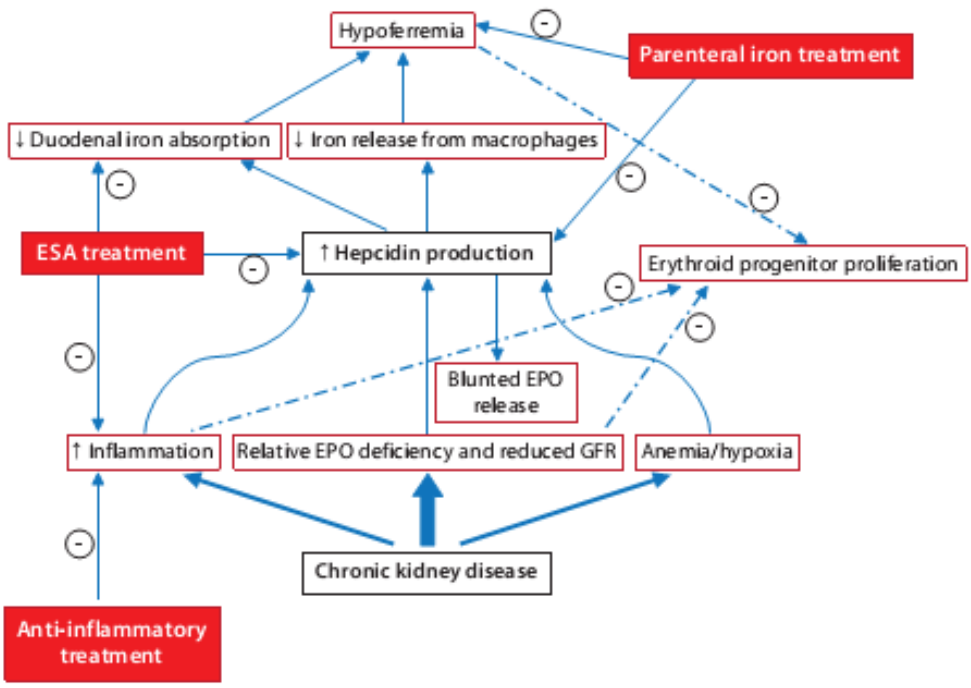

Figure 3 – The correlation between hepcidin, inflammation and ESA in CKD patients. From Yilmaz *et al.* (2011).

EPO/ESA can also alter the production of hepcidin and *vice-versa*. In fact, evidence strongly suggests that hepcidin levels inversely correlate with ESA dose/EPO levels (Fig. 3). Patients receiving high doses of ESA or initiating ESA therapy presented reduced hepcidin levels [86-88]. Srai *et al.* (2010) study showed that nephrectomized rats have a great increase in hepcidin levels [89]. Furthermore, mice with an over-expression of hepcidin had a blunted response to ESA [32]. Patients with lower levels of hepcidin responded better to ESA and IV iron than the other ones with higher levels [90]. Animal models with hepcidin overexpression have an impaired response even to supraphysiological doses of ESA [91]. The amelioration of patients "inflammatory state" by ESA might explain, partially, the lowering action of ESA therapy on hepcidin levels.

c) Overcoming resistance to ESA therapy and functional anemia in CKD

The best way to treat the anemia of CKD patients and diminish the required ESA doses (and, thus, the adverse effects) is probably by targeting the factors that are the origin of the problem: hepcidin and inflammation. Scientific community and pharmaceutical industry are evaluating a number of possible targets and trying to develop various drugs to address the expression and activity of these factors (Fig. 4).

Anti-hepcidin antibodies

Anti-hepcidin antibodies are one of the new strategies in the goal of overcoming resistance to ESA therapy and functional anemia. Amgen showed that an anti-hepcidin monoclonal antibody (mAb2.7) could improve hemoglobin levels and enhance iron availability when combined with ESA treatments in a mouse model of anemia with inflammation [91]. However, when administered alone the same effect couldn't be achieved.

