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Modulation of Iron Metabolism and Hepcidin Release by *HFE* Mutations in Chronic Hemodialysis Patients: Pathophysiological and Therapeutic Implications

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1. Introduction

Routine monitoring of body iron stores is an essential component of the management of patients with end-stage renal disease (ESRD) receiving chronic hemodialysis treatment (CHD). Maintenance of adequate iron stores is important for the prevention of iron overload as well as for the treatment of iron deficiency and anemia, and this goal is generally achieved by intravenous iron administration. Despite regular iron supplementation, anemia due to renal failure, blood losses related to the procedure, and chronic inflammation is a typical finding in CHD patients, and is associated with increased mortality, reduced physical and mental function, and poor quality of life. Treatment often requires the administration of erythropoiesis stimulating agents (ESAs), but many patients do not respond adequately and/or require high doses of these medications, with potential adverse cardiovascular and infective events.

A correct iron balance is required for the functionality of catalytic enzymes and proteins crucial for DNA synthesis, transport and storage of oxygen via hemoglobin and myoglobin, transport of electrons and cell respiration, oxidative phosphorylation, tricarboxylic acid cycle, and many other biochemical pathways. On the other hand, excess free iron is toxic to the cells due to its ability to catalyze free radical generation. Therefore, specialized transport systems and membrane carriers have evolved to keep iron in a soluble state that is suitable for circulation into the blood and transfer across cell membranes. Additionally, the absence of a physiological excretion mechanism requires systemic iron homeostasis to be regulated by intestinal absorption and iron recycling from erythrophagocytosis of senescent red cells.

Iron metabolism is frequently altered in CHD due to decreased saturation of transferrin (TF), the plasma iron carrier, resulting in reduced iron availability for erythropoiesis, because of chronic inflammation and/or blood losses. Furthermore, inflammation and oxidative stress induce iron retention in macrophages and the transcription of ferritin, a protein with anti-oxidant activity, and hyperferritinemia indicating increased iron stores, and chronic inflammation is frequently observed in these patients. Hyperferritinemia, reflecting iron overload, oxidative stress, and genetic factors, is associated with imminent death risk, mainly related to cardiovascular events, the leading cause of death in these subjects.

A major breakthrough in our understanding of iron metabolism has been the discovery that hepcidin, a peptide secreted by the liver in response to excess iron, inhibits intestinal iron absorption and iron recycling from monocytes by binding and inactivating the iron exporter Ferroportin-1 (Fp-1). Furthermore, hepcidin is an acute phase reactant induced by inflammation, and is downregulated by anemia, hypoxia and erythropoietin. Hepcidin is increased in patients with ESRD, because of chronic inflammation and of reduced urinary excretion due to kidney injury. Hepcidin levels reduce intestinal iron absorption and iron recycling from monocytes, decreasing serum iron available for the erythropoiesis, thereby playing a causal role in the anemia of inflammation and ESRD.

Recent data are consistent with the hypothesis that iron mediated vascular damage involves hepcidin upregulation with consequent accumulation of iron in macrophages, being hepcidin associated with carotid vascular damage independently of other known risk factors in high-risk populations. Moreover, *in vitro* studies suggest that hepcidin synergizes with excess iron, by favoring intracellular accumulation of this metal in macrophages and oxidative stress, and the induction of the release of pro-atherogenic cytokines, in particular macrophage chemoattractant protein-1 (MCP-1).

Mutations of the *HFE* gene responsible for the iron overload disorder hereditary hemochromatosis are frequently observed in the general population and uncouple the regulation of intestinal iron absorption from iron stores by determining altered hepatic iron sensing and a relative deficit in hepcidin release. These physiological alterations result in an attenuation of the effects of inflammation on iron metabolism, including reduced iron absorption, and iron sequestration in macrophages. Here we review novel data suggesting that *HFE* mutations are associated with reduced hepcidin release in CHD patients, resulting in an increased sensitivity to ESAs and iron supplementation, and possibly in a reduced risk of death due to the decreased incidence of complications related to excessive iron and ESAs dosages, including cardiovascular events and infections.

These results may have important clinical implications, as targeting the HFE/hepcidin/Fp-1 axis by pharmacological treatment may further improve the long-term outcomes of CHD, by reducing the amount of iron and ESAs supplementation needed and by improving iron utilization for erythropoiesis.

2. Iron metabolism

2.1 Role of iron in the cells

Iron, like other metal ions, is an essential nutrient, playing a crucial role in vital biochemical activities, as components of enzymes and other molecular complexes. Excess iron promotes noxious free-radical reactions (Kell 2010), so that it has to be compartmentalized and maintained at a fixed level to avoid any toxic effects, largely based on its ability to catalyze the generation of radicals, which attack and damage cellular macromolecules and promote cell death and tissue injury. A delicately balanced iron homeostasis is achieved by coordinated interaction among highly evolved and regulated uptake, storage, and secretion processes (Papanikolaou and Pantopoulos 2005; Andrews 2008).

2.2 Chemical properties, biological functions and toxicity of iron

The biological functions of iron are based on its chemical properties, in particular on the ability to form a variety of coordination complexes with organic ligands in a dynamic and flexible mode, and by its favorable redox potential to switch between the ferrous, Fe(II), and

ferric, Fe(III), states. Iron is associated to heme and non-heme complexes. The hemoproteins are hemoglobin and myoglobin, cytochromes and some enzymes, such as oxygenases, peroxidases, nitric oxide (NO) synthases, or guanylate cyclase. All these proteins contain heme as prosthetic group, which is composed by protoporphyrin IX and a ferrous ion, and is involved in oxygen transport to muscles and tissues (hemoglobin and myoglobin), in the respiratory chain (e.g., cytochromes a, b and c), in the activation of substrates by oxygen (e.g. cytochrome oxidase, cytochrome P450, catalase) and as a NO sensor (guanylate cyclase). The most prevalent forms of non-heme iron are metallo-proteins with iron-sulfur clusters, which are involved in the respiratory chain (e.g., in complex III), DNA synthesis (e.g. ribonucleotide reductase), and in the inflammatory response (e.g., cyclo-oxygenases and lipoxygenase). It should also be noted that non-heme iron has a central function in a recently discovered mechanism for oxygen sensing, via the hypoxia-inducible factor (HIF), that controls the transcription of a wide array of genes involved in erythropoiesis, angiogenesis, cell proliferation/survival, glycolysis, and iron metabolism in response to oxygen availability.

The efficiency of the redox reaction between ferrous and ferric ions is a fundamental feature for many biochemical reactions. However, this very property turns iron into a potential biohazard in the cell, because under aerobic conditions, iron can readily catalyze the generation of noxious radicals. Iron toxicity is largely based on the Fenton reaction, where catalytic amounts of iron are sufficient to yield hydroxyl radicals, collectively known as reactive oxygen intermediates (ROIs), from superoxide and hydrogen peroxide. ROIs are inevitable products of aerobic respiration in mitochondria and can be also generated during enzymatic reactions in peroxisomes, in the endoplasmic reticulum or in the cytoplasm. ROIs are also produced by the membrane-bound NADPH oxidase complex, mainly expressed in phagocytic neutrophils and macrophages during inflammation, and involved in the antimicrobial defense of the organism. Free radicals are highly reactive species that promote the oxidation of proteins, peroxidation of membrane lipids, and modification of nucleic acids. An increase in the steady state levels of reactive oxygen species beyond the antioxidant capacity of the organism, so called oxidative stress, is observed in many pathological conditions, such as chronic inflammation, ischemia–reperfusion injury, or neurodegeneration. Excess of redox active iron aggravates oxidative stress and leads to an accelerated tissue degeneration. Thus, under physiological conditions extracellular iron is exclusively bound to TF, which maintains iron soluble and nontoxic.

2.3 Iron distribution and absorption

The human body contains approximately 3–5 grams of iron (45–55 mg/kg of body weight in adult women and men, respectively). Approximately 60–70% of body iron is employed within hemoglobin (Hb) in circulating red blood cells; other iron-rich organs are the liver and muscles. Approximately 20–30% of body iron is stored in hepatocytes and in macrophages, to a large extent within polymers of ferritin. The remaining is primarily localized in myoglobin, cytochromes, and iron-containing enzymes. A healthy individual absorbs daily 1–2 mg of iron from the diet, which is utilized to compensate non-specific iron losses by desquamation of enterocytes and epidermis and, in childbearing aged women, by period. Erythropoiesis requires approximately 30 mg of iron per day, mainly provided by the recycling of iron via macrophages, which ingest senescent red blood cells (RBCs) and release iron, which binds to circulating TF.

An average daily Western diet contains approximately 15 mg of iron, from which only 1–2 mg is absorbed in the jejunum: in spite of such an apparently low requirement, dietary deficit of iron is a worldwide problem. Two thirds from absorbed iron derives from heme, mainly from myoglobin and hemoglobin of animal origin. The inorganic iron is not efficiently absorbed, but each transmembrane transport step is mediated by specific set of transport proteins and accessory enzymes that change the oxidation state of iron to facilitate the transport process. The low gastric and duodenal pH dissolves ingested inorganic iron and facilitates its enzymatic reduction to the ferrous form by the ferrireductase on the enterocyte brush border (DCYTB, duodenal cytochrome b), whose expression increases during iron deficiency. Ferrous iron is transported across the apical membrane by DMT1 (divalent metal transporter-1). Inside absorptive enterocytes, heme iron is enzymatically released by heme oxygenase-1 (HO-1), after having been transported by heme-carrier protein-1 (HCP-1), and follows the fate of inorganic iron: it is either stored in ferritin or transported across the basolateral membrane to plasma TF. The transport across basolateral membrane is mediated by Fp-1, also known as IREG1 (iron regulated transporter 1), or SLC40A1. Fp-1 is expressed in Kupffer cells and on the basolateral membrane of enterocytes, macrophages, placental cells and hepatocytes, where it works as iron exporter in association with the plasma ferroxidase ceruloplasmin (Cp), even if enterocytes depend heavily on the expression of an analogous transmembrane protein called hephaestin. Ferrous iron crosses the plasmatic membrane through a facilitate diffusion mechanism and Cp / hephaestin converts it to ferric state while it is still associated to the transporter protein. Ferric iron bounds to plasmatic apotransferrin (the iron-free form of TF, a glycoprotein synthesized in liver, retina, testis and brain) to form ferric iron-TF complex, which is the major type of iron present in blood. A small amount of iron circulates bound to albumin or other small molecular weight ligands, such as citrate salts. The TF complex facilitates the transport of iron to cells that express transferrin receptors, including erythroid progenitors, and limits the ability of iron to generate toxic radicals. Transferrin saturation % (TSAT) varies according to diurnal cycle and local circumstances. For example, TSAT is high in portal circulation and low in the blood leaving bone marrow, and is strongly influenced by inflammation, which decreases plasma iron availability in order to prevent iron utilization by pathogens for their replication.

