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HYPOXIA-DEPENDENT HEPCIDIN DOWN-REGULATION: *IN VITRO* AND *IN VIVO* STUDIES

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INDEX

ABSTRACT **5**

INTRODUCTION **7**

IRON METABOLISM *8*

REGULATION OF IRON HOMEOSTASIS *12*
CELLULAR IRON REGULATION: THE IRE/IRP SYSTEM 12
SYSTEMIC IRON REGULATION: THE HORMONE HEPCIDIN 14

HEPCIDIN REGULATION *16*
POSITIVE REGULATORS 17
NEGATIVE REGULATORS 21

IRON-RELATED DISEASES *25*
IRON OVERLOAD 25
IRON DEFICIENCY 28

AIM **29**

MATERIALS AND METHODS **33**

STUDIES WITH HUMAN SUBJECTS *34*

“MONTE ROSA” STUDY DESIGN AND PROCEDURES *34*

HUMAN IRON AND OTHER INDICES *35*

CELL CULTURE AND SERUM TREATMENT *35*

DUAL-LUCIFERASE REPORTER ASSAY *36*

ANIMAL CARE AND IN VIVO STUDIES *37*

HYPOXIA STUDIES IN MICE *37*

BONE MARROW FLUSHING *37*

RNA EXTRACTION AND cDNA SYNTHESIS *38*

REAL-TIME QUANTITATIVE-PCR *38*

PROTEIN ISOLATION AND WESTERN BLOT ANALYSIS. *39*

LIVER IRON CONTENT (LIC) AND SPLEEN IRON CONTENT (SIC) EVALUATION *39*

STATISTICAL ANALYSIS *40*

RESULTS **41**

STUDIES WITH HUMAN SUBJECTS *42*
BIOCHEMICAL DATA 42

HYPOXIA CHALLENGE 42
DUAL-REPORTER LUCIFERASE ASSAY 43
GENE EXPRESSION ANALYSIS 44

STUDIES WITH MICE MODELS 47
GENE EXPRESSION ANALYSIS IN THE LIVER 47
GENE EXPRESSION ANALYSIS IN BONE MARROW AND SPLEEN 49
TREATMENT WITH α-PDGF-BB AND AG1296 51
CREB/H WESTERN BLOT 52
LIVER AND SPLEEN IRON CONTENT MEASUREMENT 53

DISCUSSION 54

REFERENCES 59

ABSTRACT

Hepcidin is the main regulator of systemic iron homeostasis, acting by inhibiting macrophages iron release and intestinal iron absorption. During hypoxia hepcidin expression is inhibited to allow iron requirement to bone marrow to sustain erythropoietic expansion, but mechanisms of hypoxia-induced hepcidin down-regulation are partially unknown. Our aim was to improve our knowledge on hypoxia modulation of hepatic hepcidin, investigating the role of erythroid and iron-related pathways using *in vitro* and *in vivo* studies. We firstly studied the effect of hypoxia on healthy volunteers living at high altitude for 24h and 72h, confirming that hypoxia is a strong inhibitor of hepcidin production and that this inhibition followed EPO induction. Indeed s-hepcidin was strictly down-regulated at 72h of hypoxia while EPO levels were already markedly increased at 24h slightly decreasing thereafter. We then analyzed the effect of sera of these subjects on hepcidin expression in HuH-7 cells using both luciferase assay and RT-PCR. *HAMP* wild-type promoter activity decreased after exposure to hypoxic sera, suggesting that circulating factors present in sera may modulate hepcidin expression. However, analysis of the transcripts gave less homogenous results. In fact only a subgroup of hypoxic sera significantly inhibited hepcidin. These sera also induced a significant *ID1* down-regulation indicating that inhibitory factor(s) seems to act through the SMADs pathway. Our results also suggest that a cellular model cannot reproduce what really happens *in vivo* that might depend to complex events involving both circulating and tissue factors. Therefore, we took advantages from a mouse model. For hypoxia challenge, C57BL/6 mice were housed in a hypoxic chamber at 10% O_2 for 6h and 15h. mRNA levels of erythroid genes were assessed in liver, spleen and bone marrow by RT-PCR. *Hamp1* levels were slightly increased after 6h of hypoxia and strongly down-regulated after 15h. After 6h we already observed a marked activation of transcription of all the erythroid genes analyzed (*Pdgf-b*, *Fam132b*, *Gdf-15* and *Twsg-1*) in bone marrow (but not in spleen) that return to baseline levels after 15h. To better analyze the role of PDGF-BB, normoxic and hypoxic mice were treated with PDGF-BB neutralizing antibody

or PDGF-BB receptor inhibitor immediately prior to hypoxic challenge. Inactivation of PDGF-BB or its receptor suppressed the hypoxia-induced hepcidin inhibition confirming the importance of PDGF-BB in hepcidin regulation. However, no changes in CREB/H protein were observed suggesting that this molecule is not involved in hepcidin regulation in this setting. In conclusion, our studies demonstrated that hypoxia-induced hepcidin regulation is a quick event secondary to erythropoietic activation. Indeed hepcidin decreased after up-regulation of transcription of several erythroid genes that return to baseline when hepcidin reached the maximum inhibition. Our studies indicate that PDGF-BB is a good candidate as hepcidin erythroid regulators but also suggest that hypoxia induced-hepcidin suppression is the result of cooperation of multiple signals that need to be dissected.

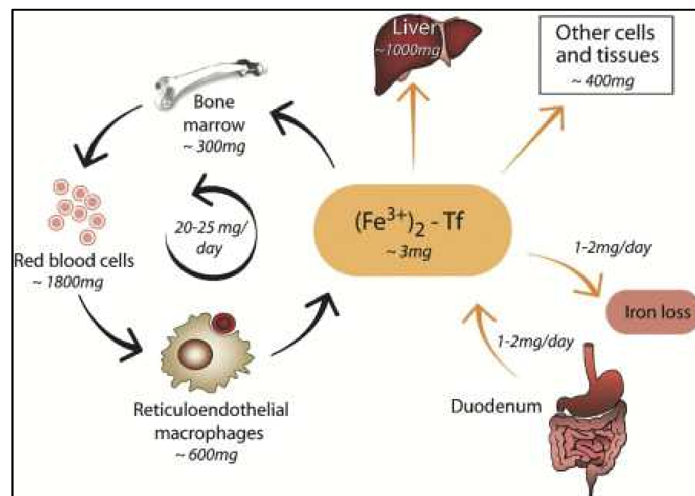
INTRODUCTION

IRON METABOLISM

Iron is a fundamental element for many biological processes as oxidative metabolism, DNA synthesis and proper erythropoietic function; indeed, iron deficiency may lead to cell growth arrest or death (Andrews, 2000). Conversely, because it readily accepts or donates electrons, free iron is highly reactive and toxic. Indeed, excess of iron might cause cellular damage through the formation of free radicals. Thus iron metabolism must be strictly regulated to prevent adverse effects from either iron deficiency or iron overload (Yun and Vincelette, 2015).

An adult human contains 3-4 g iron (Ganz, 2013). More than 80% (2-3 g) is used to sustain erythropoiesis in the bone marrow and it is incorporated in the heme group of hemoglobin in erythroid precursors and mature red blood cells; about 10% is present in the myoglobin of muscle fibers (300 mg) or in the prosthetic groups of enzymes or cytochromes. All cells contain smaller concentration of iron-containing proteins essential for energy production and other important functions. The surplus iron is stored mainly in liver hepatocytes and macrophages (200-1500mg) (Andrews, 2000). To support erythropoiesis and other metabolic processes an adult human requires approximately 25 mg iron daily. Iron is released in circulation from duodenal enterocytes and from macrophages. Normally, only 1-2 mg of iron per day are absorbed from duodenal enterocytes to compensate for iron losses due to sloughing of intestinal epithelial cells, blood loss or sweat. The majority of the daily iron needs (20-25 mg/day) are obtained through internal recycling of senescent erythrocytes by reticuloendothelial macrophages (Figure 1). Iron absorption can be enhanced when the needs are higher or suppressed in iron overload (Hentze, et al., 2010). There are no active mechanisms for iron excretion, hence intestinal absorption has to be modulated to provide enough, but not too much, iron to keep stores replete and erythroid demands met.

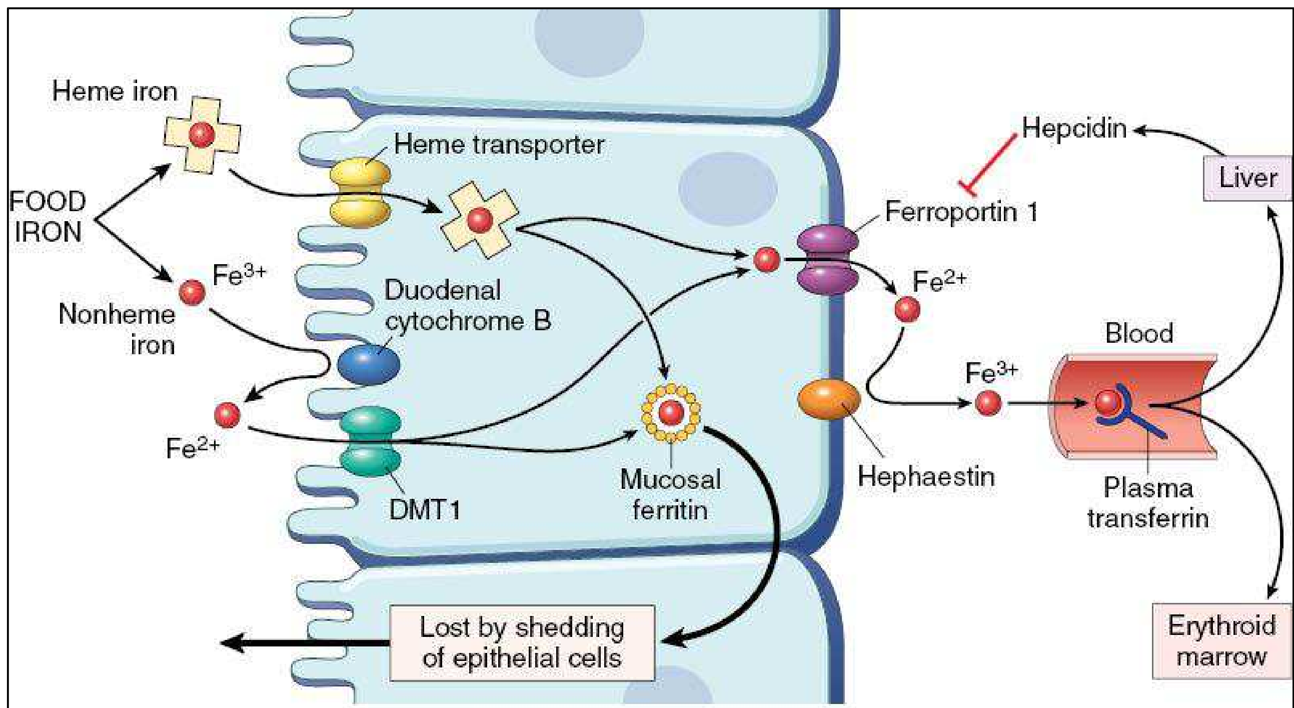
Figure 1. Systemic iron homeostasis (Hentze, et al., 2004).



The three key cell types involved in iron homeostasis are duodenal enterocytes that absorb dietary iron; macrophages that recycle iron from erythrocytes and hepatocytes that store iron and can release it when needed. Extracellular iron (2-4 mg) circulates in the plasma bound to transferrin (Tf) which has two binding sites for ferric iron (Fe^{3+}); in this form iron is nonreactive and in a safe form. Intestinal cells take up dietary non-Tf bound iron directly. Iron in the diet is found in two different forms: heme and non-heme iron. Non-heme food iron exists primarily in the bio-unavailable Fe^{3+} form. In this form it cannot be absorbed from duodenal enterocytes, because at neutral pH is almost insoluble in water and is not able to cross membranes. Fe^{3+} must first be reduced into ferrous iron (Fe^{2+}) by the cytochrome ferrireductase DcytB (Cybrd1) (McKie, et al., 2001) and then is absorbed at the brush border of duodenal enterocytes via the divalent metal transporter 1 (DMT1) (Gunshin, et al., 1997). However the majority of food iron is complex in heme, but the mechanisms of its absorption are still poorly defined. The Heme Carrier Protein 1 (HCP1) has been proposed to be the heme iron transporter, however it has been demonstrated that it carries mostly folate than iron itself (Qiu, et al., 2006) and it remains to be proven whether it is a "bi-functional" transporter or not.

Heme iron is then released intracellularly by the hemoxygenase 1 (HOX1) and then enter the normal pathway of non-heme iron. (Figure 2)

Figure 2. Intestinal iron uptake (Kumar and Robbins, 2007).

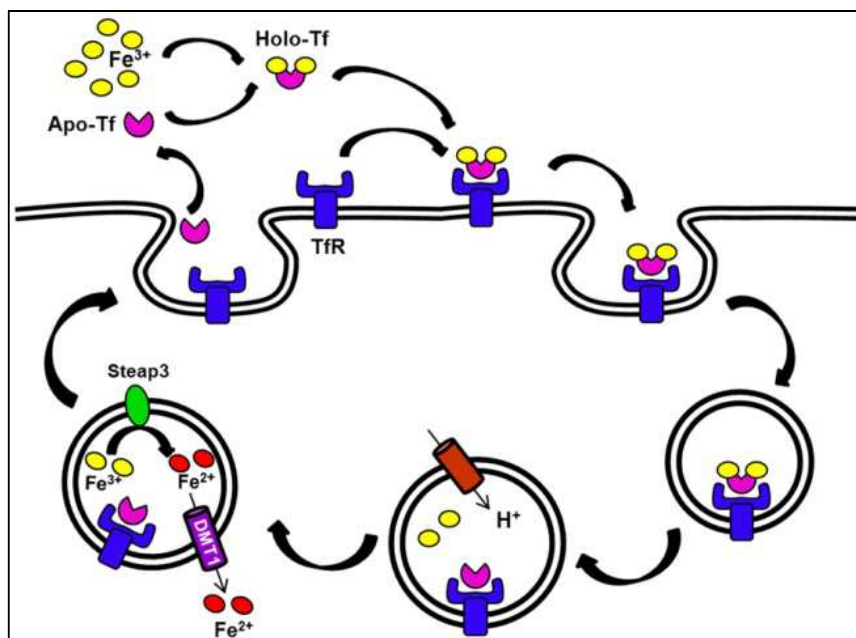


Once inside the enterocyte, the portion of iron of the Labile Iron Pool (LIP) that is not needed for immediate use is stored by ferritin, to avoid the toxic effects of free iron (Torti and Torti, 2002). Ferritin is a ubiquitous protein that consists of two subunits, the Light chain (L-ferritin) and the Heavy chain (H-ferritin) that assemble in different proportion to form apo-ferritin shells. Ferritin nanocages are made of 24 subunits of L and H chains that creates a cavity where intracellular iron is collected. They can bind, oxide and store up to 4500 atoms of Fe^{2+}, preventing cell damages triggered by free iron toxicity (Arosio and Levi, 2010). When iron is needed, enterocytes release iron in the bloodstream via Ferroportin (FPN), the only putative iron exporter identified, located at the basolateral membrane of cells (Abboud and Haile, 2000; Donovan, et al., 2000; McKie, et al., 2000). FPN mediated iron exporter in concert with Hephaestin, a ferroxidase that converts Fe^{2+} to Fe^{3+} that is then loaded onto Tf for transport in the plasma (Vulpe, et al., 1999). Iron export from

non-intestinal cells requires a ferroxidase homologous to Hephaestin named Ceruloplasmin (Cp) (Harris, et al., 1999) (Figure 2).

When non-intestinal cells need iron, they can acquire plasma iron via transferrin-dependent mechanism. Transferrin has two binding sites for iron, therefore in plasma it exists in three forms: apo-transferrin, monoferric-transferrin and diferric-transferrin (Tf-Fe_2). In physiological conditions, iron occupies 30-40% of its binding sites. Diferric-transferrin is absorbed via Transferrin Receptor (TFR) 1-mediated endocytosis (Cheng, et al., 2004). Once transferrin and iron complex binds to TFR1 on the cell surface, the complex Tf-Fe_2/TFR1 is internalized by clathrin-dependent endocytosis and form early endosome (Cheng, et al., 2004). The acidic environment inside the endosome induces conformational changes of transferrin and TFR1 inducing the dissociation of iron (Cheng, et al., 2004). Fe^{3+} in the endosome is reduced to Fe^{2+} by members of STEAP family of metalloreductases (Ohgami, et al., 2005) and then transferred in the cytosol via DMT1 (Fleming, et al., 1998). Apo-transferrin and its receptor are then recycled to the cell surface (Figure 3).

Figure 3. The transferrin (Tf) cycle (Veuthey and Wessling-Resnick, 2014).



Another transferrin receptor, TFR2, homologous to TFR1 was identified (Kawabata, et al., 1999). TFR2 is a type II membrane glycoprotein mainly expressed in liver hepatocytes and erythroid precursors (Forejtnikova, et al., 2010; Kawabata, et al., 2004). Two different isoform of TFR2 were identified, α and β. TFR2α is very similar to TFR1 (66% of homology and 45% of identity of the extracellular portion) (Kawabata, et al., 1999) however its presence is not redundant (Levy, et al., 1999) and its affinity for TF-Fe_2 is 25-30 times lower than TFR1 (West, et al., 2000). The proper role of TFR2 in iron homeostasis is not fully elucidated yet; however, it seems that it could have a role as a sensor of plasma iron content rather than intracellular iron transporter.