This may be attributed to high hepcidin daily production by organism (7.6 nmol/kg/h), which demands very high doses of these antibodies. In fact, the same authors performed another study with another antibody (Ab12B9m) alone, and high quantities (300 mg/kg per week) were needed to achieve an effective decrease in hepcidin levels (although the high affinity and long half-life of referred antibody) [92]. *Eli Lilly and Company* are also testing an anti-hepcidin monoclonal antibody and already demonstrated that could prevent hypoferremia for up to 12 hours [93].

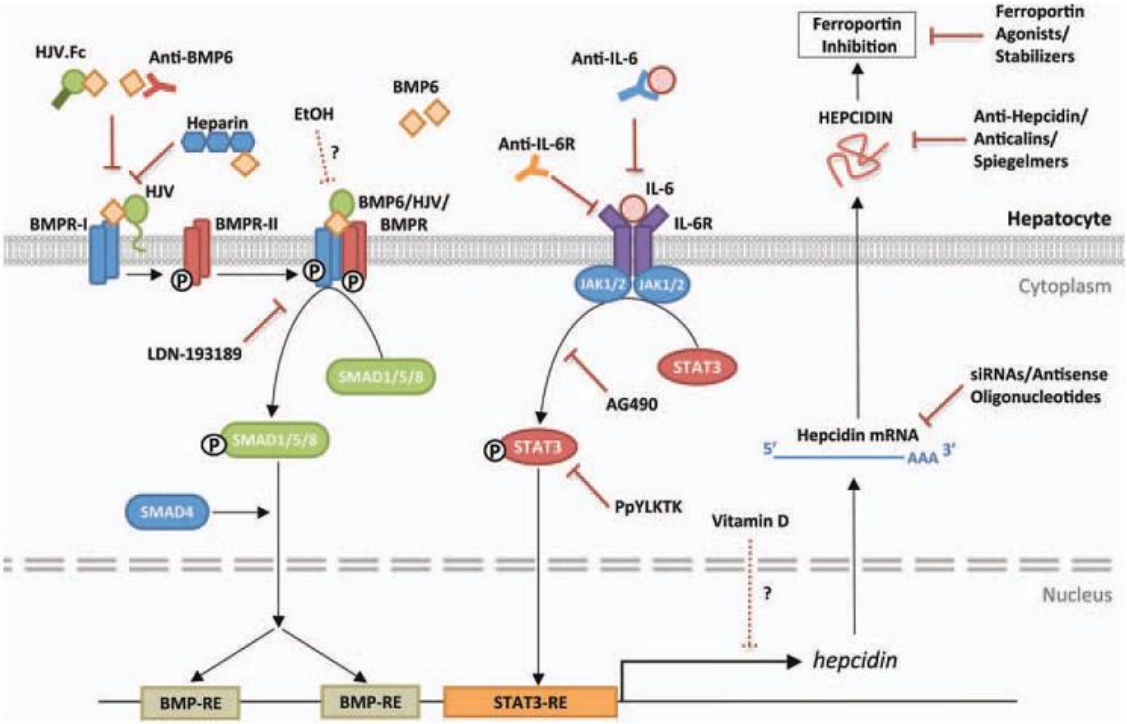

Figure 4 – Therapeutic targets to lower hepcidin. BMP 6 (bone morphogenetic protein 6) , BMP receptors Type I (BMP-RI) and Type II (BMP-RII), hemojuvelin (HJV), BMP-responsive elements (BMP-REs), STAT3-responsive element (STAT3-RE). From Sun *et al*. (2012).

Short interference RNA and antisense oligonucleotides against hepcidin

Short interference RNA (siRNA) and antisense oligonucleotides against hepcidin can induce a profound inhibition of hepcidin translation and transcription [4]. Studies conducted by Amgen used a short hairpin that corrected anemia, alone, in mice, when inducing a great suppression in hepcidin expression [91]. Although this technology seems very promise there's still a great concern with its adverse effects as it's essential to limit genetic inhibition to target gene, ensure biocompatibility and a proper vector to introduce the siRNA [94]. Death in mice was reported with short hairpin RNA against hepcidin, delivered by AAV8 capsid serotype virus system [91]. The same type of technology against hepcidin and regulators (such as HJV) is being researched by *Xenon Pharmaceuticals and ISIS Pharmaceuticals* [4].