2.4 Delivery of iron to tissues and intracellular transport

Iron uptake occurs primarily by the endocytic pathway, which involves the interaction between TF and transferrin receptors (TFR). Two different TFR are known, namely TFR-1, which is found in all cells and shows an elevated affinity for circulating TF, and TFR-2, mainly expressed in the liver and in the hematopoietic cells, which bind the TF complex with a lower affinity. Once the TF complex binds to TFR-1 at the cell surface, it is internalized in clathrin-coated pits: in the endosome the luminal pH is maintained at about 5.5 by a vacuolar H^+-ATPase (V-ATPase). This acidification process induces conformational changes in the TF-TFR-1 complex, with consequent release of iron in the endosome, while different ferrireductases operate the transformation of ferric iron into the ferrous state. The endosomal transporter DMT1 allows then the passage of ferrous iron in the cytoplasm. The acidic endosomal pH maintains apotransferrin bound to the TFR-1 and this complex is recycled on the cellular surface. At the more neutral plasmatic pH, apotransferrin dissociates and it can bind to plasmatic iron again. Not much is known about the mechanisms of intracellular iron transport

to organelles, but there is strong evidence for the existence of a transit pool of iron in the cytosol (Petrat, de Groot et al. 2002), which presumably remains bound to low-molecular-weight chelates, such as citrate, ATP, AMP, or pyrophosphate. It is believed that the levels of this elusive chelatable or "labile iron pool" (LIP) reflect the overall iron status of the cell and its variations activate adequate homeostatic responses for iron availability. The LIP constitutes also a source of iron susceptible of redox-state variations, with consequent generation of free radicals toxic for cells.

2.5 Iron storage and recycling

Not all absorbed iron is utilized in metabolic processes, but it is partly stored as reserve, both for use when iron levels are low, and to prevent toxic effects of free iron in the cell. Stored iron accounts for 20-30% of body iron in physiological conditions, and the major part of it is bound to ferritin. This is a ubiquitous multimer of 24 subunits with a central core that contains up to 4,500 atoms of iron, and that possesses an important ferroxidase activity (the H form, whereas the L form is the storage subunit) that facilitates the oxidation of the cytosolic ferrous iron to the ferric state. Iron is also stored in an insoluble form into a protein, named haemosiderin, likely derived from the lysosomal degradation of ferritin. Under iron overload conditions, ferritin levels increase dramatically, particularly in liver, pancreas and heart. Intestinal absorption accounts for only a fraction of TF-bound iron in the circulation. Recovery of iron from senescent erythrocytes also plays an important role in iron maintenance. At the end of their lifespan, human erythrocytes undergo surface alterations that mark them to be phagocytosed and digested by reticulo-endothelial macrophages in the spleen and liver. In the macrophages iron is predominantly recovered from heme by the action of HO-1.

2.6 Regulation of intracellular iron homeostasis

Regulated expression of proteins essential for cellular iron homeostasis, such as TFR-1 and ferritin, is achieved by a post-transcriptional mechanism, which is dependent on biologically active iron levels. The messenger RNAs of these peptides present regulatory sequences named IRE (iron responsive elements) in the untraslated regions (UTRs), the target of iron regulatory proteins (IRP-1 and IRP-2), which are rapidly degraded in iron-rich condition. Under iron deficiency conditions, IRPs actively bind multiple IREs localized at the 3'UTR of some mRNAs, such as TFR-1 mRNA, determining mRNA stabilization and increased translation of the protein, and simultaneously decrease the translation of ferritin mRNA by binding to 5'UTR IRE sequences, thereby maximizing the uptake and availability of iron in the cell. Conversely, when the iron levels are high, decreased IRE binding facilitates efficient translation of ferritin mRNA and decreases the stability of TFR-1 mRNA, leading to iron sequestration over uptake.

2.7 Hepcidin and systemic iron homeostasis

Systemic iron homeostasis is achieved by modulation of the amount of iron absorbed, since physiological mechanisms for the regulation of iron excretion are presently unknown. Intestinal iron absorption is regulated in response to iron need and availability and erythropoiesis activity. Stewart et al first disclosed the so-called mucosal block, detecting that a large oral dose of iron reduced the absorption of smaller dose of iron administered several hours later (Stewart, Yuile et al. 1950). These observations suggested that enterocytes

receive signals from other tissues or cells that are involved in either utilization (erythroid precursors) or storage (hepatocytes, macrophages) of iron. Signals that originate from storage sites to balance intestinal absorption were termed "storage regulators", while "erythroid regulators" signal when the consumption demand for iron in bone marrow, erythroid precursors and circulating erythrocytes, exceeds the amount present in stores. On the other hand, "inflammatory regulators" communicate signals in response to infection or inflammation, resulting in accumulation of iron in macrophages. Iron homeostasis is also influenced by hypoxia regulatory signals. Adding to the complexity, these diverse regulatory signals may not be completely independent of each other and elicit quantitative differences in response to common molecules. The amount of body storage modulates iron uptake: it is well established that in iron-deficient conditions, iron absorption is significantly stimulated by two- to three-fold compared to basal conditions, which are restored when iron storage are reconstituted. The erythropoietic regulation participates when iron demand for Hb synthesis increases, independently of body iron stores. This mechanism can explain the pathological iron accumulation observed in disorders characterized by ineffective erythropoiesis (such as thalassemia syndromes, congenital dyserythropoietic anemias, sideroblastic anemias).

A small antimicrobial peptide synthesized by the liver (and to a lesser extent by macrophages and adipocytes), named hepcidin (Pigeon, Ilyin et al. 2001), is now retained to be the principal effector of the modulation of iron availability by iron stores and inflammation in humans. The human hepcidin gene (*HAMP*; OMIM 606464), located on chromosome 19q13.1, encodes a precursor protein of 84 amino acids (aa). During its export from the cytoplasm, full-length pre-prohepcidin undergoes enzymatic cleavage, resulting in the export of a 64 aa prohepcidin peptide into the endoplasmic reticulum lumen (Wallace, Jones et al. 2006). Next, the 39 aa pro-region peptide is post-translationally removed by a furin-like pro-protein convertase, resulting in mature bioactive hepcidin-25. In human urine, hepcidin-22, a N-terminally truncated isoform of hepcidin-25 considered the urinary degradation product of hepcidin-25, can be detected. Structural analysis of human synthetic hepcidin by nuclear magnetic resonance spectroscopy revealed that this 8 cysteine-containing peptide forms a hairpin-shaped molecule with a distorted beta-sheet, which is stabilized by four disulfide bridges between the two anti-parallel strands. One of the disulfide bridges is located in the vicinity of the hairpin loop which points to a possible crucial domain in the activity of the molecule (Hunter, Fulton et al. 2002). Structure-function *in vivo* and *in vitro* studies on synthetic hepcidin have shown that the iron regulating bioactivity is almost exclusively due to the 25 aa peptide, suggesting that the five N-terminal amino acids are essential for this activity. *In vitro* experiments have shown that especially human hepcidin-20 (the other N-terminally truncated isoform detectable in human serum) exerts anti-bacterial and anti-fungal activity in a concentration range 10- fold higher than that measured in healthy individuals. Therefore, it is not clear whether *in vivo* hepcidin levels can reach values in which it can be anti-microbial, or whether this function is of biologic importance or only rudimentary in its evolutionary origin from the antimicrobial peptides of the defensin family. Hepcidin is excreted in the urine; in a murine model, its effect starts within 4 hours and lasts for more than 48 hours (Rivera, Nemeth et al. 2005). Fractional excretion of hepcidin-25 in 3-5%, because is not freely filtered or it is reabsorbed by the tubules like other small peptides.

Hepcidin mediated regulation of iron metabolism has been demonstrated to depend upon its ability to bind Fp-1 on cellular surface blocking its iron transport activity, and to increase Fp-1

endocytosis, JAK2 mediated phosphorylation, and consequently its degradation by lysosomes (Nemeth, Tuttle et al. 2004). In enterocytes, Fp-1 internalization on the basolateral surface causes the retention of absorbed iron with subsequent loss by desquamation, while the same process in macrophages causes the failure to release iron (Pietrangelo, 2007). The final effect is the reduction of plasma iron availability. Besides liver hepcidin controlled reduction of iron uptake and release at systemic level, there is also evidence for local production of hepcidin by macrophages, fat cells and cardiomyocytes, suggesting a different regulatory mechanism related to hepcidin to control iron balance (Theurl, Theurl et al. 2008). Indeed, mutations of *HAMP*, the hepcidin gene, cause severe early-onset HH in humans, whereas deletion of *Hamp* in animal models brings to severe iron accumulation (Lesbordes-Brion, Viatte et al. 2006). Conversely, hepcidin producing hepatic adenomas in patients and overexpression of hepcidin in animal models lead to hyposideremia and iron deficiency anemia.

Importantly, hepcidin is upregulated by both increased iron stores and inflammation. Indirect hepcidin inductions by IL-6 or LPS in humans displayed the same fast response in urinary hepcidin excretion, thereby acting like an acute phase protein with a peak value after 6 hours, followed by a steady decrease. When hepcidin is administered orally, a peak in urinary excretion appear in less than one day, suggesting a fast clearance of this peptide from the circulation, with a paradoxical sustained inhibitory effect on iron uptake, shown by the iron parameters which remained unchanged over the following days (Nemeth, Rivera et al. 2004). Hepcidin secretion is reduced in response to signals that cause an increase in iron release from cells, such as iron deprivation, and stimulus to erythropoiesis, whereas it rises when iron secretion is inhibited, as for iron load or for a flogistic state. Thus, hepcidin can represent the common effector of the homeostatic regulation of intercellular iron fluxes in response to the iron stores, erythroid, and inflammatory regulators.

It is still not clear how systemic iron demand modulates hepcidin release by the liver. A number of molecules have been implicated in different ways of transduction of the signal: the alteration of each of these molecules causes an insufficient release of hepcidin resulting in a deregulated iron flux from macrophages and enterocytes, which finally brings to iron overload in blood circulation and finally in tissues. Hepcidin release may be impaired by genetic factors, i.e. mutations inactivating *hepcidin*, *HJV*, *HFE* or *TFR-2*, or by non-genetic factors: alcohol abuse (that inhibits hepcidin transcription), viral infections such as chronic HCV hepatitis, and acute liver insufficiency and cirrhosis (because of the reduced hepcidin synthesis by hepatocytes). In addition, mutations in the *Fp-1* gene, which bring to insensitivity to hepcidin action, are as well responsible for hereditary forms of overload.

The small size of hepcidin, the compact and complex structure of the molecule, and the highly conserved sequence among species, make problematical the quantitative assessment of serum levels of hepcidin in humans. Furthermore, about 90% of serum hepcin-25 appears to be bound to circulating proteins, mostly α2-macroglobulin. Recently, laboratory assays for hepcidin-25 (the biologically active form of the hormone) in serum and urine has been developed: they include competitive ELISAs using biotinylated or radio-iodinated hepcidin as tracers, and several mass-spectrometry-based assays using as internal standards isotopically labeled hepcidin or shorter hepcidin mutants. Interestingly, hepcidin levels were correlated with ferritin in patients with chronic kidney disease (Tomosugi, Kawabata et al. 2006; Kato, Tsuji et al. 2008; Kroot, Laarakkers et al. 2010). In contrast, measurement of serum prohepcidin, an immature form of hepcidin measured by ELISA, was found to be increased in patients with ESRD (Kulaksiz, Gehrke et al. 2004), but have not been found to

correlate with mature biologically active hepcidin and with iron stores (Kato, Tsuji et al. 2008; Swinkels, Girelli et al. 2008).