REGULATION OF IRON HOMEOSTASIS

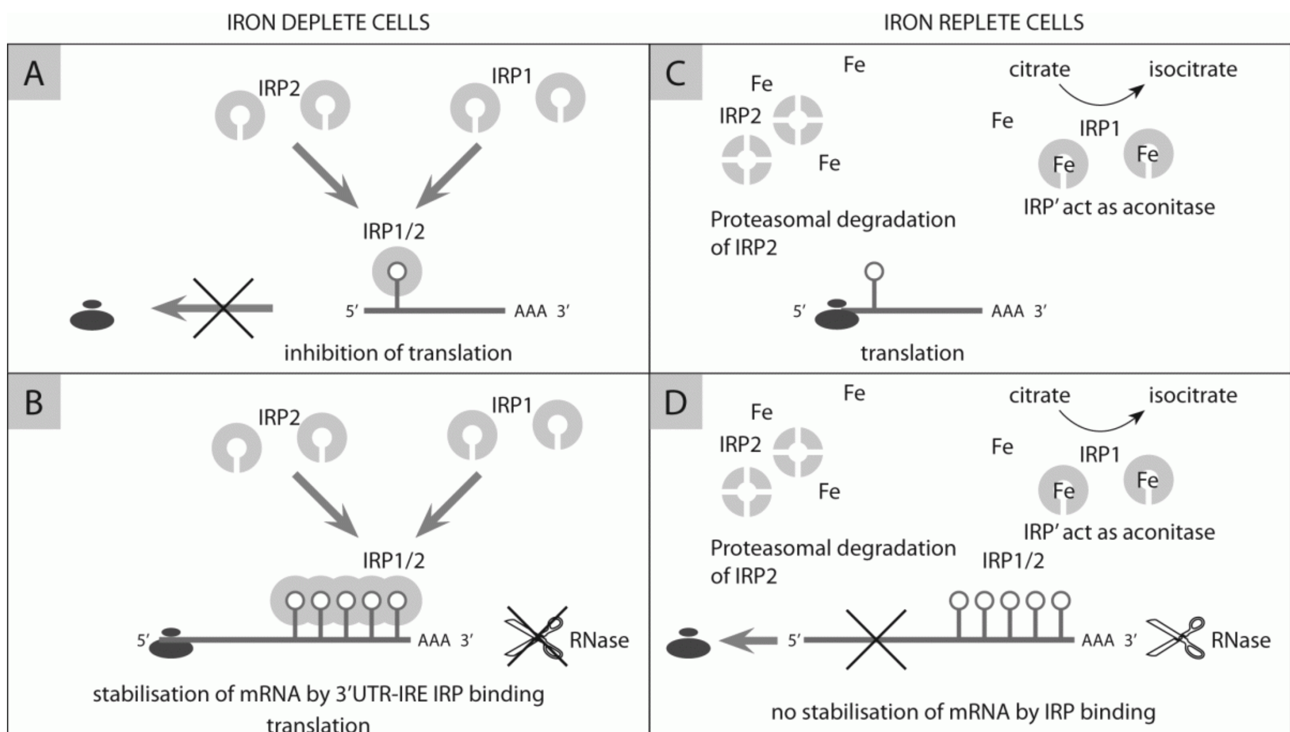
Since there are no active mechanisms for iron excretion, intestinal iron absorption must be strictly regulated to maintain physiological homeostasis. Humans possess two control mechanisms to maintain iron homeostasis by regulating iron absorption, iron recycling, and mobilization of stored iron. Iron absorption is regulated locally by iron-regulatory proteins (IRPs) and systematically by hepcidin, the central iron regulatory hormone.

CELLULAR IRON REGULATION: THE IRE/IRP SYSTEM

Iron Regulatory Protein (IRP) 1 and 2 post-transcriptionally regulates cellular iron homeostasis. IRP1 and IRP2 (also known as ACO1 and IREB2 respectively) act by binding to regulatory stem-loop structures known as Iron Responsive Elements (IREs) in 5' or 3' untranslated regions (UTRs) of target mRNAs (Cazzola and Skoda, 2000). Single IREs are located in the 5' UTRs of mRNAs encoding H and L ferritin and ferroportin while multiple IREs are present in the 3' UTR of TFR1 mRNA (Donovan, et al., 2000; Hentze and Kuhn, 1996; McKie, et al., 2000). A single IRE was

identified also in the 3' UTR of DMT1, however its mechanism of action is not well understood (Gunshin, et al., 2001). IRPs' binding to 5' UTR IREs inhibits translation initiation while their binding to IREs within 3' UTR prevents mRNA degradation. IRPs are capable of sensing cellular iron status. In iron-deplete cells both IRP1 and IRP2 bind IREs with high affinity whereas in iron-replete cells IRPs dissociate from IREs. In particular when iron concentration is high IRP1 (but not IRP2) acquires a 4Fe-4S cluster that precludes IRE binding and functions as cytosolic aconitase (Haile, et al., 1992) whereas IRP2 is targeted for degradation by the proteasome (Iwai, et al., 1998). In iron-deficient cells, IRP1 loses its Fe/S cluster and IRP2 accumulates in the cytosol, allowing the binding to targets IREs (Figure 4).

Figure 4. Regulation of cellular iron homeostasis by IRE/IRP system (Tandara and Salamunic, 2012).



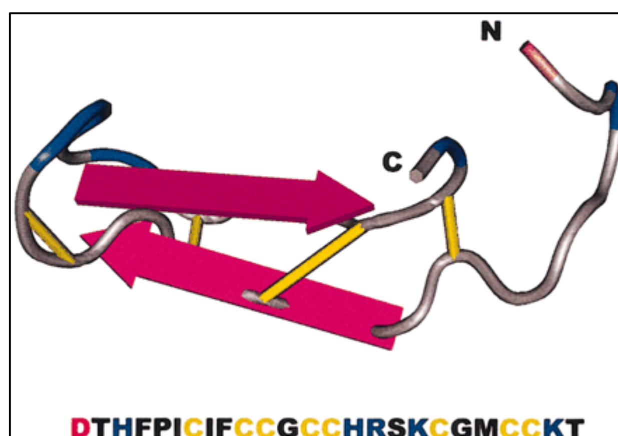
This mechanism allows a rapid response to every minimum change in cellular iron content. When cells have adequate iron, TFR1 expression decrease and ferritin levels augments, to store the excess.

When cellular iron is low TFR1 levels increase to import more iron from outside and ferritin expression decreases, to release iron from stores.

SYSTEMIC IRON REGULATION: THE HORMONE HEPCIDIN

Hepcidin was discovered in 2001 by Park *et al.* as an acute phase protein during liver inflammation (Park, et al., 2001). Pigeon and his group however were the first that connected hepcidin to iron metabolism (Pigeon, et al., 2001). Hepcidin is encoded by *HAMP* gene and synthesized in the liver as a pre-propeptide of 84 amino-acids containing in the N-terminal a signal sequence of 24 amino-acids recognized from the endoplasmic reticulum which is cleave to yield the intermediate prohepcidin (Wallace, et al., 2005). In the C-terminus there is a sequence recognized by the pro-protein convertase furin that generates the mature peptide of 25 amino-acids by cleaving the precursor (Figure 5) (Park, et al., 2001).

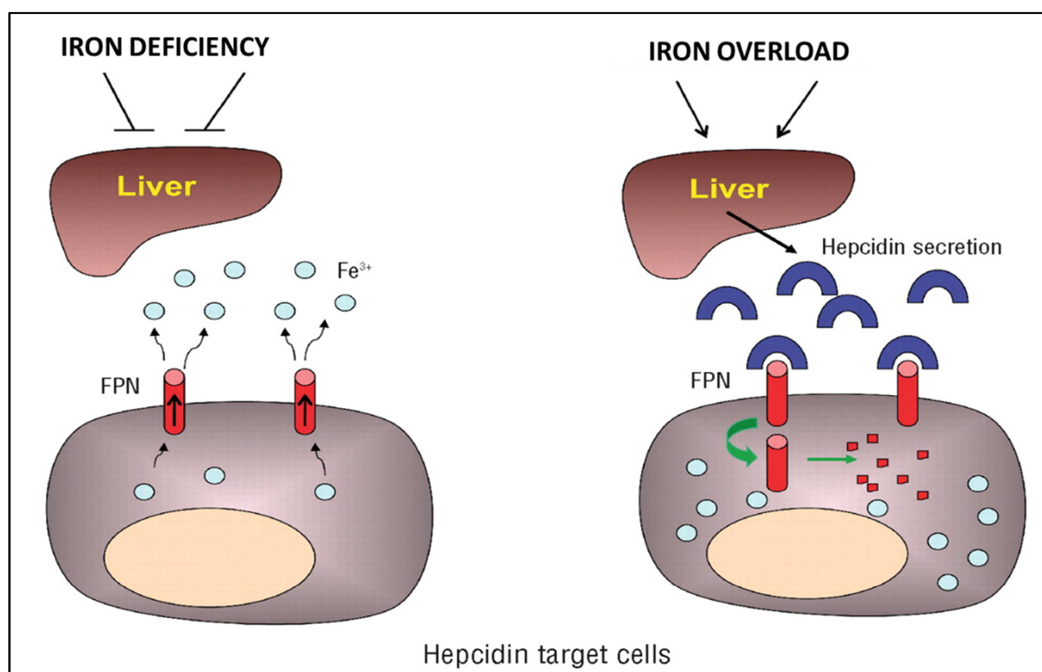
Figure 5. Amino-acid sequence and a model of 25 amino-acids human hepcidin (Modified from (Ganz, 2003).



The protein is mainly secreted by hepatocytes (Krause, et al., 2000), circulates in the plasma mostly free except for weak binding to α2-macroglobulin (Peslova, et al., 2009) and is then excreted through the kidney (Park, et al., 2001). Hepcidin is the main negative regulator of iron homeostasis, in fact it acts by binding to ferroportin mediating its internalization and degradation (Nemeth, et al.,

2004b). Hepcidin binds to the extracellular region of ferroportin via its N-terminal domain, causing its phosphorylation mediated by JAK2. FPN is in turn internalized, ubiquitinated and then degraded by lysosomes (Ward and Kaplan, 2012). The last six amino-acids of hepcidin are highly conserved and essential for its iron-regulatory function and interaction with ferroportin (Nemeth, et al., 2006). Hepcidin expression increases in case of excess iron and decreases during iron-deficiency. The protein maintains body iron balance by restricting iron absorption and macrophages iron release. Its action prevents excess iron absorption and maintains normal iron levels within the body. In particular, in case of iron deficiency, when cells and tissues need iron, hepcidin expression is inhibited, allowing iron absorption or release. On the other hand, during iron overload condition, hepcidin expression is stimulated to avoid additional iron accumulation (Figure 6).

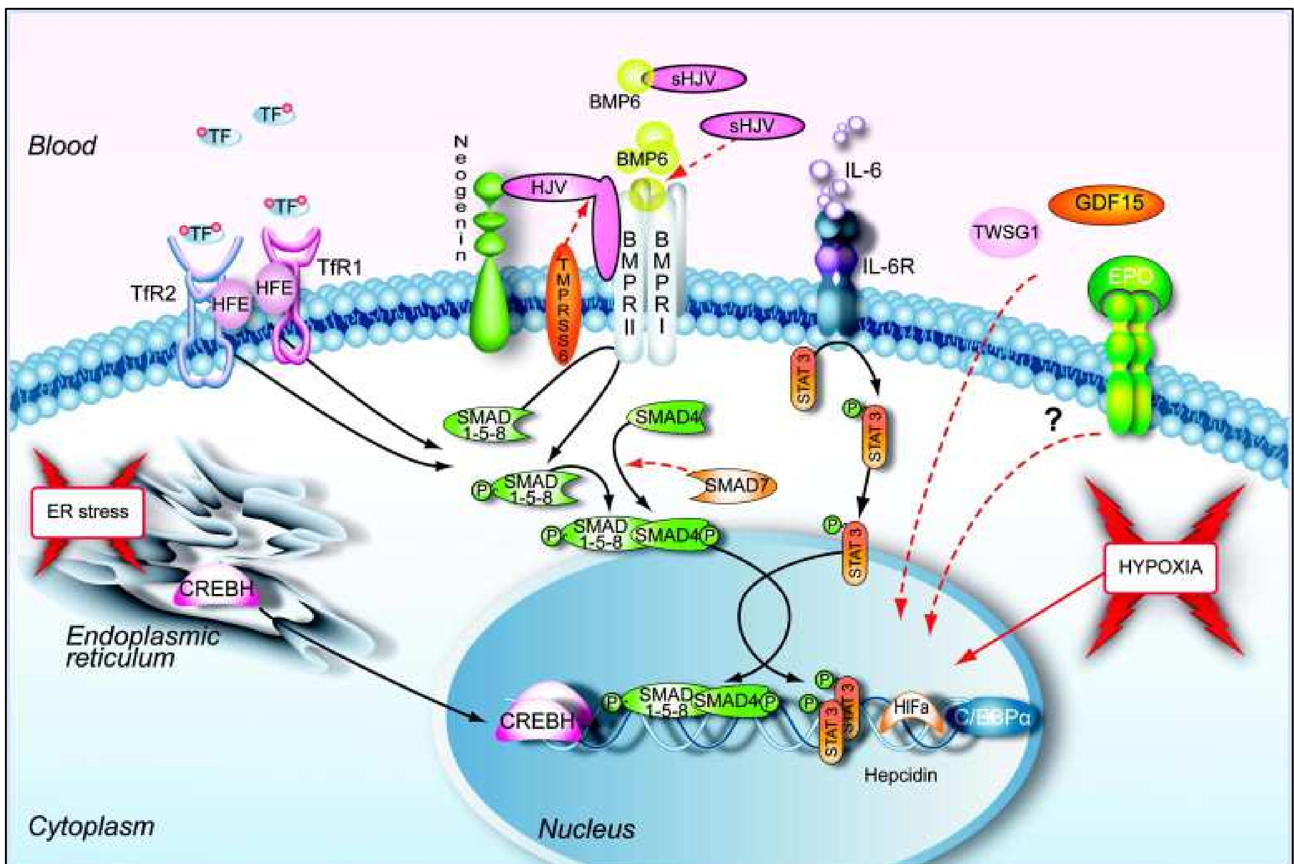
Figure 6. Mechanism of action of hepcidin. (Modified from (Bergamaschi and Villani, 2009)



HEPCIDIN REGULATION

Hepatocyte hepcidin production under physiological conditions is modulated by different stimuli that up or down regulated its transcription (Piperno, et al., 2009). Body iron content and inflammation increase hepcidin expression through two different pathways, BMPs/SMAD4 (Knutson, 2010) and IL6/STAT3 (Fleming, 2007) respectively. Anemia, erythropoietic activity (Pak, et al., 2006; Vokurka, et al., 2006) and hypoxia (Nicolas, et al., 2002) inhibit hepcidin transcription, however, how hypoxia regulates hepcidin expression is still unknown (Figure 7). To date, the only known mode of hepcidin regulation is trascriptional.

Figure 7. Signals and pathways controlling hepcidin expression in the liver (Pietrangelo, 2011).



POSITIVE REGULATORS

IRON STORE

Hepcidin expression is mainly regulated by plasma and tissue iron (Ganz, 2013). The most important pathway that controls hepcidin transcription in response to an increased in tissue iron levels is the BMP/SMADs pathway (Parrow and Fleming, 2014). BMPs (Bone Morphogenetic Proteins) are members of Transforming Growth Factor-β (TGF-β) superfamily. BMP receptors are dimers with serine/threonine kinase activity formed by two subunits, type I and type II. Once BMPs bind to receptor, a signal cascade is activated: the receptor phosphorylates the proteins SMAD1/5/8. SMAD1 or 5 interact with and phosphorylated SMAD4 that translocates into the nucleus and activates gene transcription (Figure 8) (Wang, et al., 2005). Phosphorylation of SMAD1/5/8 is inhibited and modulated by the inhibitory SMAD6 and 7 (Mleczko-Sanecka, et al., 2010). On *HAMP* promoter have been identified three potential BMP-responsive elements, BMP-RE1 (proximal), BMP-RE2 and BMP-RE3 (distal) (Casanovas, et al., 2009) critical for hepcidin transcription activated by BMPs/SMAD pathway. In liver cells are expressed BMP2, BMP4 and BMP6, but central to the regulation of iron homeostasis is BMP6. Indeed BMP6 knock out mice show hepcidin deficiency and tissue iron overload (Andriopoulos, et al., 2009; Meynard, et al., 2009). BMP6 is positively regulated by iron but it is not clear yet how the expression of *BMP6* mRNA increased in response to the increase of iron. The membrane form of the GPI-linked protein hemojuvelin (m-HJV) is a BMP6 co-receptor essential to activate the pathway by enhancing BMP signaling (Babitt, et al., 2007). HJV, codifies by *HFE2* gene, is essential to control hepcidin expression, indeed mutation in *HFE2* gene cause a juvenile and severe form of Hereditary Hemochromatosis, a recessive inherited disease characterized by progressive iron overload in liver and parenchymal organs (Hentze, et al., 2010). HJV exists also in a soluble form (s-HJV) that is generated by furin-mediated cleavage of HJV in the ER (Lin, et al., 2008). s-HJV antagonizes BMP-dependent hepcidin activation competing with m-HJV in binding BMP6. Furin mRNA levels are moreover regulated by iron, in particular during iron-deficiency it is up-regulated, inhibiting

hepcidin production (Silvestri, et al., 2008a). The protein neogenin (NEO) appears to stabilize m-HJV to enhance BMP signaling and hepcidin expression (Lee, et al., 2010). HJV also interact with Matriptase-2 (MT-2), a type II transmembrane serine protease encoded by *TMPRSS6* gene. MT-2 is a suppressor of hepcidin expression, in fact it can interact with m-HJV when they are both present on plasma membrane and cleaves it in fragments, abolishing HJV function and inhibiting hepcidin activation (Silvestri, et al., 2008b). MT-2 does not act on soluble form of HJV. Mutations in *TMPRSS6* gene cause Iron-Refractory Iron Deficiency Anemia (IRIDA) by stimulating excessive hepcidin synthesis that leads to sequestration of iron in macrophages and decreased dietary iron absorption (Ganz, 2013).