Lipocalins

Lipocalins are a set of proteins that have high plasticity and can be engineered to block specific molecules, such as hepcidin [95]. Scientists at the *Technical University Munich and Pieris AG* developed one, called anticalin PRS-080, that neutralized hypoferremia in mice with injected synthetic hepcidin [4].

BPM-SMAD pathway inhibitors

Many substances are being researched to actively inhibit the BPM-SMAD pathway, which is known for its importance in regulating hepcidin expression. Molecules that inhibit BPM receptors, inhibit HJV and mimic soluble HJV (differently than HJV, soluble HJV binds competitively to BMP receptor inhibiting the signaling pathway) are all potencial therapeutics. One of the most promising is dorsomorphin. It acts by inhibiting kinase activity

of BMP type I and also has some inhibitory effects in IL-6 stimulated hepcidin expression, diminishing hepcidin levels and raising serum iron concentration [96]. However, dorsomorprhin is a non-specific kinase inhibitor and inhibits AMP kinase bringing concerns about the possible adverse effects [96]. LDN-193189, a derivate of dorsomorphin, has shown greater potency and selectivity, lowering hepcidin levels and enchanting serum iron levels, thus improving functional anemia in mice [97]; but when tested in human kinases, inhibited vascular endothelial growth factor (VEGF) and components of the mitogen-activated protein kinase (MAPK)–extracellular signal-regulated protein kinease (ERK) pathway [98].

The BMP pathway can also be targeted by soluble HJV linked to the constant region of IgG1 (HJV.Fc) [99]. It has been shown to decrease hepcidin levels (and hepcidin mRNA) and increase serum iron levels, both in vitro and in mice [99]. Its adverse effects are yet to be investigated.

Other possible future therapeutics able to inhibit this pathway and reduce hepcidin levels include anti-BMP6 monoclonal antibody, heparin and alcohol [4].

IL-6 pathway inhibitors

Inflammation is a potent promoter of hepcidin expression and high levels of the pro-inflammatory cytokine IL-6 are found in CKD patients. IL-6 pathway inhibitors are a very promising class of drugs that can be very effective in the treatment of anemia in CKD. Anti-IL-6 receptor antibodies were successfully used in patients with Castleman's Disease (rare multi-systemic inflammatory disease complained by anemia, caused by a lymphoproliferative disorder) [100] and in monkeys with collagen-induced arthritis [35]. In fact, 5 of 6 patients with Castleman's disease treated with an anti-IL-6 receptor antibody (tocilizumab) had their hepcidin levels rapidly decreased. The treatment lasted for 6–12 months and, not only

lowered serum hepcidin contents, but also normalized hemoglobin levels and even disease symptoms where diminished (reduced fatigue, increased weight and alleviation of fever) [100]. Another way to block IL-6 is through its direct inhibition. Anti-IL-6 chimeric monoclonal antibody, called siltuximab, lowered hepcidin levels and increased hemoglobin by 2.1 g/dL in a phase 1 study in Castleman's Disease patients [101]. Of course that inhibiting IL-6 may increase the risk of infections [102]. For example, tuberculosis was associated with the use of tocilizumab in rheumatoid arthritis patients. But maybe this is due, in part, to low hepcidin levels, as hepcidin has *in vitro* antimicrobial activity [14].

Inhibition of JACK/STAT signaling is other possible mechanism to reduce hepcidin. siRNA directed to inhibit JACK/STAT and curcumin suppressed hepcidin expression [103]. AG490 and synthetic peptide inhibitor of STAT3 (PpYLKTK) are being studied and, although there aren't yet *in vivo* experiments, Faith *et al.* (2010) showed a downregulation of IL-6 mediated hepcidin expression and a decrease in STAT3 phosphorylation in mouse liver culture system models [103].