3. *HFE* mutations and iron overload

The transcription and secretion of hepcidin by the liver is regulated by a mechanism of body iron sensing and is finely regulated by a group of proteins, including the hereditary hemochromatosis protein called HFE, TFR-2, hemojuvelin (HJV), bone morphogenetic protein 6 (BMP6), matriptase-2 (TMPRSS6) and TF. Mutations in *HFE*, *TFR-2*, *HJV* and the hepcidin gene (*HAMP*) are responsible for hereditary hemochromatosis (HH), a common iron overload disorder characterized by a deficit of hepcidin release or activity (Camaschella 2005; Pietrangelo 2007).

HFE mutations represent the most frequent cause of HH in Caucasian adults (Feder, Gnirke et al. 1996). HFE structure resembles MHC class I molecules and forms a heterodimer with β2-microglobulin. TF and HFE seem to compete in vitro for binding to TFR-1, which is hypothesized to sequester HFE thereby altering iron sensing and hepcidin expression. In this way, increased iron-loaded TF levels would result in the release of HFE with possibility to interact with other proteins, in particular with TFR-2, which only binds saturated TF at physiological concentrations. However, the exact mechanisms underpinning HFE regulation of iron metabolism through SMAD (son of mother against decapentaplegic) and possibly ERK (extracellular signal-regulated kinases) signaling are still not clear. The most common *HFE* mutation responsible for HH is a single nucleotide substitution that causes the substitution of a cysteine with a tyrosine at position 282 (C282Y). The homozygous genotype is very frequent in Caucasians, particularly in people from Northern Europe (frequency 1/300-400), whereas the prevalence decreases towards Southern Europe. This substitution brings to HFE mysfolding, resulting in failed interaction with β2-microglobulin and cell surface expression. A second and most frequent mutation is a substitution at position 63 of a histidine with an aspartate (H63D), which likely interferes with the ability of HFE to interact with TFR-1. This is a very common polymorphism in the general population, as 25-30% of the population carries the H63D variant, but its contribution to the pathogenesis of HH and iron overload syndromes is negligible, with the exception of compound heterozygosity with the C282Y. The penetrance of HH depends on age, gender, environmental factors, and on the role of the so-called modifier genes (Wood, Powell et al. 2008): for example, iron overload and clinical phenotype are more serious in patients with beta-thalassemic trait (Piperno, Mariani et al. 2000; Valenti, Canavesi et al. 2010).

Mutations of *TFR-2* cause a rare recessive form of HH, clinically similar to HFE HH, which is consistent with the hypothesis that HFE and TFR-2 might be part of the same signaling pathway. Indeed, it has been hypothesized that TFR-2 binds iron-loaded TF, but not iron-depleted TF. Thus, TFR-1 and TFR-2, by binding iron bound and not bound to TF respectively, may contribute to modulate iron sensing and hepcidin release by the liver. However, deletion of *Tfr-2* and *Hfe* had additive effects on iron overload in mice, thus suggesting that the pathways through which these two genes regulate iron metabolism might not be entirely overlapping. In addition, TFR-2 was discovered to be associated to erythropoietin receptor (Epo-R) in the EpoR complex, and thus to exhibit an extra-hepatic function, being required for efficient erythroid differentiation and erythropoiesis (Forejtnikova, Vieillevoye et al. 2010). Furthermore, very recently it has been demonstrated that also HFE is expressed in erythroblast and plays a role in the regulation of erythroid

differentiation, and that HFE deficiency is associated with increased erythropoiesis partly due to enhanced iron absorption and delivery to the erythron due to decreased hepcidin levels and increased TSAT, and partly due to a direct effect of HFE on the modulation of iron uptake in erythroid cells (Ramos, Guy et al. 2011). These new exciting findings suggest that the TFR-2/HFE complex is directly involved in the regulation of erythropoiesis independently of hepcidin levels, and thus that genetic variations of HFE and TFR-2 may influence RBCs production and the development of anemia in conditions characterized by reduced iron availability, such as CHD.

Many other molecules have been implicated in the regulation of hepcidin secretion. Binding of the iron-regulated BMP6 ligand, a bone morphogenetic protein of the TGFβ superfamily, to its threonine/serine kinase receptors (BMPR-I and BMPR-II) activates a signaling cascade leading to hepcidin transcription via phosphorylation, nuclear translocation, and binding to the hepcidin promoter of SMAD 1/5/8 effectors (Babitt, Huang et al. 2006). HJV, a GPI-linked membrane protein synthesized by the hepatocytes, is a BMP6 coreceptor, which is required for its regulatory functions on iron metabolism. The critical role of the BMP6/HJV/SMAD pathway in iron homeostasis is supported by the loss of hepcidin expression and massive parenchymal iron overload observed in *BMP6*-/- and *HJV* -/- mice as well as in mice with targeted liver deletion of SMAD4 (Andriopoulos, Corradini et al. 2009), and by the fact that *HJV* mutations represent the major cause of juvenile HH. Furthermore, iron overload and increased TSAT have been associated with specific upregulation of BMP6 in hepatocytes and *in vivo*. However, *BMP6* mutations have not been associated with HH so far. Interestingly, it has been shown that BMP6 levels are increased in HFE-related HH both in mice and humans, suggesting that HFE might be involved in the transduction of BMP6 signaling, or that BMP6 levels are upregulated in the attempt to compensate for the lack of HFE function, but supraphysiological doses of BMP6 were sufficient to normalize iron metabolism in experimental models. Recently, the serine protease matriptase-2 has been connected to this iron regulatory pathway because of its ability to cleave HJV (Muckenthaler 2008). Matriptase-2 is a type 2 transmembrane serine protease that is predominately expressed in the liver and was characterized as a negative regulator of hepcidin gene expression (Ramsay, Hooper et al. 2009). Matriptase-2–deficient mice have very high levels of hepcidin, which lead to the inhibition of dietary iron absorption and cause a severe iron-deficiency anemia phenotype. This anemic phenotype is mirrored in patients with matriptase-2 mutations, who present with iron-refractory, iron-deficiency anemia (IRIDA). Indeed, patients with IRIDA show inappropriately high hepcidin levels, which explain the lack of dietary iron absorption and the only partial response to parenteral iron treatment (Finberg 2009). In addition, it has been recently recognized by means of genome-wide association studies that very common genetic polymorphisms in *TMPRSS6* represent, together with *HFE* mutations, a major source of variability in serum iron and hemoglobin levels in the general population (Benyamin, Ferreira et al. 2009; Chambers, Zhang et al. 2009; Ganesh, Zakai et al. 2009; Tanaka, Roy et al. 2009).

Severe iron overload in HH involves several organs, mainly the liver, endocrine glands, and heart. However, the involvement of a specific organ varies on the entity of iron accumulation, which depends in part also on the specific mutation at the base of the disease. Usually, the more severe phenotype is observed in the juvenile forms versus adult disease (Pietrangelo 2007), and secondary iron overload in the presence of physiological upregulation of hepcidin presents with a different organ involvement (mainly macrophages), and clinical phenotype (e.g. anemia, accelerated atherosclerosis, altered immune regulation) (Pietrangelo 2004).

4. Anemia in ESRD

Anemia is a common problem in patients with ESRD and increases mortality and morbidity in these patients, especially related to cardiovascular events. The cause of anemia in patients with chronic kidney disease is multifactorial (Lankhorst and Wish 2010), but it is believed that a deficit of erythropoietin (Epo) due to kidney injury plays a major role. Indeed, it is generally advised that erythropoiesis-stimulating agent (ESAs) should be given to all patients with ESRD with hemoglobin (Hb) levels consistently (i.e. measured twice at least 2 weeks apart) below 11 g/dl, or with haematocrit < 33%, where all other causes of anemia have been excluded. However, recent evidence such as those provided by the TREAT study (Pfeffer, Burdmann et al. 2009) warn against excessive ESAs doses, as an increased risk of stroke has been reported in those patients treated with ESA with a hemoglobin target of 13 g/dl. Thus, the recommendation of an Hb level of 10 to 12g/dl in chronic kidney disease (CKD) patients seems adequate.

Epo is a glycoprotein hormone that promotes the maintenance of committed erythroid progenitors cells, specifically the burst-forming units (BFU-E) and colony-forming units (CFU-E), by binding to surface receptors and preventing them from apoptosis, and stimulating these erythroid progenitors to differentiate into reticulocytes and RBCs. Epo is mainly released by the peritubular capillary endothelial cells in the kidney. Reduced availability of oxygen for tissue metabolic needs (during anemia, hypoxemia or impaired blood flow to the kidney) stimulates Epo production through HIF, whose spontaneous degradation is inhibited in presence of decreased oxygen delivery and iron deficiency. Furthermore, it has now been clearly demonstrated that Epo administration results in reduced hepcidin levels and increased intestinal iron absorption in CHD patients, although it is still debated whether Epo directly signals through its receptor in hepatocytes to downregulate hepcidin transcription, or its effect is indirectly mediated by hypoxia through stabilization of the HIF transcription factors, or by increased erythropoiesis through the induction of erythroid regulators of hepcidin release of the TGFβ superfamily, possibly including growth and differentiation factor 15 (GDF15) and twisted and gastrulation 1 (TWSG1) (Tanno, Bhanu et al. 2007; Kato, Tsuji et al. 2008; Pinto, Ribeiro et al. 2008; Costa, Swinkels et al. 2009; Morelle, Labriola et al. 2009; Srai, Chung et al. 2010). Anyway, this mechanism seems aimed at facilitating enhanced iron delivery to the bone marrow when is needed for accelerated RBCs production, and suggests that the increase in Hb levels in CHD patients induced by Epo may be partly mediated by normalization of hepcidin levels and of iron delivery. Therefore, the process of erythropoiesis needs an adequate renal secretion of Epo, an appropriate response of bone marrow, and sufficient supply of substrates for Hb synthesis, such as iron, folates, and cyanocobalamin. Epo levels are significantly higher in patients with advanced CKD compared to general population, but inappropriately low for the degree of anemia. In addition, the persistence of anemia despite Epo higher levels suggests a concomitant hyporesponsiveness of bone marrow in these patients.

Furthermore, in patients with CKD, RBCs life span is shortened to 60-90 days (versus 120 days), and there is an increase tendency of bleeding due to platelet dysfunction, both for the presence of uremic toxins. Other causes of anemia include chronic blood loss from gastroenteric system and blood trapping in dialyzers, dietary restrictions, loss of taste for iron-rich foods, secondary hyperparathyroidism (which is associated with bone marrow fibrosis), increased hemolysis and decreased erythropoiesis, nutritional deficiency, and accumulation of inhibitors of erythropoiesis related to uremia.

A major condition that contributes to the establishment of anemia in CKD is the inflammatory state, in which the pro-inflammatory cytokines decrease EPO production and induce in this way apoptosis in CFU-E. In the anemia of inflammation, high levels of acute-phase proteins, such as C-reactive protein (CRP), ferritin and in particular of hepcidin are detected. Recent studies unequivocally demonstrate a high prevalence of chronic systemic inflammation in dialysis patients. This condition has been associated with adverse clinical outcome (Stenvinkel, Ketteler et al. 2005), including accelerated atherosclerosis, malnutrition, and pronounced anemia. Most importantly, chronic inflammation and cytokines can worsen anemia by reducing iron availability for hematopoiesis, shorten erythrocyte life span, and directly inhibit erythrocyte progenitor proliferation. Inflammation and cytokines are independent predictors of Epo hyporesponsiveness (Gunnell, Yeun et al. 1999), whereas anti-inflammatory cytokines might be associated with less severe anemia (Stenvinkel and Barany 2002).