Key molecules involved in hepcidin regulation in response to levels of circulating iron are hereditary hemochromatosis protein (HFE), TFR1 and TFR2. HFE is a membrane protein homologous to MCH class I that can form heterodimer with β_2-microglobulin. Mutations in *HFE* cause type 1 Hereditary Hemochromatosis. TFR1 is able to sense circulating iron levels by binding diferric transferrin. HFE can bind TFR1 competing with Tf-Fe_2. TFR2 also interact with HFE protein (Goswami and Andrews, 2006) but different to TFR1, the binding of HFE to TFR2 does not interfere with holo-transferrin binding site (Chen, et al., 2007). Actually, the binding with HFE augments TFR2 stability and the up-take of holo-transferrin (Waheed, et al., 2008). TFR2, TFR1 and HFE form an iron-sensor complex and contributes to hepcidin regulation (Gao, et al., 2009). At basal conditions TFR1 sequester HFE preventing its action in activating hepcidin. When levels of iron-loaded transferrin increase, Tf-Fe_2 binds to TFR1 competing with HFE and HFE can therefore interact with TFR2. TFR2-HFE complex, sensing the iron status, can activate hepcidin expression through a pathway that is partially still unknown (Figure 8) (Chen and Enns, 2012; Gao, et al., 2009). *TFR2* mutations in human or mice result in iron overload and Type 3 hereditary hemochromatosis (Chen and Enns, 2012; Fleming, et al., 2002). Some evidences suggest that HFE-TFR2 complex and BMPs/SMAD pathway act together to activate hepcidin transcription

(Corradini, et al., 2009; Kautz, et al., 2009) however the molecular mechanisms of this cross-talk are still poorly elucidated.

INFLAMMATION

The second major signaling pathway known to positive control hepcidin regulation is the IL6/STAT3 pathway, activated by inflammatory cytokines. Hepcidin is a type II acute phase protein, as it was induced by treatment with Interleukin 6 (IL6) but not with cytokines involved in type I response (Nemeth, et al., 2004a; Nemeth, et al., 2003). In mammals hepcidin levels are also increased after bacterial infections, treatment with lipopolysaccharide or turpentine (Heinrich, et al., 2003). The increased in hepcidin levels in response to infection seems to be a defense mechanism to protect the host from infections, thus most microorganisms require iron for their growth. STAT3 is the primary transcription factor that mediates IL6 induction of hepcidin (Pietrangelo, et al., 2007) by binding a STAT-binding motif on hepcidin promoter (Fleming, 2007). When IL6 is released from Kupffer cells it binds its receptor on the hepatocytes' surface. IL6 receptor is formed by two subunits: a subunit α of 80-kDa (IL6-R) which bind the ligand and a subunit β of 130-kDa (gp130) that is able to transduce the signal. The binding of IL6 with its receptor induces the homodimerization of gp130 which in turn recruits the cytoplasmic proteins JAK kinase (Oliveira, et al., 2009). The phosphorylation of gp130 activates downstream two distinct pathways. The first involves the activation of the cascade of MAPK kinases. The second is the STAT pathway. After phosphorylation of gp130 by JAK, STAT proteins (STAT1 and STAT3) bind to a separate domain of gp130 and are themselves phosphorylated. Once detached from the receptor, STATs dimerize and translocate to the nucleus where are able to bind specific cis-activating genomic elements affecting the transcription of those genes that participate in the acute phase response, including *HAMP* (Figure 8) (Verga Falzacappa, et al., 2007). It has been suggested that there is a cross talk between the BMPs/SMAD pathway and IL6/STAT3 pathway, indeed the activation of STAT3 also

requires the presence of SMAD4 (Wang, et al., 2005). Moreover the BMP-responsive element in hepcidin promoter is required to control hepcidin expression in response to IL6 (Verga Falzacappa, et al., 2008). Recently it has been demonstrated that hepcidin expression is regulated by inflammation also through another member of TGF-β superfamily, Activin B (*INHBB* gene). LPS challenge increased Activin B and *HAMP* expression with increased of SMAD1/5/8 phosphorylation independently of BMP6 and other BMPs molecules present in the liver. Moreover, induction of Activin B and HAMP by inflammation occurs independently of IL6 (Besson-Fournier, et al., 2012).

Figure 8. Intracellular signaling pathways for positive hepcidin expression (Poli, et al., 2014).

Hepcidin expression is also increased by endoplasmic reticulum (ER) stress. This regulation is likely driven by CREB/H (transcription factor cyclic AMP response element-binding protein H)

(Vecchi, et al., 2009) or by the stress-induced transcription factors CHOP and C/EBPα (Oliveira, et al., 2009).

NEGATIVE REGULATORS

HYPOXIA

Iron metabolism, oxygen homeostasis and erythropoiesis are tightly interconnected. During hypoxia erythropoiesis is strongly stimulated and iron requirement by the bone marrow increases to allow erythroid progenitor maturation and proliferation (Liu, et al., 2012). Thus, iron absorption and release from stores must increase to sustain the erythropoietic drive (Finch, 1994). This is accomplished by a marked suppression of hepatic hepcidin transcription (Nicolas, et al., 2002). It was clearly demonstrated *in vivo* that hypoxia exposure inhibits hepatic hepidin production (Piperno, et al., 2011; Ravasi, et al., 2014; Talbot, et al., 2012), however mechanisms of hypoxia-induced hepcidin down-regulation are still partially unknown. Mammals primary sense hypoxia by the O_2-regulated Hypoxia Inducible Factors HIF-1 and HIF-2 (Semenza, 2001). They consist of an O_2-sensitive α subunit (HIF-1α and HIF-2α) and a constitutively expressed β subunit, HIF-β (ARNT) (Liu, et al., 2012). During normoxic conditions α subunits are modified by prolyl dehydrogenases (PHDs) 1, 2 or 3 and subjected to proteasomal degradation by von Hippel Lindau (VHL) factor. In oxygen-deprived conditions, PHDs are inactivated and α subunits are stabilized. HIF-α accumulates, translocates into the nucleus and binds the β subunit forming a heterodimer. The heterodimers then bind to Hypoxia Responsive Elements (HREs) in the DNA and regulate hypoxia-specific target genes (Greer, et al., 2012). Indeed, HIF activate or repress genes involved in adaptations to hypoxia itself such as erythropoietin (EPO) (Semenza, 2001) but also genes involved in iron metabolism such as TMPRSS6 (Lakhal, et al., 2011) and furin (Silvestri, et al., 2008a). Studies on hepatocyte-specific HIF-1α or VHL knockout mice (Peyssonnaux, et al., 2007) and patients with Chuvash polycythemia homozygous for the VHL R200W mutation (Gordeuk, et al.,

2011) demonstrated that VHL/HIF-1α axis has an important role in hypoxia-mediated down-regulation of hepcidin expression. In fact, if HIF-1α is knocked out in mice, animals fail to down-regulate hepcidin in response to dietary iron-deficiency (Peyssonnaux, et al., 2007), while if HIF-1α is overexpressed (VHL knockout mice) hepcidin levels are constitutively reduced (Peyssonnaux, et al., 2007). It was also demonstrated that HIF could bind HRE on hepcidin promoter, directly regulating hepcidin transcription (Peyssonnaux, et al., 2007). However HIF-2α seems to have more important role than HIF-1α in hypoxia-mediated hepcidin regulation. In fact, it was demonstrated that HIF-2α regulated EPO expression in the liver (Rankin, et al., 2007) and iron absorption in the duodenum (Mastrogiannaki, et al., 2009). Moreover Mastrogiannaki *et al.* demonstrated that in iron deficient condition HIF-2α triggered to hepcidin down-regulation through hepatic EPO-mediated activation of erythropoiesis (Mastrogiannaki, et al., 2012). However a lot of controversies exists: indeed some studies demonstrated that HIFs do not directly decrease *in vitro* hepcidin expression (Chaston, et al., 2011; Volke, et al., 2009). Volke *et al.* demonstrated that HepG2 and Huh-7 cells cultured under hypoxic conditions variable regulated hepcidin mRNA levels and concluded that reductions in hepcidin expression during hypoxia are not mediated by HIF (Volke, et al., 2009). EPO is the primary signals that trigger erythropoiesis in hypoxic conditions. Hence, it was hypothesized that EPO itself could directly regulate hepcidin transcription. It has been demonstrated *in vitro* that EPO could signal through EPO-R directly on hepatocytes downregulating C/EBPα expression and in turn inhibiting hepcidin transcription (Pinto, et al., 2008). However it has been demonstrated that if erythropoiesis is inhibited, EPO-mediated hepcidin down-regulation was blocked (Pak, et al., 2006; Vokurka, et al., 2006), suggesting that other erythropoietic factor/s downstream of EPO is/are implicated in hepcidin inhibition. Several studies have provided evidences that hepcidin may be regulated by hypoxia through the BMP/SMAD pathway. Indeed hypoxia also increase *furin* (Silvestri, et al., 2008a) and *TMPRSS6* (Lakhal, et al., 2011) mRNA levels, that hence increase s-HJV and decrease m-HJV levels, downregulating hepcidin expression

inhibiting BMP6 action. Moreover it has been suggested that the transcription factor ATOH8 could regulate hepcidin expression during hypoxia by modulating phosphorylated SMAD1/5/8 levels (Patel, et al., 2014). However, these results are not fully confirmed yet.

ERYTHROPOIETIC SIGNALS

During erythropoiesis, a lot of iron is required to the bone marrow to sustain the formation of new red blood cell. Hence, during erythropoiesis hepcidin must be inhibited to allow iron mobilization from stores and to augment iron absorption. It has been clearly demonstrated that if erythropoiesis is inhibited, EPO-mediated hepcidin down-regulation was blocked (Pak, et al., 2006; Vokurka, et al., 2006). The most likely hypothesis is that EPO-activated bone marrow releases erythroid soluble factor/s that acts directly on the liver inhibiting hepcidin production. Different molecules were supposed to be the hepcidin “erythroid regulator”.

GDF15 is a component of TGF-β superfamily secreted from erythroblasts during late stages of human erythropoiesis (Tanno, et al., 2007). High levels of GDF15 were found in β-thalassemic patients and associated with hepcidin down-regulation. These results were confirmed also in an *in vitro* study in which hepatoma cells treated with β-thalassemic sera expressed low levels of *HAMP* mRNA (Tanno, et al., 2007). However recent works about hepcidin regulation by hypoxia *in vivo* in humans and mice demonstrated that GDF15 is not required for balancing iron homeostasis in stress erythropoiesis (Casanovas, et al., 2013; Piperno, et al., 2011; Ravasi, et al., 2014).

TWSG1 is mainly produced from immature erythroid precursors during the early stages of erythropoiesis. This protein appears to suppress hepcidin production via inhibition of BMPs/SMAD (Tanno, et al., 2009) in *in vitro* models of hepatoma cells; its expression is also increased in animal models of thalassemia. However correlations between expression of TWSG1 and hepcidin levels have to be studied in humans yet.

Very recently, a new TNFα-like molecule named erythroferrone (ERFE) has been proposed as the erythroid regulator of hepatic hepcidin transcription. ERFE, produced by bone marrow erythroblasts, seems to be activated by EPO through the JAK2/STAT-5 pathway, directly acting on hepatic hepcidin expression through a still undefined mechanism that seems not to involve the BMP/SMAD pathway (Kautz, et al., 2014). This evidence was obtained in animal models whose erythropoiesis was induced by phlebotomy or EPO injections, but not in hypoxia models in which mechanisms of hepcidin suppression might be more complex. Indeed, ERFE seems not to be directly regulated by hypoxia (Kautz, et al., 2014).

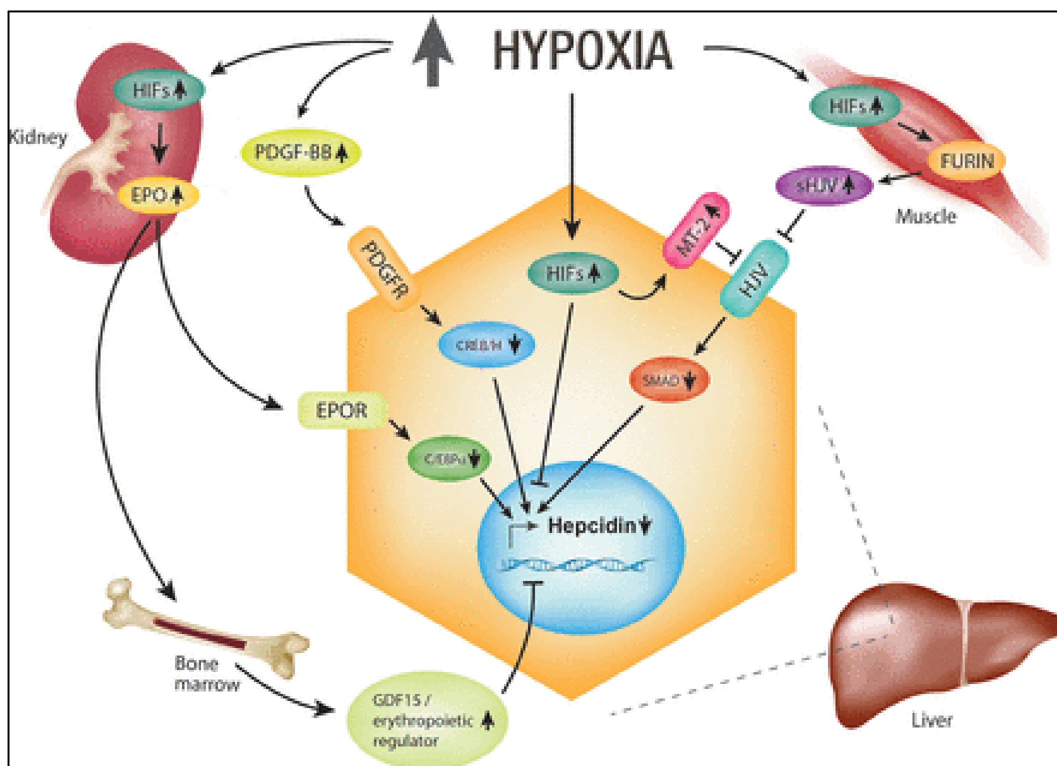
GROWTH FACTORS

Platelet-Derived Growth Factors (PDGFs) are family of molecules involved in many metabolic processes including angiogenesis, cell proliferation and fibrosis. These factors are encoded by four genes *PDGF-A*, *B*, *C* and *D* that dimerize forming five molecules: PDGF-AA, PDGF-BB, PDGF-CC, PDGF-DD and PDGF-AB. They act by binding their tyrosine-kinase receptors PDGF-Rs α and β. Recently, serum levels of PDGF-BB were found to be increased in volunteers exposed to normo-baric hypoxia chamber for 6 hours while performing physical exercises (Sonnweber, et al., 2014). Although Authors could not define which tissue was the involved in the hypoxia-induced release of PDGF-BB, their study strongly suggested that PDGF-BB could be an hypoxia-induced inhibitor of hepatic hepcidin transcription acting through C/EBPα, CREB and CREB/H signaling pathway (Sonnweber, et al., 2014). PDGF-BB is indeed a target of HIF (Schito, et al., 2012) and its possible involvement in hepcidin inhibition further supports the hypothesis that hypoxia-induced hepcidin suppression is mediated by circulating factors.

Moreover also Hepatocyte Growth Factor (HGF) and Epidermal Growth Factor (EGF) have been shown to inhibit BMP6-mediated induction of *HAMP* during chronic liver failure by decreasing

amount of pSMAD1/5/8 and hence hepcidin promoter activation (Goodnough, et al., 2012). It seems that these factors are not involved in hypoxia-mediated hepcidin down-regulation.

Figure 9. Proposed mechanisms for hypoxia-mediated regulation of hepcidin (Subramaniam and Wallace, 2014).



IRON-RELATED DISEASES

IRON OVERLOAD

Iron-overload is characterized by systemic accumulation of iron mainly caused by inadequate hepcidin production compared to reserves of body iron. Two types of iron-overload can be described: primary (or genetic) and secondary (or acquired) (Andrews, 2000). Genetic forms of

iron-overload are caused by reduced hepcidin levels due to mutations in genes that regulate *HAMP* expression or that affect *HAMP* itself, as in Hereditary Hemochromatosis (HH). Acquired forms of iron-overload are caused by increased or ineffective erythropoiesis, as in β-thalassemia.