Pentoxifylline may be used as an adjuvant therapy to treat anemia. Ferrari *et al.* (2010) demonstrated a decrease in IL-6 levels and an increase in hemoglobin when stage 4-5 CKD patients received pentoxifylline. It also changed iron kinetic values, suggesting an improvement of iron release [104]. Thus, this xanthine derivative may inhibit IL-6 pathway but further investigation is required.

Statins are known for their beneficial effects on inflammation [105, 106]; so, there may be an application for them in functional anemia. In fact, studies done so far, namely by Sirken *et al.* (2003), revealed a decrease of 25% in ESA requirement dose for hemodialysis patients with statins [107]. Another study also demonstrated that fluvastatin diminished CRP, LDL-cholesterol and hepcidin levels [108]. However, a trial with simvastatin, by Li et al.

(2010), did not show the same effect as the shown above [109]. Nevertheless, statins can have some role in the inhibition of IL-6 pathway and studies should carry on.

Ferroportin agonists

Hepcidin prevents iron absorption and iron release from reserves by binding with the iron exporter protein, ferroportin, inducing conformational changes and promoting its internalization to be degraded by lysosomes. Agents that stabilize ferroportin (preventing conformational changes and thus endocytosis) or block its interaction with hepcidin (like thiol modifier compounds or antiferroportin molecule (this one developed by *Eli Lilly* [110]) can become therapeutic weapons to treat functional anemia.

It was discovered that thiol form of Cys326 is essential to the interaction between hepcidin and ferroportin [24]. In fact, in a study using human embryonic kidney cells expressing fluorescent ferroportin, thiol modifiers compounds were able to block hepcidin-ferroportin interaction, proving their therapeutic capacity [111]. The same study also identified other molecules, like cardiac glycosides, that avoided internalization of hepcidin-ferroporin complex [111]. These strategies are yet to be tested in humans in concern with their efficacy and safety [4].

Other therapeutics

Human studies showed that **HIF** (the molecule that suppresses hepcidin and enhances erythropoiesis) can presumably be used in therapeutics for anemia. In humans, prolyl hydrolylase inhibitors raised HIF levels, lowering hepcidin and promoting erythropoiesis. However, tumor growth was enhanced in some models [14, 55].

Aptamers are a new class of oligonucleotides that have high affinity to molecules and can, therefore, block their action [112]. Spiegelmers are "mirror image aptamers" and are being developed by an enterprise, *NOXXON Pharma*, to become specific to hepcidin [113]. Promising results have been obtained, like inhibiting ferroportin degradation *in vitro* or raising iron levels in experiments with cynomolgus monkeys [114]. Safety and effectiveness are being tested in humans, after being tested in animals without major problems [115-119]. However, the accumulation of oligonucleotides in macrophages is a matter of concern [120].

Finally, **vitamin D** can also become a therapeutic adjuvant. Vitamin D has a wide range of functions throughout the body and some of them might benefit patients with functional anemia. In fact, vitamin D deficiency is associated with higher prevalence of anemia in elderly people and it occurs in many patients with CKD [121, 122]. Some studies, demonstrated that vitamin D diminished the required EPO doses of patients with functional anemia and CKD [123] and others showed it lowered hepcidin levels, even in healthy people [124]. It can also diminish inflammation [125].

VI. Measurement of hepcidin and its diagnostic and prognostic value

a) Methods to measure hepcidin levels

Measurement of hepcidin can open new windows to a more efficient diagnosis and follow-up of iron anemia disorders, namely of CKD patients. Scientific community is analyzing many features through the measure of hepcidin: one of the most important is the hepcidin levels in healthy controls and hemodialysis and non-hemodialysis CKD patients; others encompasses the relation between hepcidin, hemoglobin, iron stores, ferritin and CRP levels, effects of dialysis in hepcidin levels, difference in hepcidin levels between ESA responders and non-responders, hepcidin levels variation with ESA therapy and IV iron, amongst others.