5. Iron metabolism in ESRD

ESRD, and in particular CHD, are typically associated with alteration in iron metabolism parameters, that is most frequently characterized by decreased TSAT, resulting in reduced iron availability for erythropoiesis, and is supposed to be related to chronic inflammation and/or blood losses (Kalantar-Zadeh, McAllister et al. 2004). Hyperferritinemia is also frequently observed, and although in the most severe cases excess iron administration is believed to play a role, in the majority of cases it is thought to reflect the malnutrition-inflammation-cachexia syndrome (Kalantar-Zadeh, Rodriguez et al. 2004), referring to the state of malnutrition, chronic inflammation and wasting frequently reported in CHD patients, which is associated with a greater risk of cardiovascular disease and finally with a worse outcome (Kalantar-Zadeh, Regidor et al. 2005). It has been shown that inflammation and oxidative stress induce iron retention in monocytes/macrophages, and the transcription of ferritin, a protein with anti-oxidant activity (Scaccabarozzi, Arosio et al. 2000; Torti and Torti 2002). However, recent data highlight that ferritin levels reflect also iron stores in CHD patients. Indeed, in these subjects ferritin correlates with TSAT, with the presence of common mutations of the *HFE* gene (Valenti, Valenti et al. 2007), and with bone marrow iron stores (Rocha, Barreto et al. 2008).

In CKD patients, three types of situation related to iron-metabolism are observed: 1) absolute iron deficiency occurs due to decreased total body iron stores. It is associated with serum ferritin levels <100 ng /ml and with TSAT <20%. Due to very low iron stores, serum hepcidin is relatively low. This situation is common in patients who undergo hemodialysis, due to low-grade but frequent blood losses (typically 1-3 g of iron/year). 2) Functional iron deficiency is associated with serum ferritin levels higher than 100 ng/ml but TSAT less than 20%. Iron stores are normal or even increased, but the Epo-stimulated bone marrow needs more iron from TF than the iron output from tissue stores, resulting in ESA resistance (observed in 10-20% of cases). Increased hepcidin can aggravate functional iron deficiency by decreasing the release of stored and macrophage iron and intestinal iron aborption, through Fp-1 downregulation. There is also a third type of iron deficiency-related anemia, the most severe and intractable form, historically termed 3) "reticuloendothelial blockage", which usually occurs in the setting of acute or chronic inflammation / infection. It can be considered as an extreme form of functional iron deficiency and is associated with increased CRP levels, TSAT <20%, and normal to very high levels of ferritin. Reticuloendothelial iron

stores are locked, likely by hepcidin, and there is no release of iron to transferrin. Resistance to Epo therapy easily develops, especially if iron administration is limited by adherence to the "official" opinion-based upper cutoff (see below). Thus, the pattern of anemia, hyposideremia, ESA resistance, and high serum ferritin is frequently observed in CHD patients.

As mentioned above, increased hepcidin levels are likely involved in mediating the effect of inflammation on iron metabolism in CHD patients. Over-expression of hepcidin, stimulated by inflammatory cytokines (Nemeth, Valore et al. 2003) and by the innate immune response through Toll-like receptor-4 (TLR4) (Peyssonnaux, Zinkernagel et al. 2006) would result in sequestration of iron into macrophages, decreased intestinal iron absorption and recycling, and finally in the decrease of iron availability for erythropoiesis. Studies in mice moderately overexpressing hepcidin indicate that hepcidin can also induce blunted erythropoietic response to Epo (Nemeth and Ganz 2009). The molecular pathway activated by the inflammatory response which brings to hepcidin overexpression involves the binding of interleukin-6 (IL-6), the major hepatic regulator of the acute phase response to inflammatory stimuli, to its receptor, that eventually leads to the translocation of phosphorylated signal transducer and activator of transcription 3 (STAT3) to the nucleus and binding to the hepcidin promoter, resulting in up-regulation of hepcidin transcription. It has been further proposed that STAT3 activation itself, without inflammation, can regulate hepcidin levels (Wrighting and Andrews 2006). Other pro-inflammatory cytokines may also be involved in modulating iron balance in chronic diseases, such as interferon-γ (IFN- γ), interleukin-1, and tumor necrosis factor-α (TNF-α), but the exact mechanisms are still not clear. Furthermore, recent evidences proved that also macrophages, in addition to hepatocytes, express hepcidin at low levels upon stimulation of the STAT3 pathway by IL-6, induced by lipopolysaccharide (LPS), or by TLR4 mediated pathways (Peyssonnaux, Zinkernagel et al. 2007; Theurl, Theurl et al. 2008). In addition, activation of endoplasmic reticulum stress response by oxidative stress due to inflammation and iron overload may also play a role (Vecchi, Montosi et al. 2009). However, whether hepcidin release by macrophages produces systemic or only localized effects at the site of inflammation or bacterial invasion remains to be determined.

6. Iron therapy in the anemia of ESRD

ESAs, the recommended therapy for ESRD related anemia, increase the need for iron, as they stimulate the synthesis of 2 million new red cells/second (Cavill 1999), so that bone marrow requests strips iron off the circulating TF faster than TF can replenish it, resulting in a relative deficit of iron that leads the reticulocytes to enter the systemic circulation with suboptimal quantities of Hb (Brugnara 2000). Evidence now prove that adequate iron availability increases erythropoiesis and reduces ESA requirements (Besarab, Amin et al. 2000).

According to K-DOQI guidelines (2006), iron status should be evaluated every month during initial ESAs treatment, at least every 3 months during stable ESAs treatment or in CHD patients not treated with ESAs. In clinical practice, no single test adequately monitors iron stores or availability. Serum ferritin is the only available blood marker of storage iron, but it is more reliable in non-dialytic patients than in those who underwent hemodialysis. Tests reflecting the adequacy of iron for erythropoiesis include TSAT, mean corpuscular volume (MCV), and mean corpuscular hemoglobin (MCH) and related indices, such as percentage of hypochromic red blood cells (PHRC) and content of Hb in reticulocytes (CHr).

MCV and MCH decrease to less than normal range only after long-standing iron deficiency and so they do not configure as good indicators of relative iron deficit. TSAT and serum ferritin are undoubtedly the most available serum test, but both show acute-phase reactivity and are poorly decodable for assessment of iron status in such state as chronic disease, malnutrition and inflammation; furthermore they exhibit also diuturnal fluctuations (Kalantar-Zadeh, Rodriguez et al. 2004). Therefore, there is an urgent requirement for novel markers (Wish 2006) more specific for iron deficit, especially in CHD patients, in which occult infections and malnutrition play a major role in determining response to therapy and influencing mortality. CHr is a measure of the amount of hemoglobin in red cells 1 or 2 days old and is a reasonably good reflection of how much iron was available to the bone marrow for incorporation into new red blood cells a few days before. The CHr compared favorably with serum ferritin and TSAT in predicting a response to intravenous iron, but the optimal cutoff value is still under investigation. Mean CHr in the general population is 32±3.3 pg, whereas levels of 26–29 pg indicate iron deficiency, which is diagnosed by the absence of stainable iron stores (Mast, Blinder et al. 2002). More recent evidence refers to a cutoff of 32 pg, and determined that a CHr cutoff of 29 pg tended to miss a number of patients who ultimately responded to intravenous iron. The PHRC is based on the Hb concentration in RBCs; it takes into account the absolute amount of Hb as well as the size of the cell, but is poorly reliable due to the alteration observed in case of long sample transport and storage times. Soluble transferrin receptor levels (sTfR) provide an esteem of the iron need of the proliferating erythron, reflecting both erythropoietic activity and iron deficit, based on the fact that erythroblasts in the bone marrow will increase the presentation of membrane TFR-1 in the setting of iron deficiency, as inadequate iron supplies in a patient stimulated with ESAs. Levels of sTfR correlate with TFR-1 membrane expression and also tend to be elevated in the presence of increased erythroid activity (Tarng and Huang 2002). However, there is not a wide consensus regarding cutoff and use of sTfR for the assessment of iron status in CHD patients.

In patients undergoing ESAs therapy, interpretation of iron status tests should incorporate consideration of the Hb level and ESAs dose, in order to provide information important to medical decision making, because they elucidate the status of both external iron balance (net loss or gain of iron) and internal iron balance (disposition of iron between stores and circulating red blood corpuscles). For example, decreasing ferritin levels in the presence of a stable or decreasing Hb level may signify external iron loss, and so iron therapy is indicated. Conversely, decreasing ferritin levels in the presence of increasing Hb denotes an internal shift in iron from stores to Hb, as would be expected in a patient responding to ESA therapy: if iron status remains within the target range, additional iron administration may not be required. Finally, an increase in ferritin levels accompanied by a decrease in TSAT suggests inflammation-mediated reticuloendothelial blockade and may be accompanied by a decrease in Hb and increase in ESA dose (2006).

KDOQI guidelines suggest that iron supplementation should be administered during ESA treatment to maintain serum ferritin >200 ng/ml and TSAT >20%, or CHr >29 pg/cell in CHD and serum ferritin >100 ng/ml and TSAT >20% in ESRD or in patients in peritoneal dialysis. The upper limit of serum ferritin besides which there is no recommendation to routinely administer iron was set as 500 ng/ml. When ferritin level is greater than 500 ng/ml, decisions regarding iron administration should weight ESA responsiveness, Hb and TSAT level, and the patient's clinical status. Indeed, iron determination by means of

magnetic susceptometry showed hepatic non–heme iron concentration greater than the upper limit of normal in patients with ferritin > 500 ng/ml (Canavese, Bergamo et al. 2004).
The preferred route of iron administration in CHD patients is by intravenous (IV) infusion, since iron absorption from the gastrointestinal tract may be impaired in uremic patients (Eschbach, Cook et al. 1970; Donnelly, Posen et al. 1991; Kooistra and Marx 1998), likely because of high hepcidin levels. In ESRD or peritoneal dialysis patients, iron can be administered either orally or IV. The lack of data from clinical trials does not permit to state if a continuous iron supplementation is better than a periodical one. Available intravenous iron formulations include iron dextran, sodium ferric gluconate, and iron sucrose: all these forms may be associated with acute adverse effects, occasionally severe, including hypotension with or without other symptoms and signs, possibly for immune-mediated mechanisms (mast cell–mediated processes leading to a clinical syndrome resembling anaphylaxis), or for the release of small amounts of bioactive, partially unbound iron into the circulation, resulting in oxidative stress and hypotension (labile or free iron reactions). The pathogenesis may differ depending on the type of IV iron: anaphylactoid reactions appear to occur more frequently with iron dextran and high molecular weight forms (Novey, Pahl et al. 1994), whereas labile or free iron reactions occur more frequently with nondextran forms of iron (Agarwal, Vasavada et al. 2004; Agarwal 2006). Thus, resuscitative medication and personnel trained to evaluate and resuscitate anaphylaxis should be available whenever a dose of iron dextran is administered (2006). As a result, FDA has issued a ‘‘black box” warning, recommending that patients undergo a 25 mg test dose the first time the drug is given. If a patient does not have an adverse reaction to this dose, he/she is less likely to have an anaphylactic reaction to the therapeutic dose of iron dextran, but fatal anaphylactic reactions still occur with an uneventful test dose (Lankhorst and Wish 2010).