Hereditary Hemochromatosis is an autosomal recessive disease that leads to iron overload in liver and other parenchymal organs. It is a heterogeneous disease with a frequency of 1: 350 in northern Europe and 1: 1.500 in Italy, with low penetrance and different clinical manifestation. Complications include liver cirrhosis, cancer, diabetes, hypogonadism, heart failure and arthritis (Hentze, et al., 2010). Most patients do not manifest any symptoms until adulthood. Patients increases levels of transferrin saturation at over 45% and serum ferritin concentrations greater than 300 μg/L in males and 200 μg/L in premenopausal women (Munoz, et al., 2009). Once diagnosed with hemochromatosis, the treatment that is still the most effective is phlebotomy (Andrews, 2000) or alternatively chelation with specific drugs (Cox and Peters, 1979). In 1996 Feder *et al.* identified the gene involved in Hemochromatosis on the short arm of chromosome 6 and called it *HFE* (Hemochromatosis gene) (Feder, et al., 1996). Initially it was believed that this single gene was responsible for the disease and that the different severity of iron overload in different patients was attributable to mutations located in different areas. However, family studies implicated in the disorder different genes, all involved in hepcidin regulation or iron transport: *HFE2* (HJV), *TFR2*, *FPN* and *HAMP* itself. The disease could be classified in 4 different categories according to the gene mutated. Mutations in *HFE* gene cause Type 1 HH that is the most common type in Caucasian population. The more frequent mutation is the p.C282Y in homozygous state (Feder, et al., 1996); other *HFE* mutations are relatively rare. Penetrance of Type 1 HH is low and the influence of modifier genes and/or environment is very important for disease expression (Beutler, et al., 2002). Clinical manifestations spread from mild to severe and occur most commonly in aged male. Mutations in *HFE2*, *HAMP* and *TFR2* cause less common but clinically more severe forms of HH. Mutations in *HFE2* or *HAMP* cause Type 2 HH, also called Juvenile Hemochromatosis (JH) since it manifests within the third decade of life. The clinical symptoms of these mutations are more severe

than *HFE* related HH (Papanikolaou, et al., 2004; Pietrangelo, 2010). Patients usually present with hypogonadism and cardiac symptoms and delay in diagnosis and treatment may lead to death from cardiac involvement (Le Gac and Ferec, 2005). Type 3 HH is due to mutations in *TFR2* genes. It may presents early, but with less severe phenotype than the juvenile form (Camaschella, 2005; Chen and Enns, 2012). *TFR2*-related forms of HH are rare however are quite common in Italy and Japan. Mutations of the ferroportin gene *SLC40A1* may cause a peculiar form of Hereditary Hemochromatosis (HH) also known as Type 4 HH or ferroportin disease (Pietrangelo, 2011). Ferroportin disease is a genetically and clinically heterogeneous syndrome with an autosominal dominant transmission and incomplete penetrance (Le Lan, et al., 2011; Pelucchi, et al., 2008; Pietrangelo, 2010). Clinical phenotypes are usually classified in two groups: the loss of function phenotype (Type A or classical FPN disease) and the gain of function phenotype (Type B or non-classical FPN disease) (Detivaud, et al., 2013). Both are characterized by hyperferritinemia, but while the former is characterized by normal or low levels of transferrin saturation and iron accumulation within the macrophages (Montosi, et al., 2001), the latter is characterized by high transferrin saturation and iron accumulation mostly within hepatocytes (Njajou, et al., 2001). Usually loss of function mutations affected the localization of FPN to the cell membrane or its iron export function (Schimanski, et al., 2005), leading to iron sequestration in the cell. Conversely, gain of function mutations affected the interaction between hepcidin and FPN leading to a continuous efflux of iron from cells to plasma.

Hepcidin levels are inappropriately low also in iron-loading anemias, such as β-thalassemia in which dysfunctional erythropoiesis inhibit hepcidin expression. Repeated blood transfusions are the major cause of this form of iron overload. Moreover, the erythropoiesis demand inhibits hepcidin production, worsening the iron accumulation. Iron that accumulates derived from red blood cells and it primarily deposited in reticuloendothelial macrophages and only after in parenchymal cells. The clinical manifestation of secondary iron-overload is similar to that of HH. The most common

clinical presentation are hepatomegaly, splenomegaly, cardiac diseases, diabetes and liver cirrhosis (Gattermann, 2009).

IRON DEFICIENCY

Iron deficiency most commonly manifests as microcytic anemia. Iron Deficiency Anemia (IDA) refers to the reduction of iron stores caused by insufficient dietary iron intake or chronic blood losses, usually due to intestinal worm colonization in developing countries. Patients with iron deficiency normally have low or undetectable levels of hepcidin.

Anemia of Chronic Diseases (ACD) is a common acquired form of anemia caused by prolonged or chronic inflammatory states. Inflammatory cytokines induced hepcidin transcription reducing iron availability for erythropoietic demand. Excessive hepcidin production is seen in patients with infections, malignancies, chronic kidney diseases, or any type of inflammation. If prolonged, it leads to ACD (Hentze, et al., 2010).

Iron-Refractory Iron Deficiency Anemia (IRIDA) is a genetic disorder caused by mutations in *TMPRSS6* gene (Du, et al., 2008). Mutations in this gene cause a constitutive high expression of hepcidin and therefore low levels of available iron and anemia. Since this disease is due to a genetic defect, IRIDA patients are usually children and unresponsive to oral and partially refractory to parenteral iron, because of inappropriately high hepcidin levels.

AIM

Iron metabolism, oxygen homeostasis and erythropoiesis are tightly interconnected. During hypoxia, erythropoiesis is strongly stimulated and iron requirement by the bone marrow increases to allow erythroid progenitors maturation and proliferation (Liu, et al., 2012). Thus, iron absorption and release from stores must increase to sustain the erythropoietic drive (Finch, 1994). This is accomplished by a marked suppression of hepatic hepcidin transcription. Hepcidin is a small peptide hormone mainly produced by hepatocytes and codified by *HAMP* gene. It binds to ferroportin, the only known cellular iron exporter, mediating its internalization and degradation (Nemeth, et al., 2004b). Its action inhibits iron absorption from the diet and iron release from the stores (liver and spleen macrophages), preventing excess iron absorption and maintaining normal iron levels within the body. Hepcidin expression is strictly controlled by different stimuli that up or down-regulate its production through different signaling pathways (Fleming, 2007; Knutson, 2010; Piperno, et al., 2009). Although many progresses have been made in the last years, mechanisms of hypoxia-induced hepcidin regulation are still partially unknown. Mammals primary sense hypoxia by the O_2-regulated Hypoxia Inducible Factors HIF-1 and HIF-2 (Semenza, 2001) that activate or repress genes involved in adaptations to hypoxia including erythropoietin (EPO) and iron proteins (Semenza, 2001) through the VHL-PHDs pathway (Peyssonnaux, et al., 2008). Studies in hepatocyte-specific HIF-1α or VHL (Von Hippel Lindau) knockout mice (Peyssonnaux, et al., 2007) and in patients with Chuvash polycythemia, homozygotes for the VHL R200W mutation (Gordeuk, et al., 2011), demonstrated that VHL/HIF1 α axis has an important role in hypoxic down-regulation of hepcidin expression. However, some recent evidences indicate that hypoxia might act indirectly stimulating the bone marrow to release soluble factors in response to the EPO-induced erythropoiesis (Liu, et al., 2012; Mastrogiannaki, et al., 2012; Ravasi, et al., 2014; Volke, et al., 2009). Indeed different studies in animal models showed that hepcidin down-regulation was blocked if erythropoiesis is inhibited (Pak, et al., 2006; Vokurka, et al., 2006). Several erythroid mediators have been proposed, such as the Growth Differentiation Factor 15 (GDF15) (Tanno, et al., 2007) or the Twisted Gastrulation Homolog 1 (TWSG1) (Tanno, et al., 2009), released by the

expanded erythroid marrow that could interfere with the BMP-SMAD pathway (Tanno, et al., 2009). However, these results were not confirmed, yet (Casanovas, et al., 2013). Very recently two others molecules have been identified and proposed as erythroid regulators of hepcidin, Platelet-derived growth factor-BB (PDGF-BB) (Sonnweber, et al., 2014) and Erythroferrone (ERFE) (Kautz, et al., 2014). PDGF-BB is a target of HIF α and a significant increase of serum PDGF-BB levels was reported in humans and mice exposed to hypobaric hypoxia. ERFE is a recently discovered TNFα-like protein, released by the bone marrow in condition of enhanced erythropoiesis and codified by *FAM132B* gene. It seems to act directly inhibiting hepatic hepcidin expression, through a still not fully clarified mechanism. However, ERFE seems not to be directly regulated by hypoxia (Kautz, et al., 2014). It has been demonstrated that hypoxia-mediated hepcidin inhibition in humans begins since after 6 hours and within 48 hours of hypoxia exposure (Piperno, et al., 2011; Sonnweber, et al., 2014; Talbot, et al., 2012). Furthermore, in mice with enhanced erythropoietic activity due to phlebotomy or EPO injection, ERFE levels increased already after 4 hours of treatment reaching its maximum at 15 hours, thereafter decreasing (Kautz, et al., 2014). These evidences suggest that the hypoxia-induced hepcidin down-regulation occurs rapidly following erythropoiesis activation.

The aim of this thesis was to investigate hepcidin regulation mediated by hypoxia using different approaches:

1. we first investigated the modifications induced by acute exposure to hypobaric hypoxia on iron metabolism in healthy volunteers participating to an expedition on Mount Rosa in the Alps, evaluating the time course of serum hepcidin, ferritin, EPO, IL-6 and GDF-15 after exposure to acute hypobaric hypoxia;
2. we then use an already set up *in vitro* model to evaluate the effect of volunteers' sera exposed to normoxia and hypoxia on *HAMP* expression by using a promoter-luciferase

based assay and by analyzing endogenous gene expression in a human hepatoma-derive cells line (HuH-7);

3. finally, we took advantage of a mouse model of acute hypobaric hypoxia to study how hepatic hepcidin levels are modulated in connection with changes in erythroid molecules. For this purpose we first evaluated the time course of hepcidin inhibition after hypoxic challenge and then we analyzed the transcription levels of the candidate erythroid genes *Gdf15*, *Twsg1*, *Pdgf-b* and *Fam132b* in the liver, bone marrow and spleen. We then focused our attention on PDGF-BB and its regulatory pathway.

MATERIALS AND METHODS

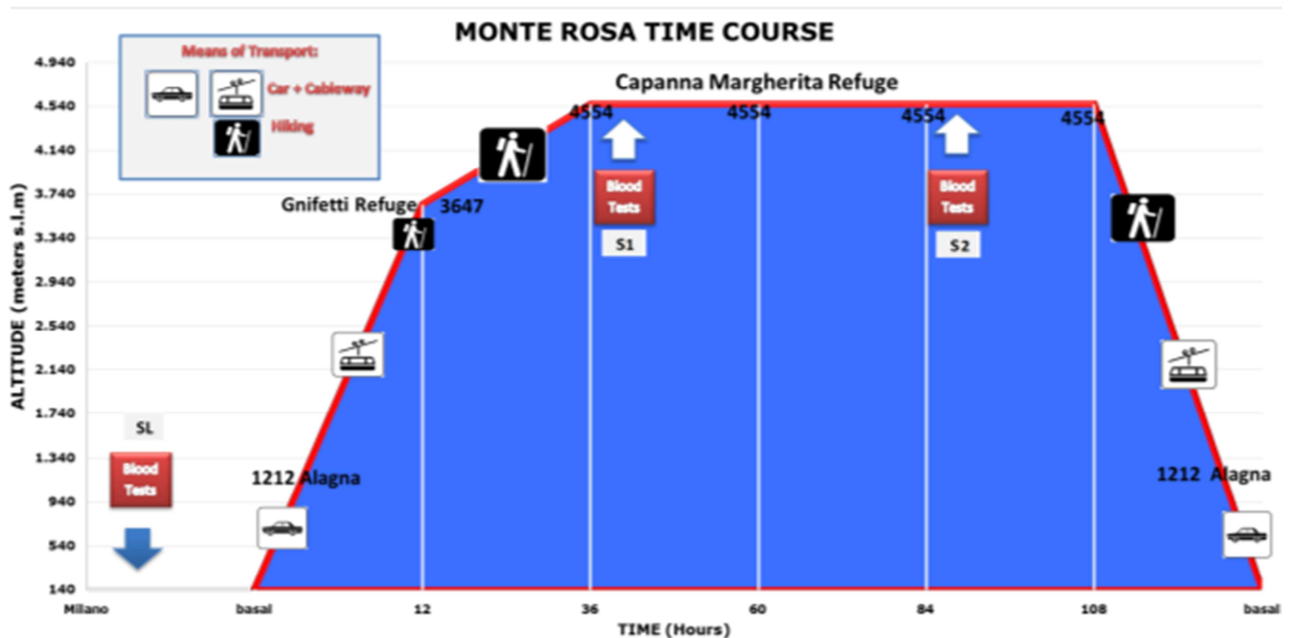
STUDIES WITH HUMAN SUBJECTS

41 healthy volunteers (21 males and 20 females) were enrolled in the “MONTE ROSA” study. They were in good general health and they live permanently at less than 500 m above sea level. Exclusion criteria were: repeated prolonged exposures to altitudes more than 3000 m above sea level in the 8 months preceding the expedition, history of severe mountain sickness, history of angioedema, and pregnancy. Professional athletes were not included in the study. Subjects underwent a general health checkup, including exercise test and echocardiography before the expedition. All subjects gave their written informed consent to the study procedures. The study protocol was approved by the Ethics Committee of Istituto Auxologico Italiano (2010_04_13_01), and the study was conducted in agreement with the Declaration of Helsinki.

“MONTE ROSA” STUDY DESIGN AND PROCEDURES

Figure 10 summarizes the different steps of the “MONTE ROSA” study. The participants traveled from sea level (Milan, 140 m) by car to Alagna (1212 m), by cableway to Indren and then by hiking to Gnifetti Refuge (3647 m) where they stayed for 24 hours. They hiked out to Capanna Margherita Refuge (4554 m) were they stayed for another 2 days, then they returned to sea level (Milan). Samples were collected at 3 time points: sea level baseline (SL) and during exposure to high altitude (4554 m) after 24 and 72 hours (S1 and S2, respectively). Blood samples were immediately centrifuged and sera stored at −80°C at sea level, whereas during the expedition they were stored in 2-mL cryogenic tubes and frozen in nitrogen vapor containers (Cryoshippers MVE Biologic Systems) for the whole period until the return, when they were definitively stored at −80°C.

Figure 10. Time-course of MONTE ROSA expedition



HUMAN IRON AND OTHER INDICES

Human serum ferritin and EPO were measured by standard methods. Serum IL-6 and GDF-15 were measured by commercial kits (ELISA assays; R&D Systems Inc. MN, USA). Serum hepcidin was measured by SELDI-TOF at the University of Verona as previously described (Campostrini, et al., 2010; Swinkels, et al., 2008).

CELL CULTURE AND SERUM TREATMENT

The human hepatoma cell line HuH-7 was grown in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% heat-inactivated fetal bovin serum (FBS), glutamine and combined antibiotics, at 37°C and 5% CO_2. To examine the effect of hypoxic sera, cells were seeded in 6-well plates (300.000 cells/well). After 24 h they were starved of FBS for 24 h, after which the medium was changed to medium containing 10% human serum as previously reported (Ravasi, et al., 2012). After an additional 48 h, cells were harvested for RNA isolation and gene expression analysis as reported below. HuH-7 cells were treated with 26 sera of volunteers collected at sea level and at S2.

DUAL-LUCIFERASE REPORTER ASSAY

A pGL2-basic reporter vector (Promega Corp., Madison, WI, USA Corp., Madison, WI, USA) harboring a 2.9Kb fragment of human hepcidin promoter (WT) (Pagani, et al., 2008) was used to analyze hepcidin promoter activity by luciferase assay. HuH-7 cells were seeded in 48-well plates at 40% of confluency. Cells were transiently co-trasfected with hepcidin promoter-luciferase construct (250ng) and pRL-TK renilla lulciferase vector (15ng) (Promega Corp., Madison, WI, USA Corp., Madison, WI, USA) to control transfection efficiency by Fugene HD transfection reagent (Promega Corp., Madison, WI, USA Corp., Madison, WI, USA). In addition, a 1207 bp fragment of hepcidin promoter was cloned in pGL4.10 basic reporter vector (Promega Corp., Madison, WI, USA Corp., Madison, WI, USA) by Polymerase Chain Reaction (PCR) with sequence specific primers (forward primer: 5'- GGCTCGAGGCTAGAATCTCAGCTCTGCCTCTGGCTG-3' and reverse primer: 5'-GG GCTAGCGTGACAGGTCGCTTTTATGGGGCCTGC -3' [modified from (Hintze, et al., 2011)) and human genomic DNA as template. The 1207 bp construct lacks the distal portion of hepcidin promoter including the two distal BMP responsive elements (BMP-RE2 and -RE3) (Casanovas, et al., 2009) and the SMAD4/7 responsive element (SMAD-R1) (Mleczko-Sanecka, et al., 2010). The PCR product was cloned upstream of the firefly luciferase gene by digestion with XhoI and NheI-HF (New England BioLabs Inc. Ipswich, MA, USA). Plasmid construct was verified by direct sequencing. Plasmid DNA was isolated by Pure Yield™ Plasmid Miniprep System kit (Promega Corp., Madison, WI, USA) for transfection. The pGL-basic plasmids were also co-transfected with pRL-TK renilla as a negative control. After 24h of transfection cells were incubated with 10% of human serum. After 48 h cells were lysed and firefly and renilla luciferase activities were measured by a Glomax Multi JR luminometer according to manufacturer's protocols (Promega Corp., Madison, WI, USA). Relative luciferase activity was calculated as the ratio between firefly (reporter) and Renilla luciferase activity. Experiments were performed in triplicate. Transfected HuH-7 cells were treated with 24 sera of volunteers collected at sea level and at S2.

ANIMAL CARE AND IN VIVO STUDIES

Wild-type C57BL/6 male mice 10 weeks old were purchased from Charles River Laboratories (Italy). They were housed at the animal facility of the University of Milano-Bicocca under pathogen-free conditions with fixed day and night cycles and free access to water and food. All animal experiments were performed in accordance with the Italian law and all experiments were approved by the Italian ethics committee (N° 009/2013).