Hepcidin measurement, however, is not easy due to its small size, difficulty of producing hepcidin antibodies and hepcidin antigen (fortunately, surpassed nowadays) and other characteristics of hepcidin that can affect measurement results [126, 127]. The first one is that, in serum, hepcidin is present in its bioactive form (hepcidin-25) but there are other isoforms in serum (such as hepcidin-20, hepcidin-22 and prohepcidine with 60 aminoacids) that seems to be without biological activity [20, 126] ; second, hepcidin is largely linked to alfa-2 microglobulin in serum, in approximately 90 % of its total [128]; finally, hepcidin has a molecular structure that makes it chemically amphipathic which enables it to adsorb to surfaces [20, 126]. All these factors should be taken in account when measuring hepcidin.

Many methods are being tested as it is shown below. These methods shall follow some criteria, to make them scientifically and clinically valid, including specificity, coefficient of variance (CV), limit of detection and of quantitation, accuracy, precision and reproducibility.

Immunodot methods to measure only urinary hepcidin were the first ones described [12]. However, with the advent of serum hepcidin assays, only-urinary measurement of hepcidin showed overpast, because of the greater variation of hepcidin values in urine compared to serum [16]. This may be due, in part, to the fact that only 3-5% [21, 129] of hepcidin is cleared by kidneys (hepcidin binding to alfa-2 microglobulin lowers its filtration) and the filtrated part is, partly, reabsorbed in proximal renal tubules. Moreover, its filtration is, probably, influenced by GFR [126]. Finally, hepcidin-22 was undetectable in serum but detectable in urine [130], which prompt us to the hypothesis of degradation of hepcidin is the nephron.

Radioimmunoassay (RIA), a very sensitive and specific method (the need for radioactive isotopes made it surpassed by ELISA) showed low CV, low cross-reactivity and low detection limit in studies by Ashby and colleagues [65, 67], Swinkels and colleagues [131] and in one commercial kit (Bachem, UK; http://www.bachem.com) [126].

Enzyme-linked immunosorbent assays (ELISA) is another method that was first developed by Kulaksizet *et al.* (2005) [132] for quantitation of prohepcidin. However, pro-hepcidin didn't show any consistent correlation with known iron storage parameters (serum ferritin, iron, or transferrin saturation [133] or iron absorption [134]). Another study, that assessed prohepcidin confirmed the same when it failed to increase in response to induced infection [34].

ELISA methods to measure hepcidin have been developed, with slight differences, by Ganz *et al.* (2008) *[21]*, Ashby *et al.* (2009) *[65]*, Zaritsky *et al.* (2009) [66], Koliaraki *et al.* (2009) [135] and by DRG (http://www.drg-diagnostics.de) [126]. Compared to RIA, ELISA methods have higher absolute values.

Ligand binding assay is a new method where radiolabelled hepcidin compete with unlabeled hepcidin to a binding domain of a synthetic peptide with the same structure as ferroportin binding site. This was developed by Domenico *et al.* (2008), and has a very low CV (lower than 5%) [136]. However, is still requires further experiments (e.g. it wasn't used in CKD and dialyzed patients) and more data (like precision, accuracy, limit of detection and limit of quantitation) to ascertain its validation.

Mass spectrometry (MS) is a method that uses various techniques (based in the same principle) to measure hepcidin. One of them is surface-enhanced laser-desorption ionization mass spectrometry with time-of-flight (**SELDI-TOF**) that was first used by Tomosugi *et al.* (2006) [137]. Swinkels *et al.* (2008) [129] and Ward *et al.* (2008) [138] also used this method (with internal standards) obtaining low CV. Matrix-assisted laser desorption ionization with time-of-flight (**MALDI-TOF**) is another method used also by Costa *et al.* (2009) and Peters *et al.* (2010) [68, 139] with similar analytical results to SELDI-TOF. Finally, liquid chromatography MS (**LM-MS**) is a highly sensitive method with low CV and low absolute values of hepcidin detected, already used by Murphy *et al.* (2007) [140], Muraoet *et al.* (2007) [141] and Kobold et al. (2008) [142] (this one used micro-LC MS/MS). Li *et al.* (2009) developed the only method that fulfills the US Food and Drug Administration (FDA) criteria for bioanalytical assays [143].