7. Iron toxicity in ESRD

Before the ESAs era, CHD patients often developed severe iron overload, frequently with ferritin levels >1000 ng/ml, due to polytransfusion. Nowadays, patients with anemia of CKD are supplemented with IV iron to correct the relative deficiency linked to the ESAs therapy. However, large doses of iron may exceed storage capacity leading to the accumulation of unbound iron in plasma, which as seen before, via the Fenton reaction, can produce reactive oxygen species (Michelis, Gery et al. 2003). In addition, different formulation of IV iron are associated with various degree of oxidative stress: iron sucrose, for example, determines a 60% greater elevation in tumor necrosis factor alpha (TNFα), a proinflammatory cytokine, compared with either iron dextran or ferric gluconate (Zager 2005).
Moreover, iron has been implicated in the pathogenesis of the accelerated atherosclerosis in CHD (Griendling and FitzGerald 2003), in addition to traditional and population specific risk factors such as anemia, hyperhomocysteinemia, hyperphosphatemia and inflammation. It has been suggested that iron overload may increase cardiovascular risk in the general population, by affecting LDL oxidation and endothelial dysfunction (Roest, van der Schouw et al. 1999; Zacharski, Chow et al. 2000; Wolff, Volzke et al. 2004) and administration of intravenous iron has been associated with increased oxidative stress (Michelis, Gery et al. 2003). In CHD patients, carotid intima media thickness (an early index of atherosclerosis and a strong predictor of cardiovascular events) and carotid plaques were positively correlated with serum ferritin and oxidative stress and reduced plasma anti-oxidant activity (Drueke,

Witko-Sarsat et al. 2002; Valenti, Valenti et al. 2007), and intima-media thickness was also associated with the dose of IV iron administered (Reis, Guz et al. 2005). Furthermore, hepcidin and TNFα levels have also been correlated with vascular stiffness, another reliable predictor of cardiovascular events in CHD (Kuragano, Itoh et al. 2011). The mechanism may involve iron trapping into endothelial cells, plaque macrophages, and vascular smooth muscle cells, with activation of the atherogenic process and progression of the plaque lesion. Indeed, a significantly higher iron content has been detected in atherosclerotic plaques than in healthy vascular tissue (Stadler, Lindner et al. 2004). Hyperferritinemia (beyond 800 ng/ml) was found to be associated with imminent death risk, and cardiovascular death in hemodialytic patients (Kalantar-Zadeh, Don et al. 2001; Kalantar-Zadeh, Regidor et al. 2005), even if this association might not reflect only iron toxicity, since hyperferritinemia associated morbidity is partially explained by non-iron-related factors, such as proinflammatory cytokine release and the malnutrition inflammation cachexia syndrome. However, even after adjustment for these confounding variables, high ferritin levels and the intravenous dose of iron were still associated with increased total and cardiovascular mortality (Feldman, Santanna et al. 2002; Kalantar-Zadeh, Regidor et al. 2005). In particular, recent data from our group confirm that in CHD patients, hyperferritinemia reflects a relative increase in iron availability and a decrease in iron-specific anti-oxidant activity, is favored by *HFE* mutations, and represents a risk factor for advanced cardiovascular damage, as evaluated by the presence of plaques, both at carotid and femoral arteries (Valenti, Valenti et al. 2007).

Recent evidence (Neven, De Schutter et al. 2011) confirm a strict relation between iron and vascular damage in CHD patients, suggesting that the mechanism underpinning this association may involve the predisposition to arterial wall calcification. Indeed, increased oxidative stress, associated with iron overload, promotes *in vitro* a shift in vascular smooth muscle cells from a contractile to an osteogenic phenotype (Byon, Javed et al. 2008), as indicated by an increased expression of Runx2, a key transcription factor for osteogenic differentiation. Meanwhile, ROIs seem to inhibit osteoblastic differentiation and mineralization in bones (the so called calcification paradox of bone-vascular axis) (Parhami, Morrow et al. 1997; Persy and D'Haese 2009); both these processes may depend on an iron-induced upregulation of ferritin and an increase in ferroxidase activity. Furthermore, iron overload itself has been associated with osteoporosis and increased bone reabsorption (Valenti, Varenna et al. 2009). Reactive species stimulates also apoptosis in vascular smooth cells, providing in this way a nidus for the deposition of calcium-phosphate crystals (Reynolds, Joannides et al. 2004; Shroff, McNair et al. 2008), which could be directly promoted by iron salts (Neven, De Schutter et al. 2011), and promoting thus vascular calcifications and an increased cardiovascular risk for ESRD patients.

Moreover, iron overload has been associated with an increased incidence and severity of infections, which, besides the known growth-promoting effect of iron on microbial pathogens, have been also related to inhibition of phagocytosis (Cieri 1999), and to inhibition of the anti-microbial molecule lactoferrin (Ellison and Giehl 1991). Both iron sucrose and ferric gluconate have been shown to result in impaired trans-endothelial polymorphonuclear leukocytes migration in vitro (Sengoelge, Kletzmayr et al. 2003). Iron is an essential nutrient for bacterial microorganisms, and several bacterial species (including *E. coli*, *Klebsiella* spp., *Pseudomonas* spp., and *Salmonella* spp) can compete with TF for unbound iron in the blood by means of bacterial iron chelators, named siderophores (Cieri 1999; Brewster and Perazella 2004). Other

bacterial species, such as *Staphylococcus aureus* and *Haemophilus influenzae* express transferrin receptors, which allow these bacteria to use iron for growth. Infection risk in CKD patients depends not only on iron, but also on the severity of anemia (Hershko, Peto et al. 1988), transfusions, secondary splenic dysfunction, and the presence of catheters for hemodialytic or endovenous therapies. Although human data are not conclusive, it is still recommended that, as a precaution, IV iron is stopped in patients with ongoing bacteraemia, as in vitro and in vivo studies demonstrated that administering IV iron during active infection may contribute to bacterial growth (Zager 2005; Zager, Johnson et al. 2005). Nevertheless, correction of anemia is effective in reducing oxidative stress and, consequently, cardiovascular risk, decreasing morbidity and mortality and also producing regression of left ventricular hypertrophy in patients with CKD (Ayus, Go et al. 2005), so that a carefully balanced management of iron supplementation is needed in ESRD patients.

8. Role of hepcidin in anemia of ESRD

Increased hepcidin levels have been hypothesized to contribute explaining many typical alterations of iron metabolism in CHD patients, and to the pathogenesis of anemia in ESRD (Kulaksiz, Gehrke et al. 2004; Tomosugi, Kawabata et al. 2006; Malyszko and Mysliwiec 2007; Kato, Tsuji et al. 2008; Kemna, Tjalsma et al. 2008), an almost universal finding associated with increased mortality (Paganini 1989; Locatelli, Pisoni et al. 2004). As mentioned above, although treatment with ESAs and IV iron formulations (Locatelli, Aljama et al. 2004) are generally prescribed, functional iron deficiency is a common finding, determining the need for high doses of ESAs and iron, both associated with adverse events. Indeed, high ESAs doses have been associated with mortality due to cardiovascular events related to hypertension and hypercoagulability (Miyashita, Tojo et al. 2004; Phrommintikul, Haas et al. 2007; Strippoli, Tognoni et al. 2007), whereas excess iron promotes vascular damage by inducing oxidative stress, and heightens the risk of infections (Seifert, von Herrath et al. 1987; Jean, Charra et al. 2002; Teehan, Bahdouch et al. 2004; Kalantar-Zadeh, Regidor et al. 2005; Valenti, Valenti et al. 2007).

The mechanism proposed to explain refractoriness to IV iron was previously related to the inhibition of erythropoiesis and iron recycling from macrophages by inflammation (Stenvinkel 2003). Increased hepcidin may be involved in mediating the effect of inflammation by reducing intestinal iron absorption and iron recycling from monocytes (Pietrangelo 2007), decreasing serum iron available for the erythropoiesis (Camaschella 2005) and the therapeutic effect of ESAs and intravenous iron (Malyszko and Mysliwiec 2007). Furthermore, in CKD the renal clearance of hepcidin diminishes gradually with the progression of kidney disease, with some studies reporting an inverse correlation between glomerular filtration rate and serum hepcidin. Thus, the severity of kidney injury directly influences hepcidin levels contributing to anemia via this mechanism. Importantly, preliminary data indicate that hemodialysis reduce serum hepcidin levels (Zaritsky, Young et al. 2010), contributing to anemia management, but whether specific dialytic techniques differentially affect hepcidin clearance is still unknown. Finally, chronic iron supplementation is a strong inducer of hepcidin also in CHD patients, thereby paradoxically hampering the optimal utilization of large doses of administered iron. Indeed, overall evidence indicate that serum ferritin and C reactive protein levels, reflecting iron stores and inflammation, are the major determinants of hepcidin levels in CHD and ESRD (Tomosugi, Kawabata et al. 2006; Kato, Tsuji et al. 2008; Valenti, Girelli et al. 2009). Supporting

a causal role of increased hepcidin in the pathogenesis of anemia, hepcidin-25 levels have been correlated with hyposideremia and the severity of anemia in patients with stable Hb values (Valenti, Valenti et al. 2008). Moreover, we recently reported that frequent mutations in the *HFE* gene, which decrease hepcidin response to iron stores (Piperno, Girelli et al. 2007; Vujic Spasic, Kiss et al. 2008), are associated with increased sensitivity to ESAs and iron in CHD patients, and may be associated with a better clinical outcome (Valenti, Valenti et al. 2008). However, in a preliminary report hepcidin-25 was not significantly associated with Epo responsiveness (Kato, Tsuji et al. 2008), and in another recent study an association between greater iron doses, increased darbepoietin resistance index, and low hepcidin levels, possibly downregulated by ESAs and anemia, was detected (Bratescu, Barsan et al. 2010), thus suggesting that other mechanisms besides increased hepcidin are involved in the pathogenesis of anemia in CHD. All in all, these recent studies suggest that hepcidin is involved in the pathogenesis of anemia in CKD and chronic diseases, but is not the only player involved.