HYPOXIA STUDIES IN MICE

Mice were put into a hypoxic chamber under hypobaric hypoxic conditions containing a fraction of 10% O_2 in nitrogen-saturated air for 6 and 15 hours. Control mice were kept under normal conditions (21% oxygen content of the air). Following hypoxic challenge, mice were anesthetized and blood was taken via intra-cardiac puncture. Mice were sacrificed via cervical dislocation and liver and spleen were collected and snap-frozen in liquid nitrogen. Femur and tibia bones were collected to immediately perform the bone marrow flushing. Tissue samples were used to extract mRNA as described below; serum was used to quantify Erythropoietin and PDGF-BB levels by ELISA Quantikine Immunoassay kit (R&D Systems, Minneapolis, MN, U.S.A.). Mice subjected to hypoxia received either an intraperitoneal injection of 500 μg of AG1296 (MMEDICAL), or 100 μg of anti-PDGF-BB antibody (R&D Systems Inc., Minneapolis, MN) each in 100 μL of PBS solvent. AG1296 and anti-PDGF-BB antibody were delivered as a single dose immediately prior to hypoxic challenge for 15 hours. Intraperitoneal injection of 100 μl of PBS is used as “mock” control.

BONE MARROW FLUSHING

For bone marrow cell suspension preparation, cells from mice’s femur and tibia were flushed with PBS-2% FBS. The cell suspension was gently passed through a 21 G needle and then through a 40 μM cell strainer (BD Bioscience, San Jose, CA, U.S.A.). The suspension was then transferred to a

50-mL conical tube and centrifuged at 2000 rpm for 5 minutes. After centrifugation, the cell pellet was used for RNA extraction as described below.

RNA EXTRACTION AND cDNA SYNTHESIS

RNA from cell cultures and from bone marrow cell suspension was extracted using ZR RNA miniprep (Zymo Research Corporation, Irvine, CA, U.S.A.) according to the manufacturer’s protocol. RNA from frozen organs was extracted using TRIzol reagent (Lifetechnologies, Foster City, CA, U.S.A.) according to the manufacturer’s protocol. RNA was quantified by spectrophotometry and its integrity assessed by non-denaturing agarose gel. Two µg of total RNA was used as a template for reverse transcription, performed using the High Capacity cDNA Archive kit (Lifetechnologies, Foster City, CA, USA), according to the manufacturer’s protocol.

REAL-TIME QUANTITATIVE-PCR

mRNA expression levels of human and mice genes were evaluated by quantitative real time PCR (qRT-PCR); *HPRT1* was chosen as housekeeping gene. The analysis were performed on an ABI 7900HT (Lifetechnologies) using the Assays-on-Demand Gene Expression Products (Lifetechnologies) according to the manufacture’s protocol. Instrument was set up with default thermal cycler protocol provided by the producer: 50°C for 2 min, 95°C for 10 min, 95°C for 15 sec and 60°C for 1 min for 40 cycles. For each PCR reaction, 25 ng of cDNA were used as a template. All analyses were carried out in triplicate; results showing a discrepancy greater than 0.3 cycles between the samples were excluded. Relative quantities present in each sample were assessed using the $2^{-(\Delta Ct)}$ method (Livak and Schmittgen, 2001). Non-retrotranscribed RNAs were included in each amplification plate, and the analysis regarded as valid if the fluorescence intensity in the no-template control was zero.

PROTEIN ISOLATION AND WESTERN BLOT ANALYSIS.

For western blot analysis, proteins were isolated by NET buffer. Samples were solubilized in NET buffer [150 mM NaCl, 5 mM EDTA, 10 mM Tris-Hcl pH 7.4, 1% Triton X-100, and 1X Protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) (pH 7.4)] on a roller mixer for 10 min 4°C and cleared by centrifugation at 13500 rpm for 10 min 4°C. Protein concentrations were measured using Bradford Assay (Sigma-Aldrich, St. Louis, MO, USA). Lysates were then analyzed by Western blotting for CREB/H. 40 μg of protein extracts were diluted in reducing LDS sample buffer and boiled for 5 min at 97 °C. Proteins were separated electrophoretically on an pre-cast SDS 12% polyacrylamide gel transferred to nitrocellulose (GE Healthcare, Amersham Biosciences Europe GmbH, Freiburg, Germany), and incubated for 1 hr 30 min in blocking buffer [5% nonfat dry milk in Tris-buffered saline with 0.1% Tween20 (TBST)]. Blots were incubated in blocking buffer containing rabbit anti-CREB/H (1:1000; Kerafast, Inc., Boston, MA, USA). After wash with TBST, blots were incubated in TBST containing a 1:3000 donkey anti-rabbit secondary antibody conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). To confirm equivalent loading blots were re-probed with rabbit anti-actin (1:5000; Sigma-Aldrich, St. Louis, MO, USA) followed by HRP-conjugated donkey anti-rabbit secondary antibody (1:10000, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) After washes with TBST bands were visualized by using enhanced chemiluminescence (GE Healthcare, Amersham Biosciences Europe GmbH, Freiburg, Germany) and X-ray film.

LIVER IRON CONTENT (LIC) AND SPLEEN IRON CONTENT (SIC) EVALUATION

Briefly, liver and spleen samples were stored at -80°C until analysis. Specimens were dried for 18 hours (O/N) at 65°C, weighed, transferred to a small iron-free vessel and acid-digested with 0.3 mL of a 1/1 (v/v) mixture of concentrated sulphuric and nitric acids in 3 mL total volume. A blank was

treated similarly. LIC and SIC were measured by atomic absorption spectrophotometry and expressed in mg Fe/g dry weight (Barry and Sherlock, 1971).

STATISTICAL ANALYSIS

Data were expressed as median or geometric mean and range. Paired t test was used to compare values at baseline and after hypoxia exposure. One-way Anova and Tukey or Dunn test post-hoc analysis were used to compare values among more than two groups. All tests were two sided and with a significance level of α equal to 0.05. Analyzes were carried out by the GRAPHPAD PRISM statistical analysis software (version 3.02) (GraphPad Software, Inc., La Jolla, CA, USA).

RESULTS

STUDIES WITH HUMAN SUBJECTS

BIOCHEMICAL DATA

Table 1 shows the characteristics of volunteers at baseline according to sex. As expected, hematocrit, hemoglobin, serum hepcidin and serum ferritin were lower in women than men. 15 volunteers (3 males and 12 females) were excluded because of pre-latent iron-deficiency (low serum ferritin) due to menstrual blood loss or blood donations. They showed undetectable hepcidin levels at baseline and throughout the entire study. Thus, the subsequent analyses were performed in the remaining 26 volunteers (18 males and 8 females).

Table 1. Characteristics of 41 subjects of Monte Rosa group at basal level (SL).

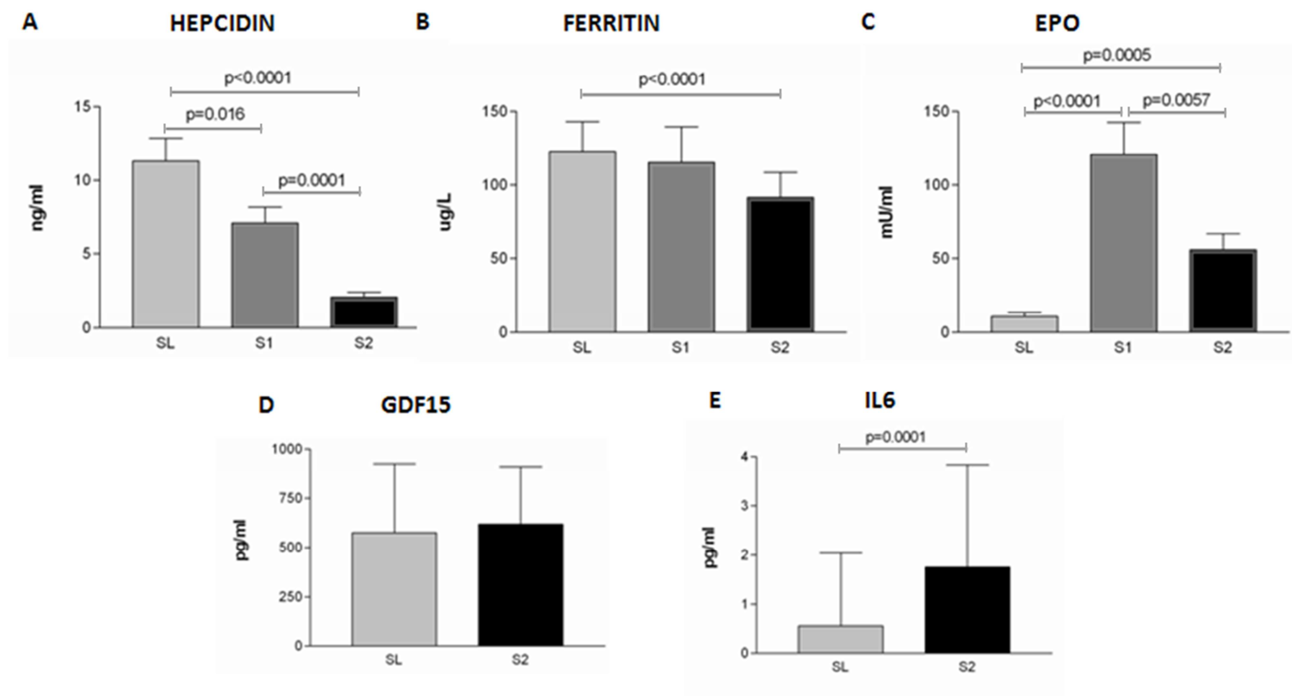
	Females (N=20)			**Males (N=21)**			
	Mean	**Median**	**1st-3rd quartile**	**Mean**	**Median**	**1st-3rd quartile**	**P value**
Age (yrs)	35.7	32	29.2-41.2	37.5	37	31-41.3	ns
Hematocrit (%)	38.6	38.4	37.1-40.3	42.9	42.5	40.7-44.8	< 0.0001
Hemoglobin (g/dL)	13.2	13.05	12.7-13.6	14.6	14.4	13.8-15.3	< 0.0001
Serum Hepcidin (ng/mL)	4.54	1.53	1.53-5.83	10.7	7.5	3.7-16.9	0.008
Serum Ferritin (ng/mL)	34.5	31.5	11.7-51	146	114	74-172	0.001
Erythropoietin (mU/mL)	10.2	9.96	6.3-13.4	12.4	8.2	5.8-12.9	ns
IL-6 (pg/mL)	1.07	0.03	0.00-0.30	0.24	0.00	0.00-0.30	ns

HYPOXIA CHALLENGE

Figure 11 reports data at baseline (SL) and after 24 (S1) and 72 (S2) hours of hypoxia exposure. Hypoxia induced significant hepcidin reduction that was mild at S1 and marked at S2, when 25 out

of 26 subjects (96.15%) showed undetectable levels. As expected, EPO markedly increased at S1, slightly decreasing thereafter, while serum ferritin decrease reaches the statistical significance at S2. IL-6 significantly increased after 72 hours of hypoxia, while GDF-15 levels did not change.

Figure 11. Serum parameters at basal level (SL) and after 24 and 72 hours exposure to high altitude (S1 and S2) in 26 healthy volunteers; A: Serum hepcidin, B: Serum ferritin, C: Erythropoietin, D: GDF-15 and E: IL-6. Data are expressed as mean + ds.

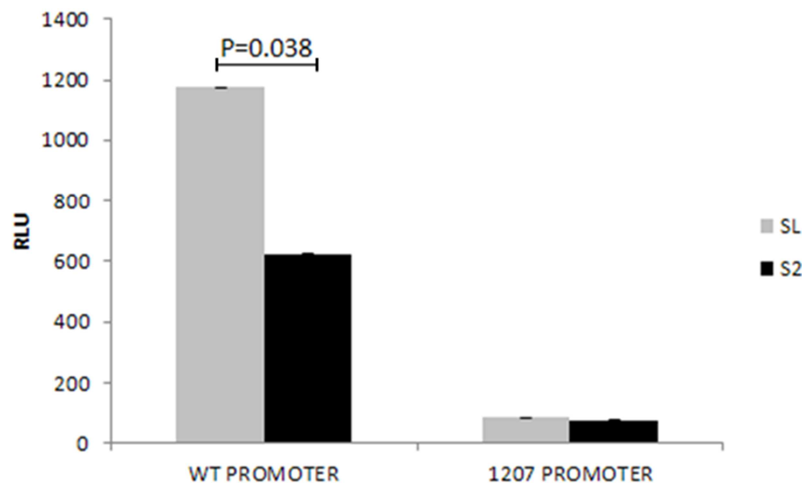


DUAL-REPORTER LUCIFERASE ASSAY

To evaluate whether hypoxia regulates hepcidin through the release of circulating factors by the activated erythroid bone marrow, we transfected HuH-7 cells with WT or 1207bp-HAMP-promoter luciferase constructs and we stimulated them with volunteers' normoxic and hypoxic sera (at S2). The 1207 bp construct lacks the distal portion of hepcidin promoter including the two distal BMP responsive elements (BMP-RE2 and -RE3) (Casanovas, et al., 2009) and the SMAD4/7 responsive element (SMAD-R1) (Mleczko-Sanecka, et al., 2010). Results of luciferase experiments are reported in Figure 12. Treatment with hypoxic sera led to a significant decrease of luciferase activity of WT-HAMP-promoter, compared to normoxic sera (p=0.038). After transfection with the

1207bp-HAMP-promoter we observed a reduced activation of luciferase gene, both with normoxic and hypoxic sera.

Figure 12. Luciferase assay in HuH-7 cell line transfected with the WT-HAMP promoter or 1207bp-HAMP-promoter and stimulated with 24 normoxic (SL) and hypoxic sera (at S2). Data are expressed as geometric mean + sd. (RLU=Relative Luciferase Unit).



GENE EXPRESSION ANALYSIS

To confirm the results obtained using the hepcidin promoter luciferase assay, we then evaluated whether exposure of HuH-7 cells to medium containing normoxic or hypoxic sera modified endogenous *HAMP* transcription, as well as the mRNA levels of *HAMP* regulators (*BMP6* and *TMPRSS6*) and markers of the BMPs-SMADs pathway (*ID1*) (Meynard, et al., 2011) and of the inflammatory pathway (*INHBB*) (Besson-Fournier, et al., 2012). Different to the hepcidin-promoter luciferase data, results were not uniform regarding hepcidin expression. In fact, compared to basal sera, the hypoxic sera of 14 volunteers (Group A) down-regulated endogenous *HAMP* mRNA levels. In the same group of subjects, also *ID1* mRNA was significantly down-regulated by hypoxic sera (Table 2). Hypoxic sera of the remaining 12 volunteers (Group B) either up-regulated or did not change both *HAMP* and *ID1* mRNA levels. mRNA levels of *BMP6* (*HAMP* activator), *TMPRSS6* (*HAMP* repressor) and *INHBB* (marker of inflammatory pathway) did not change in both group A and B after treatment with hypoxic sera.

Table 2. *HAMP, ID1, BMP6*, *TMPRSS6* and *INHBB* mRNA expression in HuH-7 cells treated with normoxic and hypoxic (S2) sera of 26 volunteers. Values are expressed as median and range.

	Group A (N=14)			**Group B (N=12)**		
	Sea Level (SL) (141m)	**High Altitude (S2) (4554m)**	**P value**	**Sea Level (SL) (141m)**	**High Altitude (S2) (4554m)**	**P value**
***HAMP* mRNA (2^-ΔCt)**	0.15 (0.02-0.57)	0.08 (0.02-0.33)	0.0008	0.07 (0.04-0.21)	0.12 (0.04-0.22)	0.018
***ID1* mRNA (2^-ΔCt)**	17.9 (1.8-34.5)	13.1 (2.1-26.1)	0.005	8.7 (5.6-28.8)	12.5 (3.8-30.7)	ns
***BMP6* mRNA (2^-ΔCt)**	0.004 (0.0006-0.006)	0.003 (0.0009-0.007)	ns	0.003 (0.00-0.007)	0.002 (0.0005-0.005)	ns
***TMPRSS6* mRNA (2^-ΔCt)**	0.035 (0.005-0.13)	0.027 (0.007-0.11)	ns	0.014 (0.004-0.04)	0.012 (0.003-0.03)	ns
***INHBB* mRNA (2^-ΔCt)**	0.146 (0.005-0.843)	0.151 (0.006-0.558)	ns	0.215 (0.086-0.547)	0.184 (0.073-0.510)	ns

Trying to find explanations for the differences in hepcidin expression between the two groups, we evaluated serum levels of IL-6, EPO, s-FERR and GDF-15, but no significant differences were observed either at sea level or high altitude (Table 3). In each group GDF15 did not change, s-Hepcidin and s-FERR decreased, while IL-6 and EPO significantly increased after hypoxia exposure and the magnitude of changes were similar in both groups.

Table 3. Biochemical parameters differences between groups A and B of 26 volunteers. Values are expressed as median and range

	Group A (N=14)			Group B (N=12)		
	Sea Level (SL) (141m)	**High Altitude (S2) (4554m)**	**P value**	**Sea Level (SL) (141m)**	**High Altitude (S2) (4554m)**	**P value**
Serum Hepcidin (ng/mL)	7.30 (2.6-35.6)	1.53 (1.53-7.7)	<0.01	13.10 (1.8-20)	1.53 (1.53-5.35)	<0.0001
Serum Ferritin (ng/mL)	69.5 (17-338)	47.5 (10-252)	<0.01	110.5 (34-445)	72.0 (14-393)	<0.0001
Erythropoietin (mU/mL)	7.35 (3.5-13.2)	38.45 (15.4-123)	<0.001	9.0 (2.3-73.1)	34.4 (7.8-271)	<0.05
GDF-15 (pg/mL)	429.2 (210.6-1265.3)	580.4 (357.1-1147.6)	ns	492.6 (75.6-2278.2)	496.3 (231.0-1350.0)	ns
IL-6 (pg/ml)	0.0 (0.0-7.4)	0.71 (0.0-9.6)	<0.01	0.1 (0.0-0.74)	1.26 (0.0-4.7)	<0.01

STUDIES WITH MICE MODELS

To assess the erythropoietic activity of the hypoxic mice we first examined serum erythropoietin levels. EPO levels doubled at 6 hours of hypoxia exposure, reaching their highest values after 15 hours of treatment, as compared to control mice (Table 4).