Through the analysis of the data presented here hepcidin absolute levels varied, substantially, according to the type of method used (ELISA, MS or RIA) and even between studies using the same methods with different components/methodologies, with parameters like median, range, coefficient of variance and detection limit being different. However, the analytical variance and between –sample variation was generally low, which means that they all, correlate very well.

A recent published round-robin study evolving eight laboratories using LC-MS, SELDI, ELISA and RIA techniques analyzing the same seven samples showed exactly that, with Spearman correlations (*i.e.* the comparability) between methods being high, but with different absolute hepcidin values. In the same round-robin study, comparison between SELDI and LC-MS, showed that mean values for hepcidin were similar. ELISA (with one exception) showed higher CV and a seven-fold increase in absolute values when compared to RIA. In all of them, hepcidin levels where higher in CKD patients when compared to healthy ones. Finally, between, ELISA and MS, there was a 10-fold variation [144].

The differences observed between methods can be attributed to different calibrators, different methodologies, other possible analytes being measured beyond hepcidin and the own hepcidin properties (its isoforms, its adsorption to surfaces and its link to alfa-2 microglobulin) [126]. Cross-reactivity is, in fact, a problem, principally in CKD patients, where hepcidin-20 and hepcidin-22 levels are higher [68]. Besides that, probably, all of the studies are measuring only circulating hepcidin and not the one bound to hydrophobic surfaces and some studies might be measuring free hepcidin, while others alfa-2 microglobulin-hepcidin complex or both of them [126]. In agreement, standardization in hepcidin measurement is required. It is also important to validate the different methods by the guidelines produced by the International Conference of the Harmonization of analytical procedures. Another aspect that has to be noted is that MS methods are laborious; many of them are semi-quantitative and require expensive equipment [138]. However, they are generally more sensitive than ELISA, but their application in a clinical setting remains a doubt.

b) Clinical utility

Evaluation of the clinical utility of hepcidin measurement is fundamental. First of all, methods to measure urinary hepcidin only [12] and pro-hepcidin [132-134] levels have proven not to be reliable. However, almost all the other assays conducted confirm higher hepcidin values in hemodialysis CKD patients compared to healthy controls [21, 65-68, 139, 142, 145]. They also confirmed hepcidin levels in non-dialysis CKD patients intermediate between controls and dialysis patients [67, 68]. However, the coefficient of variation is still very high, mainly when compared to ferritin measures, as Ford *et al.* (2009 and 2010) demonstrated [146, 147]. Thus, maybe measurement of hepcidin to check iron status isn't superior to ferritin.

The greater variation of hepcidin levels might be attributed not only to methods used, but also to the fact that hepcidin is influenced by patients inflammatory state. In fact, Ford *et al.* (2010) [146] and Ganz *et al.* (2008) [21] showed a modest correlation between hepcidin levels and CRP. However Kato *et al.* (2008) [148] found no correlation between these two parameters. In CKD, the greater inflammatory state of patients can preclude even more the use of hepcidin as a reflection of iron status. Higher levels of hepcidin isoforms in CKD, which are biologically inactive, can enhance even more this problem. So, maybe measurement of hepcidin in anemia of CKD as a diagnosis tool isn't useful.

More promising results were obtained, concerning hepcidin, iron administration and ESA therapy. Although it's a matter of debate, whether hepcidin levels may, or may not, predict ESA responsive or hyporresponsive patients [148], as well as predict response to iron administration, many studies have reported a rapid variation in hepcidin levels with ESA and iron therapy [65, 67, 148]. The exception was the study of Ford *et al.* (2010), that reported no correlation between hepcidin, ESA and iron [146]. The positive results suggest that

measurement of hepcidin can be used to monitor patient's response to these therapies in an early stage of the process. In the study by Kato *et al.* (2008) [148] in 33 patients, using a combination of weak cation exchange chromatography and TOF mass spectrometry, the administration of ESA (50 IU/kg/week) produced a significant decrease in hepcidin-25 levels with an increase in reticulocytes. After 6 months hemoglobin values increased [148]. Thus, regarding hepcidin as a parameter to predict patient resistance to ESA therapy, more studies have to be realized. A study evaluating ESA dose response in two groups of patients, one with high hepcidin levels and other with low, would be a good trial [148].