9. Influence of *HFE* mutations on iron metabolism regulation, hepcidin levels, and erythropoiesis in CHD patients

9.1 *HFE* mutations, iron metabolism, and erythropoiesis

As in the general population, in CKD patients iron metabolism homeostasis and iron stores depend from the interaction between genetic and environmental factors, including iron administration. Among the candidate genetic factors that have been considered, the effect of *HFE* genotype on iron overload in CHD patients was analyzed in a few small studies with inconsistent results. In a recent study from our group, the presence of the C282Y or H63D *HFE* mutations was associated with increased iron stores in Italian patients (Valenti, Valenti et al. 2007), whereas previously other Authors found only a nonsignificant, modest effect of the more common H63D mutation on serum ferritin (Pericole, Alves et al. 2005), and lower iron requirement in the few subjects carrying the C282Y *HFE* mutation (Canavese, Bergamo et al. 2002). Since the C282Y and H63D *HFE* mutations uncouple the regulation of intestinal iron absorption from iron stores, by modifying hepatic iron sensing and defective release of hepcidin (Gehrke, Herrmann et al. 2005), we hypothesized that these genetic variants may also influence iron mobilization from macrophages after therapeutic iron infusion and ESAs treatment by influencing hepcidin release. Consistently with previous data (Canavese, Bergamo et al. 2002), we showed that the two common C282Y and H63D mutations, present in about one third of subjects, were associated with a lower requirement of ESAs and a trend to a lower requirement of iron (Valenti, Valenti et al. 2008). The lower baseline ESAs and iron requirement in mutation carriers indicate that the alterations in iron metabolism include not only increased iron stores, but possibly also iron handling by macrophages after infusion, and iron availability to the erythron. *HFE* mutations attenuate the effects of inflammation on iron metabolism, including reduced iron absorption, iron sequestration in macrophages, and erythroblast resistance to Epo, and protect against iron-related damage by favoring the delivery of intravenously administered iron to the erythron. Importantly, in this study iron stores were not lower, and the requirement of iron and ESAs were not higher in *HFE* H63D/wt compared to C282Y/wt and H63D/H63D patients. In subjects without ESRD, chronic inflammation, and liver disease, simple heterozygosity for the H63D or the C282Y *HFE* mutations was not found to be associated with either iron overload (Adams, Reboussin et al. 2005) or reduced hepcidin release (Bozzini, Campostrini et al. 2008), and previous data

obtained in a very limited number of CHD patients did not support an influence of increased hepcidin on Epo requirement (Kato, Tsuji et al. 2008). It could therefore be speculated that chronic inflammation and blood losses of CHD may provide enough environmental pressure to magnify subtle alterations in cellular iron handling in carriers of the milder and more common H63D mutation, which thus reach clinical significance, unlike to what we observed in general population (Adams, Reboussin et al. 2005), and that the effect of *HFE* mutations might not be limited to modulation of serum hepcidin levels. Thus, in the setting of HDT paradoxically *HFE* mutations protect against iron related damage by favoring the delivery of intravenously administered iron to the erythron.

9.2 *HFE* mutations and survival

Furthermore, although due to the limited number of patients considered and the inclusion of prevalent patients in the analysis results should interpreted with caution, our data strikingly highlighted that the presence of *HFE* mutations was associated with a reduced hazard ratio of death. An updated mortality curve for cardiovascular disease and infection in 127 Italian CHD patients subdivided according to the presence or not of the C282Y and H63D *HFE* mutations is shown in figure 1. It is worthy of note that, in patients negative for *HFE* mutations, we observed a higher mortality due to sepsis, previously associated with a higher iron dosage (Jean, Charra et al. 2002; Teehan, Bahdouch et al. 2004), and due to cardiovascular disease, possibly linked to hypertension and thromboembolic events related to ESAs (Miyashita, Tojo et al. 2004; Phrommintikul, Haas et al. 2007) and oxidative stress related to iron (Valenti, Valenti et al. 2007).

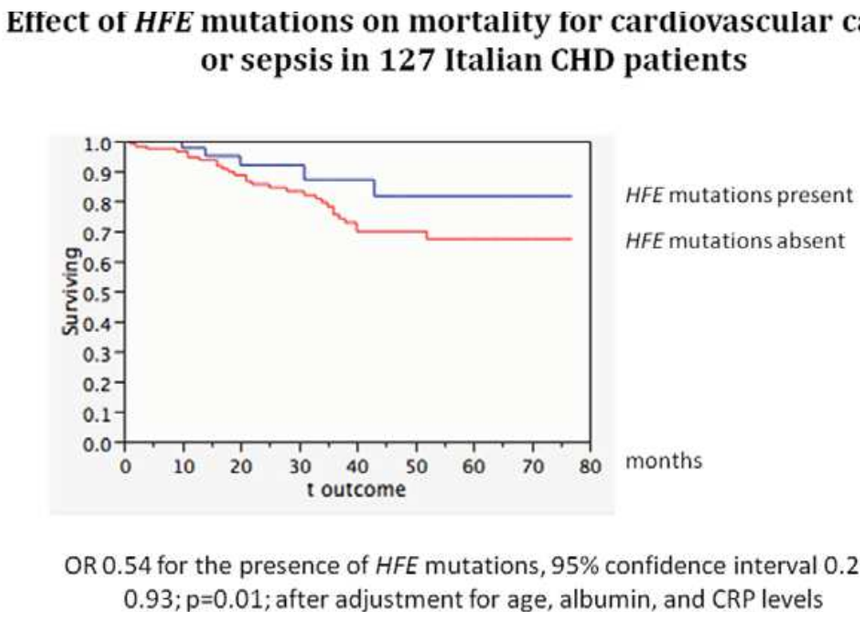


Fig. 1. Effect of *HFE* mutations on mortality for cardiovascular causes or infections in 127 Italian CHD patients.

9.3 *HFE* mutations and hepcidin release

In the hypothesis that altered regulation of hepcidin release by iron stores might explain the apparent protective role of the *HFE* mutations on cardiovascular complication and on the

response to ESAs therapy (Valenti, Girelli et al. 2009), we recently investigated in the largest series of CHD patients analyzed for serum hepcidin levels to date, and the first one in which a quantitative assay was used, whether the effect of common *HFE* gene mutations on hepcidin-25 could be involved in the pathophysiology of the alterations of iron metabolism and anemia. Our data clearly confirmed and extended previous reports based on a semiquantitative assay in smaller series, suggesting substantial hepcidin-25 upregulation in CHD patients compared to controls (Tomosugi, Kawabata et al. 2006; Kemna, Tjalsma et al. 2008), and preserved regulation of hepcidin-25 by iron stores and inflammation in this setting (Tomosugi, Kawabata et al. 2006; Kato, Tsuji et al. 2008). They also provided evidence of a modulating effect of *HFE* mutations, both the C282Y and the H63D mutations, on hepcidin-25 regulation by iron stores. First, we showed that hepcidin-25 levels are higher in patients receiving CHD than in healthy controls. Notably, while the groups studied were matched for gender, that is a major determinant of serum hepcidin levels (Ganz, Olbina et al. 2008; Swinkels, Girelli et al. 2008), CHD patients were significantly older than controls. However, this is unlikely to represent a substantial bias, since previous studies did not show a consistent increase in hepcidin levels with age in the general population (Ganz, Olbina et al. 2008). These data suggest that ESRD itself plays a role in hepcidin-25 accumulation in this setting. Also, we showed preserved regulation of hepcidin-25 levels by iron stores, as demonstrated by the very strong correlation with serum ferritin, and modulation by inflammation, as detected by CRP levels. Again, these data match closely those previously obtained showing a correlation of hepcidin-25 with ferritin and IL-6, but not CRP, in CHD, and, anecdotally, an increase in hepcidin-25 after IV iron administration (Tomosugi, Kawabata et al. 2006; Kato, Tsuji et al. 2008). Moreover, for the first time we were able to demonstrate a negative correlation between hepcidin-25 and serum iron, and we found that in a subgroup of patients with stable disease, selected to avoid the confounding effect of the frequent presence of acute inflammation, blood losses, cancer, and recent variation in the dosage of therapy, hepcidin-25 negatively correlated with Hb levels, and with a trend towards a negative correlation with lower serum iron. Since anemia and hyposideremia should rather decrease hepcidin levels, these findings suggest that hepcidin-25 plays a causal role in determining anemia by reducing iron availability to the erythron. To our knowledge, this is the first evidence supporting the theory that hepcidin is involved the pathogenesis of the anemia of CHD. These results imply that in CHD excessive iron administration may paradoxically hamper iron utilization for erythropoiesis by trapping iron in phagocytes, because of excessive hepcidin-25 induction favored by chronic inflammation, and that the effect of inflammation on altered iron metabolism and erythropoiesis may be mediated by increased hepcidin levels. They also suggest, together with the survival data in this same cohort shown above, that pharmacological downregulation of hepcidin (Babitt, Huang et al. 2007) may be beneficial in CHD. The subtle effect of *HFE* mutations on hepcidin release is likely magnified in CHD patients by the environmental pressure determined by chronic inflammation, and exposure to high amounts of iron and ESAs, thereby reaching clinical significance.

However, the protective effect of *HFE* mutations might not be limited to enhanced erythropoiesis related to relatively lower hepcidin levels. Indeed, as reported above, HFE protein seems to be directly implicated in the maturation of erythroblasts, and lack of functional HFE in erythroid precursors favors erythropoiesis and is associated with increased Hb levels independently of the effect on hepcidin levels (Ramos, Guy et al. 2011). In addition, another recently discovered role of HFE is the regulation of iron uptake and

inflammatory response in monocytes and macrophages. It has indeed been demonstrated that in the presence of mutated *HFE* human macrophages have a deficient response to inflammatory stimuli, which is likely related to an impaired ability to retain iron, NFκB activation, and cytokine release, and that hepcidin upregulation by inflammation is also impaired in the absence of functional HFE (Roy, Custodio et al. 2004; Wang, Johnson et al. 2008; Valenti, Dongiovanni et al. 2011; Wallace, McDonald et al. 2011).

9.4 Iron, *HFE* mutations and atherogenesis

Contrasting evidence suggest that iron deposition in the arterial wall may favor atherogenesis (Sullivan 2007). As a consequence of reduced hepcidin levels and lower inflammation, *HFE* mutations may play a protective role also in vascular damage related to atherosclerotic process, the major cause of mortality in CHD patients. In a large series of patients with metabolic syndrome alterations (Valenti, Swinkels et al. 2010), we observed that serum ferritin, reflecting iron stores, was an independent predictor of vascular damage, but only in patients negative for *HFE* genotypes predisposing to iron accumulation due to a relative decrease in the release of hepcidin. These data suggest that ferritin may represents a new marker of vascular damage, and support the controversial hypothesis that increased hepcidin favors atherosclerosis by inducing iron accumulation in arterial wall macrophages (Sullivan 2007). We found that ferritin levels were independently associated with common carotid arteries intima-media thickness, reflecting early vascular damage and a strong predictor of cardiovascular events, and very strongly associated with the presence of carotid plaques, possibly because of an additional effect of iron on the promotion of the complication of atherosclerosis by favoring endothelial damage and thrombosis (Day, Duquaine et al. 2003). The association between ferritin and vascular damage may thus be explained by the atherogenic effect of increased iron stores (Lee, Shiao et al. 1999; Duffy, Biegelsen et al. 2001; Wolff, Volzke et al. 2004; Lapenna, Pierdomenico et al. 2007), possibly mediated by increased hepcidin levels that determine iron trapping into monocytes, thus promoting transformation into foam cells in the presence of an atherogenic environment (Yuan, Li et al. 2004; Kraml, Klein et al. 2005; Sullivan 2007). Following this hypothesis, *HFE* mutations would be protective by decreasing hepcidin release and favoring iron egress from macrophages. Our data are consistent with the hypothesis that iron mediated vascular damage involves hepcidin upregulation, a mechanism that could be implied also in the enhanced atherosclerotic process in CHD patients, in which as explained before, hepcidin levels are more elevated than in general population.