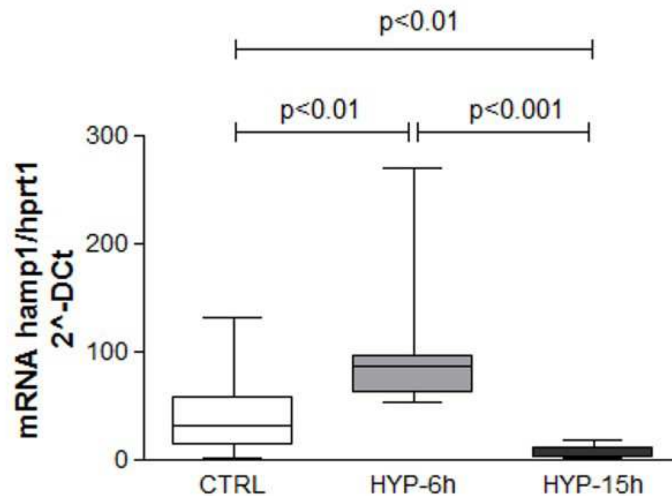
Table 4. Erythropoietin levels of normoxic (CTRL, N=7) and hypoxic (6h, N=6 and 15h, N=8).

	CTRL (N=7)	**HYPOXIA EXPOSURE 6 h (N=6)**	**HYPOXIA EXPOSURE 15 h (N=8)**	**P VALUE**
S-EPO (pg/ml)	627 (131-1675)	1202 (742-2433)	1351 (244-8584)	Ns

GENE EXPRESSION ANALYSIS IN THE LIVER

We evaluated the modulation of hepatic hepcidin production in response to increased erythropoietic activity measuring *Hamp1* mRNA levels. As reported in Figure 13, *Hamp1* mRNA expression increased after 6 hours of hypoxic challenge compared to controls, possibly due to temporary inflammatory activation (Hartmann, et al., 2000) and then significantly decreased after 15 hours.

Figure 13. *Hamp1* mRNA expression in the liver of normoxic (CTRL, N=17) and hypoxic mice (HYP) after 6h (N=8) and 15h (N=11). Boxes denote lower quartile, mean and upper quartile, and whiskers show maximum and minimum ranges.



Hepatic mRNA levels of *Gdf15* did not significantly change while *Twsg1* significantly decreased after 15 hours of treatment (Table 5). We also analyzed Hepatocyte Growth Factor (*Hgf*) and Epithelial Growth Factor (*Egf*) mRNA levels to assess whether they could have a role in hepcidin inhibition during hypoxia, because previous study suggested they could both inhibit hepcidin expression in chronic liver failure (Goodnough, et al., 2012). However, we did not observe changes between hypoxic and control mice at 6 hours. However, at 15 hours *Hgf* mRNA was lower in hypoxic mice than in controls, and *Egf* mRNA decreased significantly compared to 6-hours hypoxic mice. (Table 5)

Table 5. *Gdf15*, *Twsg1*, *Hgf* and *Egf* mRNA expression in the liver of normoxic (CTRL, N=12) and hypoxic mice (HYP) after 6h (N=6) and 15h (N=11). Results were expressed as median (range).

	CTRL (N=12)	**HYPOXIC CHALLENGE 6 hours (N=6)**	**HYPOXIC CHALLENGE 15 hours (N=11)**	**P value**
LIVER *Gdf15* mRNA (2^-ΔCt)	0.195 (0.023-0.737)	0.067 (0.014-0.161)	0.043 (0.012-0.103)	ns
LIVER *Twsg1* mRNA (2^-ΔCt)	0.469 (0.240-0.776)	0.428 (0.087-1.111)	0.182 (0.044-0.519)	<0.05 CTRL vs 15h
LIVER *Hgf* mRNA (2^-ΔCt)	0.06 (0.02-0.18)	0.03 (0.01-0.08)	0.019 (0.007-0.60)	<0.05 CTRL vs 15h
LIVER *Egf* mRNA (2^-ΔCt)	0.01 (0.002-0.68)	0.03 (0.01-0.06)	0.008 (0.001-0.023)	<0.01 6h vs 15h

GENE EXPRESSION ANALYSIS IN BONE MARROW AND SPLEEN

We analyzed mRNA expression of candidate genes *Gdf15, Twsg1, Pdgf-B* and *Fam132b* (ERFE), in bone marrow and spleen. In bone marrow, all genes were significantly up-regulated after 6 hours of hypoxia exposure and then returned to basal level after 15 hours of treatment with the exception of *Fam132b* whose mRNA levels decreased at 15 hours, but not enough to reach the statistical significance (Figure 14).

On the contrary, when we analyzed the same genes in the spleen, we did not observe any differences in their mRNA levels after hypoxic challenge, as compared to controls (Figure 15).

Figure 14. **A)** *Gdf15,* **B)** *Twsg1,* **C)** *Pdgf-b* and **D)** *Fam132b* mRNA expression in the bone marrow of normoxic (CTRL, N=12) and hypoxic mice (HYP) after 6h (N=6) and 15h (N=11). Boxes denote lower quartile, mean and upper quartile, and whiskers show maximum and minimum ranges.

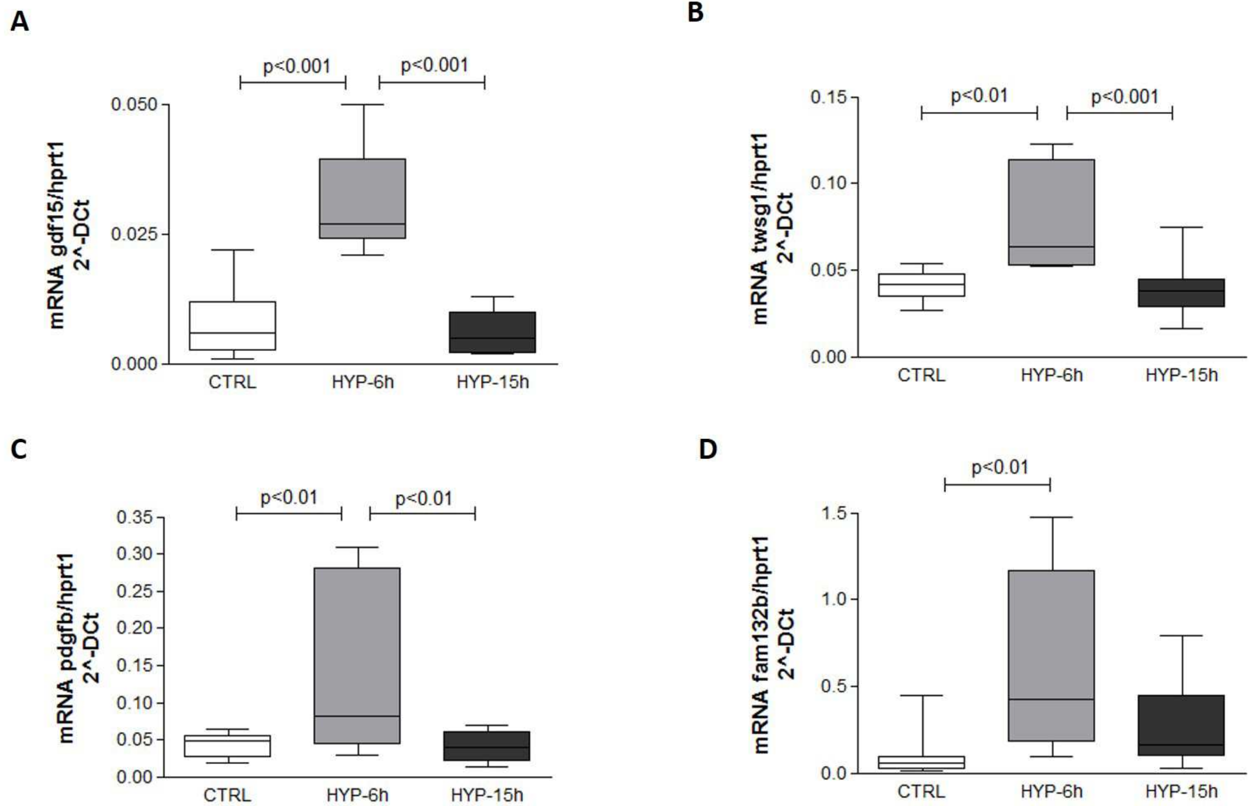
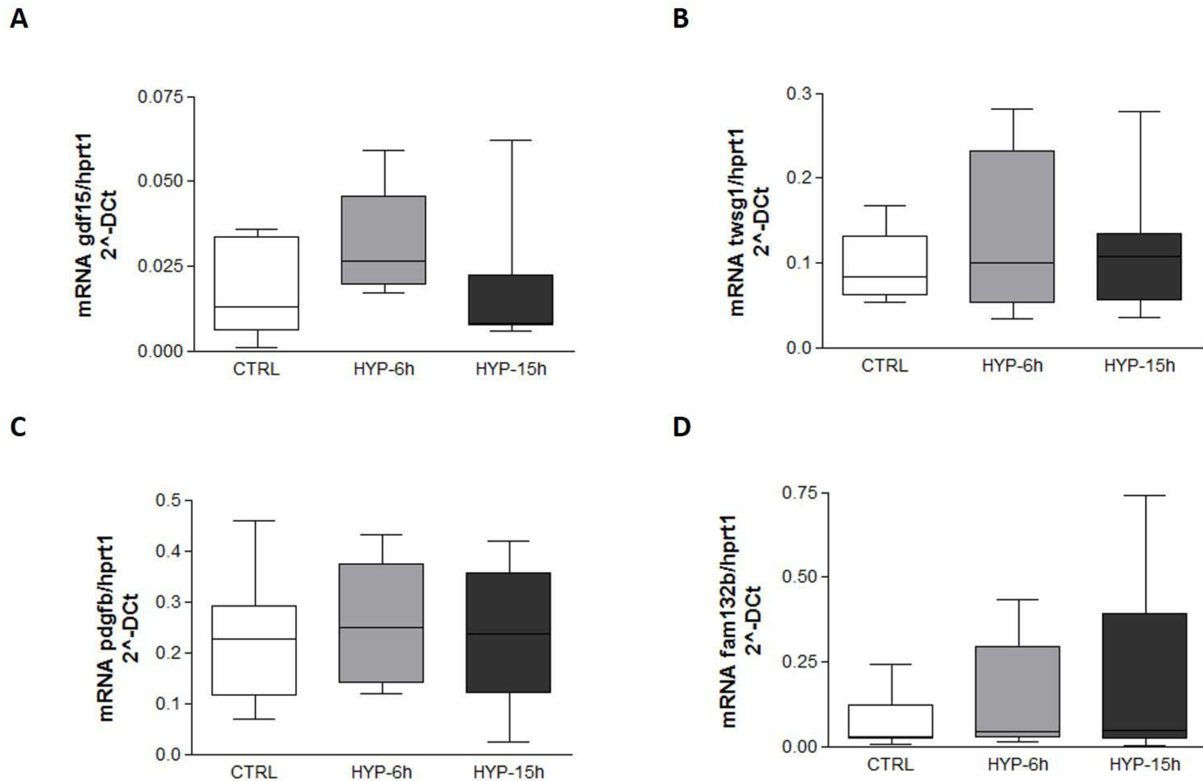


Figure 15. **A)** *Gdf15,* **B)** *Twsg1,* **C)** *Pdgf-b* and **D)** *Fam132b* mRNA expression in the spleen of normoxic (CTRL, N=12) and hypoxic mice (HYP) after 6h (N=6) and 15h (N=11).Boxes denote lower quartile, mean and upper quartile, and whiskers show maximum and minimum ranges.

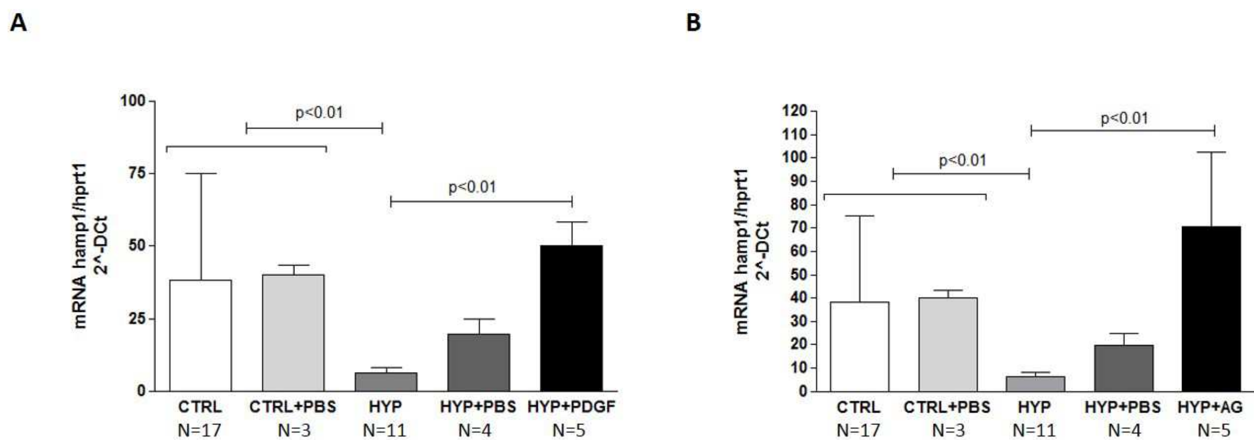


TREATMENT WITH α-PDGF-BB AND AG1296

We explored the hypothesis that PDGF-BB is responsible for hypoxia-mediated inhibition of hepcidin expression. We injected mice with the specific anti-PDGF-BB antibody or with the specific PDGF receptor (PDGF-R) kinase antagonist AG1296 (tyrphostin) and exposed them to hypoxia for 15h. Analysis of levels of serum PDGF-BB confirmed that treatment with α-PDGF-BB completely reset PDGF-BB levels while treatment with AG1296 did not change serum PDGF-BB levels respect to untreated mice, as expected (data not shown). Hypoxic mice receiving the anti-PDGF-BB antibody or AG1296 showed a hepatic hepcidin mRNA expression which was comparable with normoxic mice, and higher than in hypoxic mice untreated or treated with solvent

(figure 16 A and B). As expected, treatments of normoxic mice with α-PDGF-BB or AG1296 (data not shown) did not lead to significant changes in hepcidin expression.

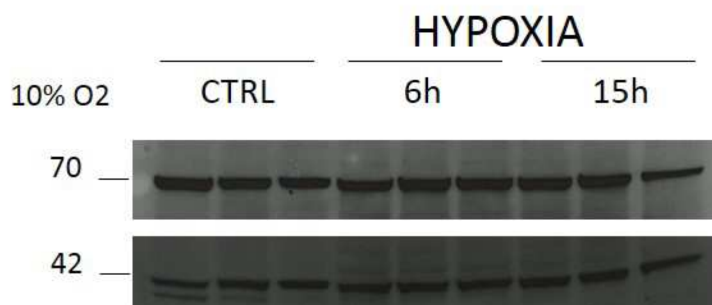
Figure 16. *Hamp1* mRNA expression of liver of normoxic untreated mice (CTRL); normoxic mice treated with PBS (CTRL+PBS), hypoxic mice after 15h of hypoxia challenge untreated (HYP) and treated with PBS (HYP+PBS), α-PDGF-BB (HYP+PDGF) (Figure 16A) or AG1296 (HYP+AG) (Figure 16B). Results are expressed as mean and standard deviation. Values of normoxic mice untreated and treated with PBS were considered as a sole group in statistical analysis.



CREB/H WESTERN BLOT

To figure out the molecular pathway involved in hepcidin inhibition PDGF-BB-mediated, based on previous work (Sonnweber, et al., 2014), we perform Western Blot analysis on CREB/H transcription factors. However, differently to Sonnweber *et al.* who reported a down-regulation of this molecule at 48 hours of hypoxic challenge, in our experiments CREB/H levels did not change after 6 and 15 hours of hypoxia exposure suggesting that it is not involved in early response of hypoxia-mediated hepcidin down-regulation (Figure 17).

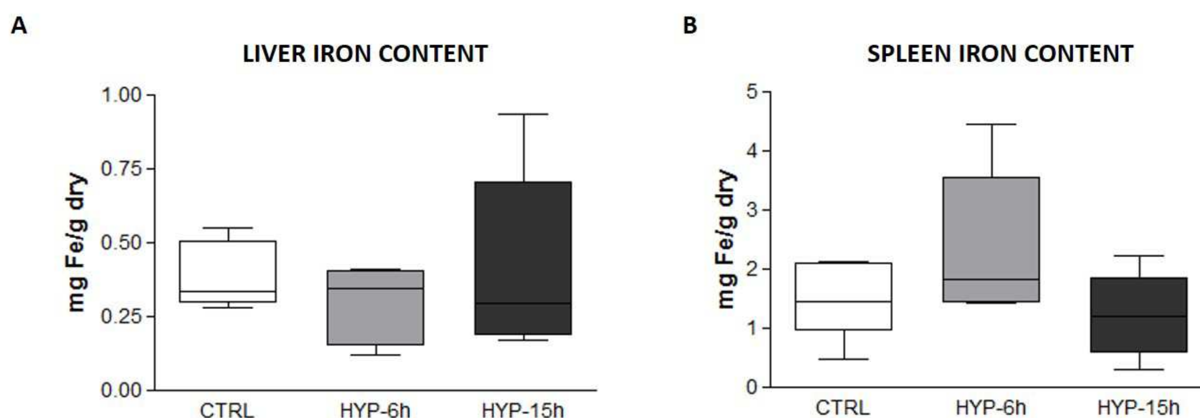
Figure 17. Western blot analysis of total lysate protein in liver of normoxic mice (CTRL) and mice subjected to hypoxia challenge for 6 and 15 hours. After protein extraction, western blot analysis of CREB/H was performed. Results were then normalized with actin expression.