There is controversial data regarding the relation of hepcidin with estimated glomerular filtration rate (eGFR). Some data, in dialysis patients, report no correlation [148], while others, in non-dialysis patients showed correlation between the two [65, 66]. Peters *et al.* (2010)., further showed that hepcidin-25 (the bioactive one) levels are independent of eGFR, while hepcidin-20 correlates inversely [68]. This means that some studies might be measuring higher levels of hepcidin in CKD patients with reduced eGFR, because their actually measuring the isoforms.

The removal of hepcidin by dialysis is yet to be proved. Some data reported lower hepcidin levels after dialysis [68, 137, 145] while other do not [65, 148]. Measurement of hepcidin can also have important role in iron overload disorders [16].

As summary, there are yet many unexplored and controversial fields in hepcidin measurement and its clinical utility. One of the most critical goals is to diminish the influence of the method used in the measurement. It is important to develop more accurate methods, reduce cross-reactivity (particularly due to hepcidin isoforms), and reduce the influence of inflammation in hepcidin values. When properly validated, hepcidin measurement might be

important as a tool to improve diagnosis and better follow-up the results of therapeutics in anemia patients.

VII. Conclusions

In the past, CKD patients suffered from anemia without any great achievements in their treatment. With the advent of ESA therapy great progresses were achieved, but high doses are still needed and some patients develop resistance to treatment and adverse effects. The recent knowledge about the role of inflammation and hepcidin in this type of anemia, and discover of the molecular pathways and interactions between them, have allowed the development of drugs targeting them.

Many ways to lower hepcidin or diminish inflammation are being researched, with some promising results in clinical trials involving humans. Some examples involve statins, pentoxifylline, IL-6 pathway inhibitors and antihepcidin antibodies. Others like BMP6–HJV–SMAD pathway inhibitors (namely, dorsomorphin), ferroportin agonists, lipocalins, short interference RNA (siRNA) and antisense oligonucleotides against hepcidin, seem to have good potential, although more studies concerning their efficacy and adverse effects, namely in humans, need to be done.

The recent advances in this field have also brought the light on hepcidin measurement as a diagnostic and follow-up tool in CKD patients. Although the urgent need of standardization, almost all the assays used correlate very well and have reported higher hepcidin levels in CKD patients compared with healthy ones. MS methods seems to be more sensitive than ELISA ones, but are more expensive and less practical in a clinical basis. The best method, therefore, is yet to be determined.

The clinical utility of hepcidin measurement it is, however, yet to be fully assessed and many aspects need to be clarified. It is still undetermined if hepcidin is superior to ferritin to check iron status, although some studies say it isn’t [146, 147]. In CKD patients, inflammation can influence, positively, hepcidin values and act as a bias to hepcidin

measurement. Additionally, in CKD patients high levels of hepcidin isoforms (inactive biologically) can undermine even more the results. Furthermore, there is still doubt if hepcidin levels can predict a patient responsiveness to ESA, although it is probable that can be used to monitor patient response to ESA and iron therapy. Finally, relation between hepcidin and dialysis, and hepcidin and eGFR is still unclear, due to disparity of results.

More studies have to be conducted to evaluate these aspects, like comparing ferritin and hepcidin levels in anemic patients with the same CRP levels through time (diminishing therefore the influence of the inflammatory state) or evaluate ESA dose response in two groups of patients, one with high hepcidin levels and other with low, testing, therefore, hepcidin as a predictor of ESA responsiveness. Of course, the methods used shall become more accurate, standardized and with low cross-reactivity to diminish their influence in results.

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