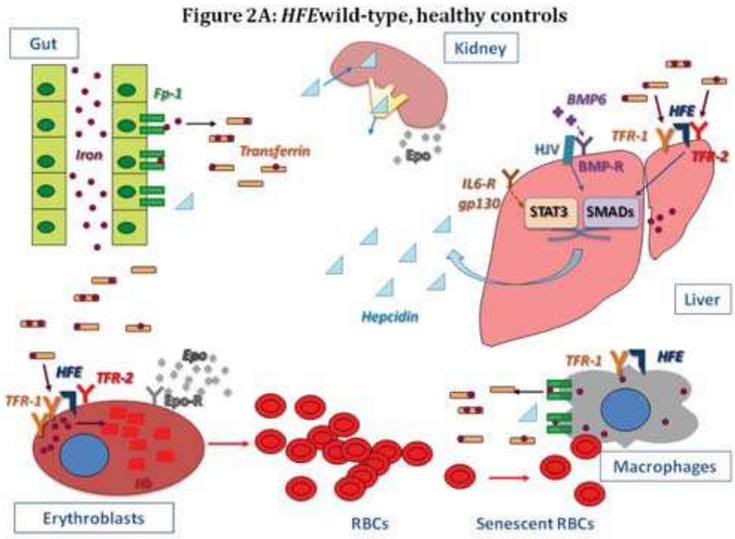
To further investigate the mechanisms involved in iron-mediated atherogenesis and explain the protective effect of *HFE* mutations, we next investigated the effect of iron treatment on the activation and secretion of atherogenic molecules in differentiating monocytes of subjects with different *HFE* genotypes. Treatment with iron salts, determining increased intracellular iron, enhanced the release of the macrophage chemoattractant protein-1 (MCP-1) and of IL-6 independently of oxidative stress in differentiating monocytes derived from patients with metabolic syndrome (Valenti, Dongiovanni et al. 2010). Furthermore, the iron-dependent induction of the MCP-1 and IL-6 was associated with the severity of vascular damage in these patients, suggesting that macrophage activation by iron may be involved in the pathogenesis of atherosclerosis progression in these patients also *in vivo*. IL-6 is a pro-inflammatory cytokine involved in the acute phase response, whose serum levels correlate with cardiovascular risk and have been linked to the inflammatory status within atherosclerotic plaques (Luc, Bard et al. 2003). MCP-1 (also known as CCL2), is a chemokine involved in macrophage recruitment at

inflammation sites released by macrophages, but also by smooth muscle cells and endothelial cells. MCP-1 plays a crucial role in both the initiation and progression of atherosclerosis, and its serum levels reflect the atherosclerotic plaque burden. Higher MCP-1 has also been reported to represent a negative prognostic factor in acute coronary syndromes (Nelken, Coughlin et al. 1991; Gu, Okada et al. 1998; Amasyali, Kose et al. 2009). A dose-dependent induction of MCP-1 transcription and release in the supernatant induced by iron treatment was confirmed in monocytes of healthy subjects with normal iron parameters, suggesting that this represents a physiological response to increase intracellular iron availability. These data are in line with previous experimental evidence, suggesting that iron may induce MCP-1 release by oxidative stress in macrophages. Indeed, intravenous iron treatment induces MCP-1 release by monocytes in mice and patients with ESRD (Zager 2005; Agarwal 2006), whereas the iron chelator deferoxamine have recently been shown to decrease NFκB and consequently MCP-1 release. Importantly, we also showed that treatment with hepcidin mimicked the effect of iron salts on MCP-1 release. This evidence led to another confirmation of the fundamental pathogenic role of hepcidin on atherogenic process. In addition, we obtained evidence that, as it occurs in the presence of homozygosity for the C282Y *HFE* mutation (Garuti, Tian et al. 2010), monocytes of patients with *HFE* genotypes predisposing to mild iron overload show reduced ability to accumulate intracellular iron. Iron induced upregulation of MCP-1 and IL-6 was prevented in monocytes of patients carrying these *HFE* genotypes "at risk", thus suggesting that the hampered upregulation of pro-inflammatory mediators may contribute to explain the lack of association between iron overload and accelerated atherosclerosis in HH patients, and the contradictory data on the effect of *HFE* mutations on vascular damage (Sullivan 2007; Engberink, Povel et al. 2010). Consistently, despite the fact that iron overload has been associated with increased serum MCP-1 levels, patients homozygous for the C282Y HFE mutation had lower levels than those whose iron overload was explained by other genetic/acquired factors and healthy controls (Lawless, White et al. 2007). We also measured serum MCP-1 levels in a series of patients at high risk for vascular disease because of metabolic syndrome, who typically display a high prevalence of altered iron metabolism (Valenti, Swinkels et al. 2010). In line with a possible induction of MCP-1 release by increased iron in monocytes/macrophages, serum MCP-1 levels, whose primary source is represented by activated macrophages, were significantly correlated with hepcidin-25, and MCP-1 levels were an independent predictor of the presence of carotid plaques, indicating an advanced atherosclerotic process. Thus, the emerging details of the physiology of hepcidin suggest a resolution of the apparent paradox of an important role of iron in atherogenesis in the absence of increased plaque burden in HH. Hepcidin, induced by iron and inflammation, acts to block iron recycling from macrophages by binding and causing internalization and degradation of ferroportin, the sole cellular iron exporter. Low hepcidin levels are observed in iron deficiency anemia and HH, both characterized by reduced macrophage iron stores. The failure of vascular wall macrophages to retain iron in HH may therefore prevent the progression of atherosclerotic plaques. *HFE* mutations could assume a protective role in the pathogenesis of atherosclerotic plaques and cardiovascular disease. These data support the hypothesis that MCP-1 release by intralesional monocytes/macrophages, which can be iron loaded because of intraplaque hemorrhage, systemic iron overload, and local inflammation is involved in the pathogenesis of iron-induced atherosclerosis.

9.5 Impact of *HFE* mutations in CKD: a model

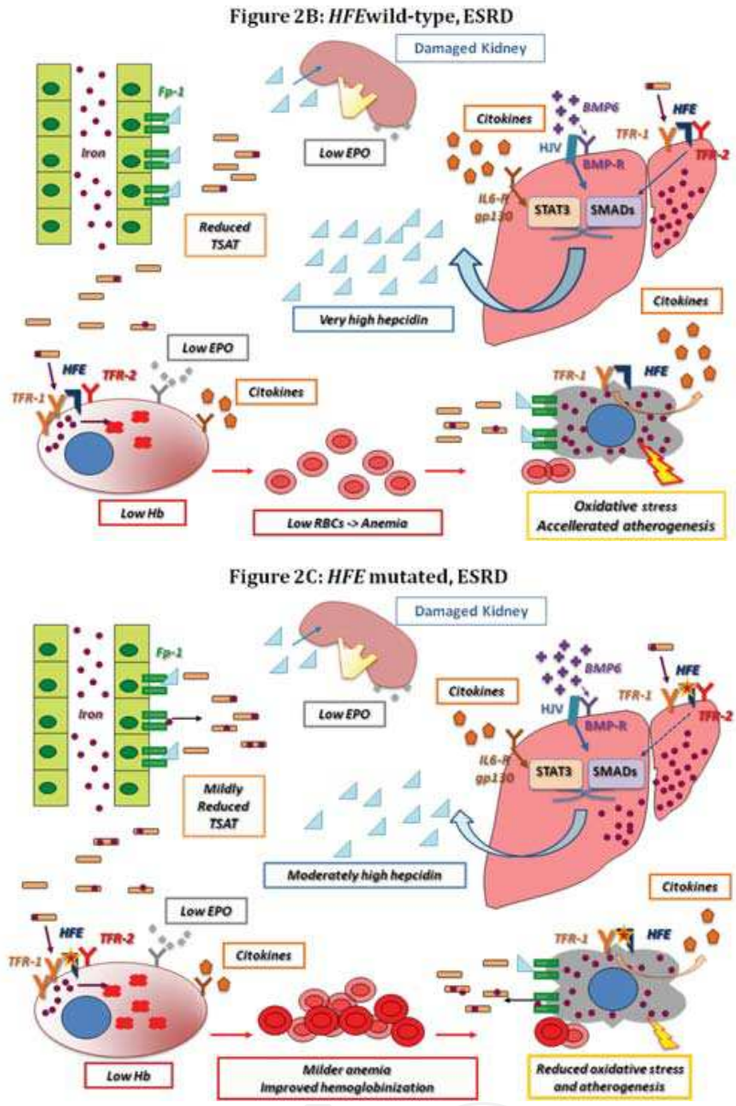
In healthy controls wild-type for *HFE* (i.e. not carrying mutations) (Figure 2A), iron absorption depends on the activity of Fp-1, regulated by hepcidin. Iron is necessary for the

production of Hb in erythroblasts, which are stimulated by Epo, secreted by the kidneys to stimulate the production of mature RBCs in response to anemia or a relative deficit of oxygen delivery to the cells. Uptake of iron-TF occurs by means of TFR-1. Iron recycled from senescent RBCs by macrophages exits these cells via Fp-1. Hepcidin, secreted by the liver and excreted by the kidneys, promotes the degradation of Fp-1, thus inhibiting enteric absorption and the release of iron from macrophages. HFE is bound to TFR- 1 and is involved in the transmission of signaling that finally brings to the finely tuned secretion of hepcidin, in agreement with iron status. This regulatory pattern involves also BMP6, HJV and many other molecules, and converges on the activation of SMADs. In patients with ESRD wild type for *HFE* mutations (Figure 2B), the presence of proinflammatory cytokines, through IL-6 release and the activation of STAT3, and the impairment of renal excretion enhance the concentration of hepcidin, and consequently cause the impairment of iron absorption from the enteric lumen and of the excretion from macrophages: iron stores are not depleted so ferritin levels are high, but few iron circulates into the blood, so that TSAT is low. The erythroblasts have not sufficient iron to support Hb production and, along with Epo deficiency due to renal dysfunction, this provokes a significant reduction in RBCs production, which consequently leads to anemia. Furthermore, iron overload in macrophages predisposes to enhanced oxidative stress and accelerated atherosclerosis, and increases the susceptibility to infections. Conversely, patients with ESRD and mutations of *HFE* gene (C282Y and/or H63D) (Figure 2C) display a milder degree of anemia, since these mutations uncouple the hepatic iron sensing and lead to a relative deficit in hepcidin secretion, to a reduced cytokines release and to an improvement of erythropoiesis. In this way, more iron is absorbed from the gut, TSAT is only moderately diminished, and less iron remains trapped into macrophages. Finally, less severe oxidative stress is observed, contributing to the possible reduction in mortality and better response to ESAs therapy.



Fp-1: ferroportin 1, EPO: erythropoietin, Epo-R: erythropoietin receptor, IL6: interleukine 6, HJV: hemojuvelin, BMP6: bone morphogenetic protein 6, BMP-R: bone morphogenetic protein receptor, TFR: transferrin receptor, HFE: hereditary hemochromatosis protein, STAT3: Signal transducer and activator of transcription 3, SMADs: small mother against decapentaplegic, Hb: hemoglobin, RBCs: red blood cells, TSAT: transferrin saturation.