LIVER AND SPLEEN IRON CONTENT MEASUREMENT

We analyzed iron content in liver and spleen of control and hypoxic mice. As reported in Figure 18 A and B, no differences were observed between normoxic and hypoxic mice.

Figure 18. A) LIC (Liver Iron Content) and SIC **B)** (Spleen Iron Content) measured in normoxic mice (CTRL, N=6) and hypoxic mice (HYP) after 6h (N=6) and 15h (N=6) of hypoxia challenge. Boxes denote lower quartile, mean and upper quartile, and whiskers show maximum and minimum ranges. p values (not significant) were determined using One-way Anova and Tukey test post hoc analysis



DISCUSSION

Hepcidin is the master regulator of iron homeostasis. Its hepatic expression is regulated by different stimuli that up- or down-regulated its levels (Piperno, et al., 2009). Anemia, hypoxia and erythropoietic drive (Nicolas, et al., 2002; Pak, et al., 2006; Vokurka, et al., 2006) inhibit hepcidin expression to meet iron demand of bone marrow to sustain erythroid expansion. Although much progress have been made in the last years, mechanisms of hypoxia-induced hepcidin regulation are still partially unknown. It has been recently demonstrated that hypoxia inhibits hepcidin expression indirectly, stimulating the bone marrow to release soluble factors in response to EPO-induced erythropoiesis (Liu, et al., 2012; Mastrogiannaki, et al., 2012; Pak, et al., 2006; Vokurka, et al., 2006; Volke, et al., 2009). To shed more light on this topic we used different approaches to investigate molecular mechanisms of hepcidin regulation mediated by hypoxia. Our work on human volunteers exposed to acute hypobaric hypoxia confirmed that hypoxia is a strong negative regulator of hepcidin expression *in vivo*. Serum hepcidin already decreased after 24 hours of hypoxia exposure (S1) and was almost fully suppressed after 72 hours (S2). These results confirm those described by Talbot *et al.* who showed almost complete hepcidin inhibition within 48 hours of exposure to high altitude in volunteers living at sea level (Talbot, et al., 2012). The time course of hepcidin inhibition indicates that it occurs rapidly but follows EPO activation, that markedly increased after 24h slightly decreasing thereafter, suggesting that factor(s) released by the expanding erythroid bone marrow might regulate hepcidin production (Piperno, et al., 2011). Although GDF-15 has been previously reported as candidate erythroid regulators of hepcidin (Tanno, et al., 2007; Tanno, et al., 2009), in the present study GDF-15 did not significantly changed in volunteers after acute exposure to hypoxia. This result confirms that GDF-15 is not required for balancing iron homeostasis in stress erythropoiesis, as also obtained by Casanovas *et al.* in mice models with genetic inactivation of *Gdf15* (Casanovas, et al., 2013).

The *in vitro* study performed using hypoxic sera of volunteers support the hypothesis that circulating factors are involved in hepcidin suppression induced by hypoxia. Indeed, a significant decrease of *HAMP* promoter activity was observed in HuH-7 exposed to hypoxic sera, a finding

that disappeared when transfecting HuH-7 cells with a 1207 bp fragment that lacks the distal portion of HAMP promoter containing the BMP responsive elements. This suggests that factor(s) present in hypoxic sera modulate a signaling cascade in HuH-7 cells leading to down-regulation of *HAMP* transcription and operating through one or more transcription factors binding sites related to the BMP-SMAD pathway located in the distal promoter region. This is also confirmed by gene expression studies. Although only a number of sera (N=14) inhibited *HAMP* mRNA levels, this down-regulation occurred together with *ID1* mRNA inhibition in HuH-7 cells, suggesting that hypoxia regulates hepcidin transcription through the SMAD pathway. It is not easy to explain the different results observed between luciferase and gene expression assays and between subgroups of sera. We excluded that the two subgroups differ according to IL-6, EPO, s-FERR and GDF-15 levels. One possible explanation is the higher sensibility of luciferase assay to detect even small differences compared to qRT-PCR. In summary, in 53% of participants (14 out of 26) there is a concordance between *in vivo* and *in vitro* analyses both by luciferase and gene expression assays. These findings allow us to conclude that: i. circulating factors are present in hypoxic sera; ii. they can modulate hepcidin expression; iii. the cellular model we used cannot fully reproduce what happens *in vivo*. It is also possible that hypoxia-induced hepcidin down-regulation depends to complex events involving both erythroid-derived and tissue (liver-related) factors.

To overcome the limits of the cellular model, we set up an *in vivo* model of acute hypobaric hypoxia to investigate erythroid hepcidin regulation, analyzing the expression of different bone marrow-derived molecules in the liver, bone marrow and spleen of mice subjected to hypoxic challenge. Different molecules have been proposed to be the hepcidin erythroid regulator, such as GDF15 (Tanno, et al., 2007), TWSG1 (Tanno, et al., 2009) and very recently PDGF-BB (Sonnweber, et al., 2014) and Erythroferrone (ERFE) (Kautz, et al., 2014). However, these results need to be confirmed. The recent study of Sonnweber *et al.* investigated the effects of hypoxia on iron metabolism in mice suggesting PDGF-BB as a new “iron-molecule” able to inhibit hepcidin during hypoxia acting through the CREB/H pathway (Sonnweber, et al., 2014). In this study mice

kept under normobaric hypoxia for 48 hours presented with a significant reduction in hepatic hepcidin mRNA and with a significant augment of serum PDGF-BB levels as compared with control mice (Sonnweber, et al., 2014). Kautz *et al.* demonstrated that ERFE, a new TNFα-like molecule produced by bone marrow erythroblasts, seems to be activated by EPO through the JAK2/STAT-5 pathway, directly acting on hepatic hepcidin expression through a still undefined mechanism. However the evidences that ERFE could be the erythroid hepcidin regulator were obtained in animal models whose erythropoiesis was induced by phlebotomy or EPO injections, but not in hypoxia models in which mechanisms of hepcidin suppression might be more complex (Kautz, et al., 2014).

In our study, EPO levels already increased after 6 hours of hypoxic treatment, suggesting a rapid activation of the erythropoietic drive, according to previous studies that demonstrated that hypoxia generates a detectable increase in serum EPO within 90 minutes, which peaks within 2 days and thereafter declines over a period of 1 to 2 weeks in volunteers exposed to high altitude (Eckardt, et al., 1989; Richalet, et al., 1994).

Hamp1 mRNA levels were slightly increased after 6 hours of hypobaric hypoxia exposure and strongly down-regulated since after 15 hours of treatment, confirming a rapid hepcidin down-regulation, as occurs in humans (Ravasi, et al., 2014; Talbot, et al., 2012). After 6 hours of hypoxia challenge we already observed a marked activation of transcription of all the erythropoietic genes analyzed in the bone marrow, which then return to baseline levels after 15 hours. These data demonstrate that hypoxia-induced hepcidin regulation occurs indirectly. Indeed, hepcidin decreases only after the up-regulation of the erythroid molecules, which return to baseline levels when hepcidin has reached the maximum inhibition. PDGF-BB and ERFE but also GDF15 and TWSG1 were up-regulated after hypoxia exposure only in the bone marrow and not in the spleen or in the liver. This indicates that hypoxia-induced transcription of these genes is tissue specific and highlights the role of EPO as the promoter of this process. Moreover, when we analyzed other hepatic growth factors such as HGF and EGF, we observed a slightly reduction of these molecules

after 15 hours of hypoxia exposure as compared to controls or 6-hours hypoxic mice. These results suggested that these factors, that inhibit hepcidin expression during chronic liver failure (Goodnough, et al., 2012), were not involved in hepcidin down-regulation hypoxia-mediated.

Neutralization experiments confirm the important role of PDGF-BB in hepcidin inhibition in this setting, indeed when PDGF-BB or its receptor are inactivated, hepcidin down-regulation is suppressed and CREB/H seems not to be involved in hypoxia-mediated hepcidin regulation. Indeed, different of that observed by Sonneweber *at al.* (Sonnweber, et al., 2014) who reported a down-regulation of CREB/H at 48 hours of hypoxia exposure, in our experiments CREB/H levels did not change during hypoxic challenge. This could be due to different time point we analyzed and suggests that this molecule is not involved in early response of hypoxia-mediated hepcidin down-regulation.

It was previously demonstrated that ferritin levels correlate with modulation of hepcidin expression during exposure to hypobaric hypoxia, suggesting that iron itself or its use in response to hypoxia can induce hepcidin down-regulation (Piperno, et al., 2011). However, our results obtained by measuring the total iron content of liver (LIC) and spleen (SIC) suggest that hepcidin inhibition after 15h of hypoxia exposure was not influenced from iron stores.

In conclusion, our results demonstrate that hypoxia-mediated hepcidin regulation follows the release of circulating factors from the erythroid bone marrow activated by EPO. This study also confirmed that the bone marrow plays a key role in the inhibition of hepcidin during hypoxia and that PDGF-BB could be a good candidate as hepcidin erythroid regulators. However, this study also suggest that multiple signals likely cooperate in hepcidin suppression during hypoxia. The results of these studies are expected to have implications for hypoxia-related acute and chronic disorders, contributing to explain the role of hepcidin in the pathophysiology of hypoxia adaptation and possibly providing markers of clinical utility for several common disorders.

REFERENCES

Abboud S, Haile DJ. 2000. A novel mammalian iron-regulated protein involved in intracellular iron metabolism. J Biol Chem 275(26):19906-12.

Andrews PA. 2000. Disorders of iron metabolism. N Engl J Med 342(17):1293; author reply 1294.

Andriopoulos B, Jr., Corradini E, Xia Y, Faasse SA, Chen S, Grgurevic L, Knutson MD, Pietrangelo A, Vukicevic S, Lin HY and others. 2009. BMP6 is a key endogenous regulator of hepcidin expression and iron metabolism. Nat Genet 41(4):482-7.

Arosio P, Levi S. 2010. Cytosolic and mitochondrial ferritins in the regulation of cellular iron homeostasis and oxidative damage. Biochim Biophys Acta 1800(8):783-92.

Babitt JL, Huang FW, Xia Y, Sidis Y, Andrews NC, Lin HY. 2007. Modulation of bone morphogenetic protein signaling in vivo regulates systemic iron balance. J Clin Invest 117(7):1933-9.

Barry M, Sherlock S. 1971. Measurement of liver-iron concentration in needle-biopsy specimens. Lancet 1(7690):100-3.

Bergamaschi G, Villani L. 2009. Serum hepcidin: a novel diagnostic tool in disorders of iron metabolism. Haematologica 94(12):1631-3.

Besson-Fournier C, Latour C, Kautz L, Bertrand J, Ganz T, Roth MP, Coppin H. 2012. Induction of activin B by inflammatory stimuli up-regulates expression of the iron-regulatory peptide hepcidin through Smad1/5/8 signaling. Blood 120(2):431-9.

Beutler E, Felitti VJ, Koziol JA, Ho NJ, Gelbart T. 2002. Penetrance of 845G--> A (C282Y) HFE hereditary haemochromatosis mutation in the USA. Lancet 359(9302):211-8.

Camaschella C. 2005. Understanding iron homeostasis through genetic analysis of hemochromatosis and related disorders. Blood 106(12):3710-7.

Campostrini N, Castagna A, Zaninotto F, Bedogna V, Tessitore N, Poli A, Martinelli N, Lupo A, Olivieri O, Girelli D. 2010. Evaluation of hepcidin isoforms in hemodialysis patients by a proteomic approach based on SELDI-TOF MS. J Biomed Biotechnol 2010:329646.

Casanovas G, Mleczko-Sanecka K, Altamura S, Hentze MW, Muckenthaler MU. 2009. Bone morphogenetic protein (BMP)-responsive elements located in the proximal and distal hepcidin promoter are critical for its response to HJV/BMP/SMAD. J Mol Med (Berl) 87(5):471-80.

Casanovas G, Vujic Spasic M, Casu C, Rivella S, Strelau J, Unsicker K, Muckenthaler MU. 2013. The murine growth differentiation factor 15 is not essential for systemic iron homeostasis in phlebotomized mice. Haematologica 98(3):444-7.

Cazzola M, Skoda RC. 2000. Translational pathophysiology: a novel molecular mechanism of human disease. Blood 95(11):3280-8.

Chaston TB, Matak P, Pourvali K, Srai SK, McKie AT, Sharp PA. 2011. Hypoxia inhibits hepcidin expression in HuH7 hepatoma cells via decreased SMAD4 signaling. Am J Physiol Cell Physiol 300(4):C888-95.

Chen J, Chloupkova M, Gao J, Chapman-Arvedson TL, Enns CA. 2007. HFE modulates transferrin receptor 2 levels in hepatoma cells via interactions that differ from transferrin receptor 1-HFE interactions. J Biol Chem 282(51):36862-70.

Chen J, Enns CA. 2012. Hereditary hemochromatosis and transferrin receptor 2. Biochim Biophys Acta 1820(3):256-63.

Cheng Y, Zak O, Aisen P, Harrison SC, Walz T. 2004. Structure of the human transferrin receptor-transferrin complex. Cell 116(4):565-76.

Corradini E, Garuti C, Montosi G, Ventura P, Andriopoulos B, Jr., Lin HY, Pietrangelo A, Babitt JL. 2009. Bone morphogenetic protein signaling is impaired in an HFE knockout mouse model of hemochromatosis. Gastroenterology 137(4):1489-97.

Cox TM, Peters TJ. 1979. The kinetics of iron uptake in vitro by human duodenal mucosa: studies in normal subjects. J Physiol 289:469-78.

Detivaud L, Island ML, Jouanolle AM, Ropert M, Bardou-Jacquet E, Le Lan C, Mosser A, Leroyer P, Deugnier Y, David V and others. 2013. Ferroportin diseases: functional studies, a link between genetic and clinical phenotype. Hum Mutat 34(11):1529-36.
Donovan A, Brownlie A, Zhou Y, Shepard J, Pratt SJ, Moynihan J, Paw BH, Drejer A, Barut B, Zapata A and others. 2000. Positional cloning of zebrafish ferroportin1 identifies a conserved vertebrate iron exporter. Nature 403(6771):776-81.
Du X, She E, Gelbart T, Truksa J, Lee P, Xia Y, Khovananth K, Mudd S, Mann N, Moresco EM and others. 2008. The serine protease TMPRSS6 is required to sense iron deficiency. Science 320(5879):1088-92.
Eckardt KU, Boutellier U, Kurtz A, Schopen M, Koller EA, Bauer C. 1989. Rate of erythropoietin formation in humans in response to acute hypobaric hypoxia. J Appl Physiol (1985) 66(4):1785-8.
Feder JN, Gnirke A, Thomas W, Tsuchihashi Z, Ruddy DA, Basava A, Dormishian F, Domingo R, Jr., Ellis MC, Fullan A and others. 1996. A novel MHC class I-like gene is mutated in patients with hereditary haemochromatosis. Nat Genet 13(4):399-408.
Finch C. 1994. Regulators of iron balance in humans. Blood 84(6):1697-702.
Fleming MD, Romano MA, Su MA, Garrick LM, Garrick MD, Andrews NC. 1998. Nramp2 is mutated in the anemic Belgrade (b) rat: evidence of a role for Nramp2 in endosomal iron transport. Proc Natl Acad Sci U S A 95(3):1148-53.
Fleming RE. 2007. Hepcidin activation during inflammation: make it STAT. Gastroenterology 132(1):447-9.
Fleming RE, Ahmann JR, Migas MC, Waheed A, Koeffler HP, Kawabata H, Britton RS, Bacon BR, Sly WS. 2002. Targeted mutagenesis of the murine transferrin receptor-2 gene produces hemochromatosis. Proc Natl Acad Sci U S A 99(16):10653-8.
Forejtnikova H, Vieillevoye M, Zermati Y, Lambert M, Pellegrino RM, Guihard S, Gaudry M, Camaschella C, Lacombe C, Roetto A and others. 2010. Transferrin receptor 2 is a component of the erythropoietin receptor complex and is required for efficient erythropoiesis. Blood 116(24):5357-67.
Ganz T. 2003. Hepcidin, a key regulator of iron metabolism and mediator of anemia of inflammation. Blood 102(3):783-8.
Ganz T. 2013. Systemic iron homeostasis. Physiol Rev 93(4):1721-41.
Gao J, Chen J, Kramer M, Tsukamoto H, Zhang AS, Enns CA. 2009. Interaction of the hereditary hemochromatosis protein HFE with transferrin receptor 2 is required for transferrin-induced hepcidin expression. Cell Metab 9(3):217-27.
Gattermann N. 2009. The treatment of secondary hemochromatosis. Dtsch Arztebl Int 106(30):499-504, I.
Goodnough JB, Ramos E, Nemeth E, Ganz T. 2012. Inhibition of hepcidin transcription by growth factors. Hepatology 56(1):291-9.
Gordeuk VR, Miasnikova GY, Sergueeva AI, Niu X, Nouraie M, Okhotin DJ, Polyakova LA, Ammosova T, Nekhai S, Ganz T and others. 2011. Chuvash polycythemia VHLR200W mutation is associated with down-regulation of hepcidin expression. Blood 118(19):5278-82.
Goswami T, Andrews NC. 2006. Hereditary hemochromatosis protein, HFE, interaction with transferrin receptor 2 suggests a molecular mechanism for mammalian iron sensing. J Biol Chem 281(39):28494-8.
Greer SN, Metcalf JL, Wang Y, Ohh M. 2012. The updated biology of hypoxia-inducible factor. Embo J 31(11):2448-60.
Gunshin H, Allerson CR, Polycarpou-Schwarz M, Rofts A, Rogers JT, Kishi F, Hentze MW, Rouault TA, Andrews NC, Hediger MA. 2001. Iron-dependent regulation of the divalent metal ion transporter. FEBS Lett 509(2):309-16.