Fig. 2. (A) A model of the impact of *HFE* mutations in chronic renal disease (see text).



Fp-1: ferroportin 1, EPO: erythropoietin, Epo-R: erythropoietin receptor, IL6: interleukine 6, HJV: hemojuvelin, BMP6: bone morphogenetic protein 6, BMP-R: bone morphogenetic protein receptor, TFR: transferrin receptor, HFE: hereditary hemochromatosis protein, STAT3: Signal transducer and activator of transcription 3, SMADs: small mother against decapentaplegic, Hb: hemoglobin, RBCs: red blood cells, TSAT: transferrin saturation.

Fig. 2. (B), (C) A model of the impact of *HFE* mutations in chronic renal disease (see text).

10. Hepcidin targeting therapies for the management of the anemia of ESRD and CHD

Overall evidence thus suggest that *HFE* mutations are associated with reduced serum hepcidin levels in CHD patients, and result in a reduced need of ESAs and iron supplementation, thus conferring a possible benefit on survival, in particular by reducing cardiovascular and infectious complications (Valenti, Valenti et al. 2008; Valenti, Girelli et al. 2009). These results may have important clinical implications, as they suggest that targeting

the HFE/hepcidin/ferroportin-1 axis by pharmacological treatment (Babitt, Huang et al. 2007), which is now under extensive investigation for the treatment of anemia of chronic disease (Nemeth 2010), may further improve the long-term outcomes of CHD, by reducing the amount of iron and ESAs supplementation needed and by improving iron utilization for erythropoiesis (Babitt and Lin 2010).

Several drugs are under development for the modulation of the release and activity of hepcidin. The approaches under study include neutralizing anti-hepcidin antibodies (Sasu, Cooke et al. 2010), inhibitors of BMP type I receptors such as dorsomorphin (Yu, Hong et al. 2008), soluble HJV, which acts as decoy receptor inhibiting BMP signaling (Babitt, Huang et al. 2007), and ESAs themselves, which can directly or indirectly inhibit hepcidin release (Pinto, Ribeiro et al. 2008).

In a mouse model of anemia of chronic disease (ACD) caused by injections of heat-killed *Brucella Abortus* (Sasu, Cooke et al. 2010), neutralizing antibodies (Abs) directed against hepcidin were able to restore the reticulocyte response and normal Hb levels in combination with ESAs, whereas ESAs alone, and importantly ESAs plus IV iron, were ineffective. Furthermore, over-expression of hepcidin in mice was sufficient to hamper the erythropoietic response to ESAs. It is therefore conceivable that administration of anti-hepcidin antibodies could restore ESAs susceptibility in the roughly 10% of CHD patients, who display ESAs resistance due to chronic inflammation and high hepcidin levels. These preliminary preclinical data suggest that it is unlikely that therapies that antagonize hepcidin would result in the control of anemia when administered alone, but they could restore susceptibility in patients with ESAs resistance or reduce the dose of ESAs and iron required to achieve anemia control, thereby minimizing side effects.

A different approach to control hepcidin activity is represented by the modulation of hepcidin expression by targeting bone morphogenetic proteins (BMPs) activity. Soluble forms of HJV (sHJV), deriving from cleavage by furin or TMPRSS6, have been detected both in cell cultures and in human sera. Administration of sHJV (HJV.Fc) has been demonstrated to inhibit BMP signaling and hepcidin expression, likely by binding to BMP6 and preventing the interaction with BMP receptors (Andriopoulos, Corradini et al. 2009), and could be possibly exploited as a treatment for anemia of inflammation (Babitt, Huang et al. 2007). It was also shown that anti-BMP6 Abs were similarly effective (Andriopoulos, Corradini et al. 2009). Notably, sHJV has been demonstrated to be downregulated by iron stores and induced by hypoxia, thus possibly being involved in hepcidin regulation by these stimuli. However, the physiological relevance of sHJV forms is still unknown; it has been hypothesized that it is released by the skeletal and cardiac muscle, where HJV is expressed, thus acting in response to iron deficiency and hypoxia to increase oxygen supply by hepcidin downregulation and increased iron availability for the synthesis of Hb, myoglobin, and mitochondrial cytochromes (Babitt and Lin 2010).

A recent and promising development was provided by the recognition of the hepcidin suppressive activity of heparin. Heparan sulfate proteoglycans (HSPGs) are expressed on the surface of various cell types and in the extracellular matrix, and modulate BMP osteogenic activity by binding BMPs, BMP antagonist, and BMP receptors. Heparin, a proteoglycan analog to HSPGs, has been reported to strongly down-modulate hepcidin release *in vitro* in hepatocytes and *in vivo* in mice, resulting in increased serum iron and decreased splenic iron levels (Poli, Girelli et al. 2011). The effect was long lasting and higher for unfractioned than for low-molecular-weight heparin and fondaparinux, and seemed to depend on the sequestration of BMP6, thereby preventing the interaction with HJV and the

induction of hepcidin transcription. Importantly, heparin not only hampered BMP6 dependent induction of SMAD phosphorylation and hepcidin transcription, but could also prevent the IL-6 and STAT3 dependent activation of hepcidin promoter, suggesting that it may overcome the effect of inflammation on hepcidin release also in patients with anemia of chronic diseases or ESRD. Due to the dose-dependent anticoagulant effects and the need for therapeutic monitoring, it is unlikely that unfractioned heparin could ever be implemented as a therapy for the anemia of chronic disease or CHD anemia in patients without thrombophilic conditions. However, it is conceivable that heparin could be modified experimentally to improve the anti-hepcidin while decreasing anticoagulant activity. This represents a very promising approach, because heparins with low anticoagulant, but preserved anti-inflammatory activity, have already been developed (Ceccarelli, Bani et al. 2009).

Another possibility consists in the downstream inhibition of BMP receptors. Dorsomorphin has been identified through a large scale *in vivo* screening approach as a selective inhibitor of BMP signaling through the antagonism of type I receptors activity (Yu, Hong et al. 2008). Interestingly, this small molecule compound was able to profoundly reduce basal hepcidin levels and increase serum iron in mice. However, due to the many roles of BMP signaling in the regulation of cell differentiation and homeostasis, it is likely that dorsomorphin, that for example effectively inhibits osteogenesis *in vitro*, would have unacceptable side effects in humans.

Anti-cytokines Abs, such as those neutralizing IL-6, a major inducer of hepcidin during inflammation via STAT3 activation, would likely reduce hepcidin transcription and inflammation-related ESAs resistance at the same time, but potential side-effects (altered immune function) will be again a limiting factor for their clinical utilization. Finally, it should be not be forgotten that large doses of ESAs can reduce hepcidin levels, even if the effect seem to require the effective induction of erythropoietic activity *in vivo*, and is therefore subjected to clinical resistance, and at the price of severe side effects.

In conclusion, the association of anti-hepcidin Abs, or possibly anti-BMP6 Abs or anti-inflammatory/anti-hepcidin heparins, to ESAs represents the most promising approach for the treatment of the anemia of CHD and chronic diseases. However, as *HFE* has been demonstrated to be directly involved in the maturation of erythroid progenitors (Ramos, Guy et al. 2011) inflammation and atherogenesis, it should be noted that the advantageous effect of the presence of *HFE* mutations on erythropoiesis in CHD patients may not be completely dependent on relatively decreased hepcidin levels (Valenti, Girelli et al. 2009). Therefore, even if the degree of hepcidin activity suppression achievable *in vivo* would likely play a major role in determining the clinical outcome, it is possible that anti-hepcidin therapies would result in a lesser improvement in anemia control than that conferred by the presence of protective *HFE* genotypes. A better comprehension of the mechanisms linking *HFE* mutations with altered protein function both related to iron sensing / hepcidin induction and to the direct control of cellular iron intake is needed to design new therapeutic approaches aimed specifically at inhibiting this molecule. Furthermore, the possible development of side effects, such as the promotion of carcinogenesis related to chronic exposure to high TSAT levels, which would confer easy access to this growth promoting and mutagen metal to susceptible cells in patients already exposed to high levels of oxidative stress, should be weighted against the potential benefits (Zacharski, Chow et al. 2008; Dongiovanni, Fracanzani et al. 2010; Fargion, Valenti et al. 2010).

11. Conclusion

Common polymorphisms in the *HFE* gene of HH represent a major determinant of iron metabolism balance in Caucasian subjects, and the subtle effect of the C282Y and H63D mutations is likely magnified in CHD patients by the environmental pressure determined by chronic inflammation, and exposure to high amounts of iron and ESAs, thereby reaching clinical significance. These genetic factors act by hampering hepcidin induction in hepatocytes in response to increased iron stores, thereby resulting in reduced serum hepcidin and inadequate inhibition of the activity of the iron exporter Fp-1, and consequently in inappropriately high duodenal iron absorption and iron recycling from erythrophagocytosis in macrophages and increased serum iron. As a consequence, homozygosity for these mutations may result in organ damage related to iron overload in parenchymal tissues.

ACD and the anemia of ESRD and CHD are conversely characterized by chronic inflammation with increased cytokines levels, resulting in increased hepcidin levels with consequent reduction in iron absorption, recycling, and availability to the erythron. This response proves advantageous in the short-term to restrain iron availability to pathogens during infections, but in the case of chronicization it leads to severe anemia, and may impair the response to ESAs and oral and even IV iron therapy. Furthermore, as hepcidin is filtered by the glomerulus, ESRD itself is a contributing factor to increased hepcidin levels in CHD. Evidence is also accumulating that *HFE* mutations directly favor erythroblast maturation and hemoglobinization independently of serum hepcidin and reduce macrophages activation in response to inflammation.

We showed that in CHD patients *HFE* mutations may confer an adaptive benefit by decreasing hepcidin release in response to iron and inflammation, thereby improving iron availability to erythropoiesis, anemia control, and the response to ESAs and IV iron therapies. This would translate in a decreased burden of side effects, mainly related to an increased susceptibility to cardiovascular events and infections, the latter limited to IV iron. Although data must be confirmed in larger prospective studies, this favorable shift in iron metabolism balance possibly results in reduced mortality, in particular because of cardiovascular and infective diseases.

These data suggest that anti-hepcidin therapies such as anti-hepcidin or anti-BMP6 Abs, which are currently under development for ACD, may improve anemia management in CHD, concomitantly sparing the doses and side-effects of ESAs and iron and resulting in better quality-of-life, and most importantly a survival advantage for these patients. However, as the beneficial effect of *HFE* mutations on iron metabolism in CHD does not seem to be fully explained by lower hepcidin levels, direct inhibition of HFE-mediated regulation of iron metabolism may represent another valuable new therapeutic target.

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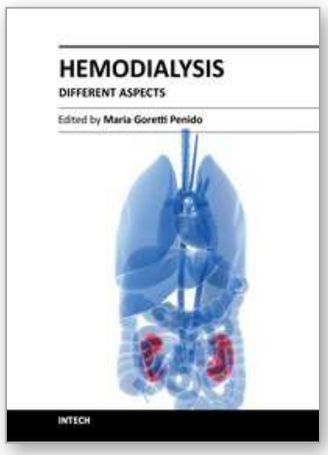

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