Gunshin H, Mackenzie B, Berger UV, Gunshin Y, Romero MF, Boron WF, Nussberger S, Gollan JL, Hediger MA. 1997. Cloning and characterization of a mammalian proton-coupled metal-ion transporter. Nature 388(6641):482-8.
Haile DJ, Rouault TA, Harford JB, Kennedy MC, Blondin GA, Beinert H, Klausner RD. 1992. Cellular regulation of the iron-responsive element binding protein: disassembly of the cubane iron-sulfur cluster results in high-affinity RNA binding. Proc Natl Acad Sci U S A 89(24):11735-9.
Harris ZL, Durley AP, Man TK, Gitlin JD. 1999. Targeted gene disruption reveals an essential role for ceruloplasmin in cellular iron efflux. Proc Natl Acad Sci U S A 96(19):10812-7.
Hartmann G, Tschop M, Fischer R, Bidlingmaier C, Riepl R, Tschop K, Hautmann H, Endres S, Toepfer M. 2000. High altitude increases circulating interleukin-6, interleukin-1 receptor antagonist and C-reactive protein. Cytokine 12(3):246-52.
Heinrich PC, Behrmann I, Haan S, Hermanns HM, Muller-Newen G, Schaper F. 2003. Principles of interleukin (IL)-6-type cytokine signalling and its regulation. Biochem J 374(Pt 1):1-20.
Hentze MW, Kuhn LC. 1996. Molecular control of vertebrate iron metabolism: mRNA-based regulatory circuits operated by iron, nitric oxide, and oxidative stress. Proc Natl Acad Sci U S A 93(16):8175-82.
Hentze MW, Muckenthaler MU, Andrews NC. 2004. Balancing acts: molecular control of mammalian iron metabolism. Cell 117(3):285-97.
Hentze MW, Muckenthaler MU, Galy B, Camaschella C. 2010. Two to tango: regulation of Mammalian iron metabolism. Cell 142(1):24-38.
Hintze KJ, Snow D, Nabor D, Timbimboo H. 2011. Adipocyte hypoxia increases hepatocyte hepcidin expression. Biol Trace Elem Res 143(2):764-71.
Iwai K, Drake SK, Wehr NB, Weissman AM, LaVaute T, Minato N, Klausner RD, Levine RL, Rouault TA. 1998. Iron-dependent oxidation, ubiquitination, and degradation of iron regulatory protein 2: implications for degradation of oxidized proteins. Proc Natl Acad Sci U S A 95(9):4924-8.
Kautz L, Jung G, Valore EV, Rivella S, Nemeth E, Ganz T. 2014. Identification of erythroferrone as an erythroid regulator of iron metabolism. Nat Genet.
Kautz L, Meynard D, Besson-Fournier C, Darnaud V, Al Saati T, Coppin H, Roth MP. 2009. BMP/Smad signaling is not enhanced in Hfe-deficient mice despite increased Bmp6 expression. Blood 114(12):2515-20.
Kawabata H, Tong X, Kawanami T, Wano Y, Hirose Y, Sugai S, Koeffler HP. 2004. Analyses for binding of the transferrin family of proteins to the transferrin receptor 2. Br J Haematol 127(4):464-73.
Kawabata H, Yang R, Hirama T, Vuong PT, Kawano S, Gombart AF, Koeffler HP. 1999. Molecular cloning of transferrin receptor 2. A new member of the transferrin receptor-like family. J Biol Chem 274(30):20826-32.
Knutson MD. 2010. Iron-sensing proteins that regulate hepcidin and enteric iron absorption. Annu Rev Nutr 30:149-71.
Krause A, Neitz S, Magert HJ, Schulz A, Forssmann WG, Schulz-Knappe P, Adermann K. 2000. LEAP-1, a novel highly disulfide-bonded human peptide, exhibits antimicrobial activity. FEBS Lett 480(2-3):147-50.
Kumar V, Robbins SL. 2007. Robbins basic pathology. Philadelphia, PA: Saunders/Elsevier.
Lakhal S, Schodel J, Townsend AR, Pugh CW, Ratcliffe PJ, Mole DR. 2011. Regulation of type II transmembrane serine proteinase TMPRSS6 by hypoxia-inducible factors: new link between hypoxia signaling and iron homeostasis. J Biol Chem 286(6):4090-7.
Le Gac G, Ferec C. 2005. The molecular genetics of haemochromatosis. Eur J Hum Genet 13(11):1172-85.

Le Lan C, Mosser A, Ropert M, Detivaud L, Loustaud-Ratti V, Vital-Durand D, Roget L, Bardou-Jacquet E, Turlin B, David V and others. 2011. Sex and acquired cofactors determine phenotypes of ferroportin disease. Gastroenterology 140(4):1199-1207 e1-2.

Lee DH, Zhou LJ, Zhou Z, Xie JX, Jung JU, Liu Y, Xi CX, Mei L, Xiong WC. 2010. Neogenin inhibits HJV secretion and regulates BMP-induced hepcidin expression and iron homeostasis. Blood 115(15):3136-45.

Levy JE, Jin O, Fujiwara Y, Kuo F, Andrews NC. 1999. Transferrin receptor is necessary for development of erythrocytes and the nervous system. Nat Genet 21(4):396-9.

Lin L, Nemeth E, Goodnough JB, Thapa DR, Gabayan V, Ganz T. 2008. Soluble hemojuvelin is released by proprotein convertase-mediated cleavage at a conserved polybasic RNRR site. Blood Cells Mol Dis 40(1):122-31.

Liu Q, Davidoff O, Niss K, Haase VH. 2012. Hypoxia-inducible factor regulates hepcidin via erythropoietin-induced erythropoiesis. J Clin Invest 122(12):4635-44.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25(4):402-8.

Mastrogiannaki M, Matak P, Keith B, Simon MC, Vaulont S, Peyssonnaux C. 2009. HIF-2alpha, but not HIF-1alpha, promotes iron absorption in mice. J Clin Invest 119(5):1159-66.

Mastrogiannaki M, Matak P, Mathieu JR, Delga S, Mayeux P, Vaulont S, Peyssonnaux C. 2012. Hepatic hypoxia-inducible factor-2 down-regulates hepcidin expression in mice through an erythropoietin-mediated increase in erythropoiesis. Haematologica 97(6):827-34.

McKie AT, Barrow D, Latunde-Dada GO, Rolfs A, Sager G, Mudaly E, Mudaly M, Richardson C, Barlow D, Bomford A and others. 2001. An iron-regulated ferric reductase associated with the absorption of dietary iron. Science 291(5509):1755-9.

McKie AT, Marciani P, Rolfs A, Brennan K, Wehr K, Barrow D, Miret S, Bomford A, Peters TJ, Farzaneh F and others. 2000. A novel duodenal iron-regulated transporter, IREG1, implicated in the basolateral transfer of iron to the circulation. Mol Cell 5(2):299-309.

Meynard D, Kautz L, Darnaud V, Canonne-Hergaux F, Coppin H, Roth MP. 2009. Lack of the bone morphogenetic protein BMP6 induces massive iron overload. Nat Genet 41(4):478-81.

Meynard D, Vaja V, Sun CC, Corradini E, Chen S, Lopez-Otin C, Grgurevic L, Hong CC, Stirnberg M, Gutschow M and others. 2011. Regulation of TMPRSS6 by BMP6 and iron in human cells and mice. Blood 118(3):747-56.

Mleczko-Sanecka K, Casanovas G, Ragab A, Breitkopf K, Muller A, Boutros M, Dooley S, Hentze MW, Muckenthaler MU. 2010. SMAD7 controls iron metabolism as a potent inhibitor of hepcidin expression. Blood 115(13):2657-65.

Montosi G, Donovan A, Totaro A, Garuti C, Pignatti E, Cassanelli S, Trenor CC, Gasparini P, Andrews NC, Pietrangelo A. 2001. Autosomal-dominant hemochromatosis is associated with a mutation in the ferroportin (SLC11A3) gene. J Clin Invest 108(4):619-23.

Munoz M, Villar I, Garcia-Erce JA. 2009. An update on iron physiology. World J Gastroenterol 15(37):4617-26.

Nemeth E, Preza GC, Jung CL, Kaplan J, Waring AJ, Ganz T. 2006. The N-terminus of hepcidin is essential for its interaction with ferroportin: structure-function study. Blood 107(1):328-33.

Nemeth E, Rivera S, Gabayan V, Keller C, Taudorf S, Pedersen BK, Ganz T. 2004a. IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin. J Clin Invest 113(9):1271-6.

Nemeth E, Tuttle MS, Powelson J, Vaughn MB, Donovan A, Ward DM, Ganz T, Kaplan J. 2004b. Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. Science 306(5704):2090-3.

Nemeth E, Valore EV, Territo M, Schiller G, Lichtenstein A, Ganz T. 2003. Hepcidin, a putative mediator of anemia of inflammation, is a type II acute-phase protein. Blood 101(7):2461-3.

Nicolas G, Chauvet C, Viatte L, Danan JL, Bigard X, Devaux I, Beaumont C, Kahn A, Vaulont S. 2002. The gene encoding the iron regulatory peptide hepcidin is regulated by anemia, hypoxia, and inflammation. J Clin Invest 110(7):1037-44.
Njajou OT, Vaessen N, Joosse M, Berghuis B, van Dongen JW, Breuning MH, Snijders PJ, Rutten WP, Sandkuijl LA, Oostra BA and others. 2001. A mutation in SLC11A3 is associated with autosomal dominant hemochromatosis. Nat Genet 28(3):213-4.
Ohgami RS, Campagna DR, Greer EL, Antiochos B, McDonald A, Chen J, Sharp JJ, Fujiwara Y, Barker JE, Fleming MD. 2005. Identification of a ferrireductase required for efficient transferrin-dependent iron uptake in erythroid cells. Nat Genet 37(11):1264-9.
Oliveira SJ, Pinto JP, Picarote G, Costa VM, Carvalho F, Rangel M, de Sousa M, de Almeida SF. 2009. ER stress-inducible factor CHOP affects the expression of hepcidin by modulating C/EBPalpha activity. PLoS One 4(8):e6618.
Pagani A, Silvestri L, Nai A, Camaschella C. 2008. Hemojuvelin N-terminal mutants reach the plasma membrane but do not activate the hepcidin response. Haematologica 93(10):1466-72.
Pak M, Lopez MA, Gabayan V, Ganz T, Rivera S. 2006. Suppression of hepcidin during anemia requires erythropoietic activity. Blood 108(12):3730-5.
Papanikolaou G, Samuels ME, Ludwig EH, MacDonald ML, Franchini PL, Dube MP, Andres L, MacFarlane J, Sakellaropoulos N, Politou M and others. 2004. Mutations in HFE2 cause iron overload in chromosome 1q-linked juvenile hemochromatosis. Nat Genet 36(1):77-82.
Park CH, Valore EV, Waring AJ, Ganz T. 2001. Hepcidin, a urinary antimicrobial peptide synthesized in the liver. J Biol Chem 276(11):7806-10.
Parrow NL, Fleming RE. 2014. Bone morphogenetic proteins as regulators of iron metabolism. Annu Rev Nutr 34:77-94.
Patel N, Varghese J, Masaratana P, Latunde-Dada GO, Jacob M, Simpson RJ, McKie AT. 2014. The transcription factor ATOH8 is regulated by erythropoietic activity and regulates HAMP transcription and cellular pSMAD1,5,8 levels. Br J Haematol 164(4):586-96.
Pelucchi S, Mariani R, Salvioni A, Bonfadini S, Riva A, Bertola F, Trombini P, Piperno A. 2008. Novel mutations of the ferroportin gene (SLC40A1): analysis of 56 consecutive patients with unexplained iron overload. Clin Genet 73(2):171-8.
Peslova G, Petrak J, Kuzelova K, Hrdy I, Halada P, Kuchel PW, Soe-Lin S, Ponka P, Sutak R, Becker E and others. 2009. Hepcidin, the hormone of iron metabolism, is bound specifically to alpha-2-macroglobulin in blood. Blood 113(24):6225-36.
Peyssonnaux C, Nizet V, Johnson RS. 2008. Role of the hypoxia inducible factors HIF in iron metabolism. Cell Cycle 7(1):28-32.
Peyssonnaux C, Zinkernagel AS, Schuepbach RA, Rankin E, Vaulont S, Haase VH, Nizet V, Johnson RS. 2007. Regulation of iron homeostasis by the hypoxia-inducible transcription factors (HIFs). J Clin Invest 117(7):1926-32.
Pietrangelo A. 2010. Hereditary hemochromatosis: pathogenesis, diagnosis, and treatment. Gastroenterology 139(2):393-408, 408 e1-2.
Pietrangelo A. 2011. Hepcidin in human iron disorders: therapeutic implications. J Hepatol 54(1):173-81.
Pietrangelo A, Dierssen U, Valli L, Garuti C, Rump A, Corradini E, Ernst M, Klein C, Trautwein C. 2007. STAT3 is required for IL-6-gp130-dependent activation of hepcidin in vivo. Gastroenterology 132(1):294-300.
Pigeon C, Ilyin G, Courselaud B, Leroyer P, Turlin B, Brissot P, Loreal O. 2001. A new mouse liver-specific gene, encoding a protein homologous to human antimicrobial peptide hepcidin, is overexpressed during iron overload. J Biol Chem 276(11):7811-9.
Pinto JP, Ribeiro S, Pontes H, Thowfeequ S, Tosh D, Carvalho F, Porto G. 2008. Erythropoietin mediates hepcidin expression in hepatocytes through EPOR signaling and regulation of C/EBPalpha. Blood 111(12):5727-33.

Piperno A, Galimberti S, Mariani R, Pelucchi S, Ravasi G, Lombardi C, Bilo G, Revera M, Giuliano A, Faini A and others. 2011. Modulation of hepcidin production during hypoxia-induced erythropoiesis in humans in vivo: data from the HIGHCARE project. Blood 117(10):2953-9.

Piperno A, Mariani R, Trombini P, Girelli D. 2009. Hepcidin modulation in human diseases: from research to clinic. World J Gastroenterol 15(5):538-51.

Poli M, Asperti M, Ruzzenenti P, Regoni M, Arosio P. 2014. Hepcidin antagonists for potential treatments of disorders with hepcidin excess. Front Pharmacol 5:86.

Qiu A, Jansen M, Sakaris A, Min SH, Chattopadhyay S, Tsai E, Sandoval C, Zhao R, Akabas MH, Goldman ID. 2006. Identification of an intestinal folate transporter and the molecular basis for hereditary folate malabsorption. Cell 127(5):917-28.

Rankin EB, Biju MP, Liu Q, Unger TL, Rha J, Johnson RS, Simon MC, Keith B, Haase VH. 2007. Hypoxia-inducible factor-2 (HIF-2) regulates hepatic erythropoietin in vivo. J Clin Invest 117(4):1068-77.

Ravasi G, Pelucchi S, Greni F, Mariani R, Giuliano A, Parati G, Silvestri L, Piperno A. 2014. Circulating factors are involved in hypoxia-induced hepcidin suppression. Blood Cells Mol Dis 53(4):204-10.

Ravasi G, Pelucchi S, Trombini P, Mariani R, Tomosugi N, Modignani GL, Pozzi M, Nemeth E, Ganz T, Hayashi H and others. 2012. Hepcidin expression in iron overload diseases is variably modulated by circulating factors. PLoS One 7(5):e36425.

Richalet JP, Souberbielle JC, Antezana AM, Dechaux M, Le Trong JL, Bienvenu A, Daniel F, Blanchot C, Zittoun J. 1994. Control of erythropoiesis in humans during prolonged exposure to the altitude of 6,542 m. Am J Physiol 266(3 Pt 2):R756-64.

Schimanski LM, Drakesmith H, Merryweather-Clarke AT, Viprakasit V, Edwards JP, Sweetland E, Bastin JM, Cowley D, Chinthammitr Y, Robson KJ and others. 2005. In vitro functional analysis of human ferroportin (FPN) and hemochromatosis-associated FPN mutations. Blood 105(10):4096-102.

Schito L, Rey S, Tafani M, Zhang H, Wong CC, Russo A, Russo MA, Semenza GL. 2012. Hypoxia-inducible factor 1-dependent expression of platelet-derived growth factor B promotes lymphatic metastasis of hypoxic breast cancer cells. Proc Natl Acad Sci U S A 109(40):E2707-16.

Semenza GL. 2001. HIF-1 and mechanisms of hypoxia sensing. Curr Opin Cell Biol 13(2):167-71.

Silvestri L, Pagani A, Camaschella C. 2008a. Furin-mediated release of soluble hemojuvelin: a new link between hypoxia and iron homeostasis. Blood 111(2):924-31.

Silvestri L, Pagani A, Nai A, De Domenico I, Kaplan J, Camaschella C. 2008b. The serine protease matriptase-2 (TMPRSS6) inhibits hepcidin activation by cleaving membrane hemojuvelin. Cell Metab 8(6):502-11.

Sonnweber T, Nachbaur D, Schroll A, Nairz M, Seifert M, Demetz E, Haschka D, Mitterstiller AM, Kleinsasser A, Burtscher M and others. 2014. Hypoxia induced downregulation of hepcidin is mediated by platelet derived growth factor BB. Gut 63(12):1951-9.

Subramaniam VN, Wallace DF. 2014. Linking hypoxia and iron homeostasis: a 'plate' full of factors. Gut 63(12):1840-2.

Swinkels DW, Girelli D, Laarakkers C, Kroot J, Campostrini N, Kemna EH, Tjalsma H. 2008. Advances in quantitative hepcidin measurements by time-of-flight mass spectrometry. PLoS One 3(7):e2706.

Talbot NP, Lakhal S, Smith TG, Privat C, Nickol AH, Rivera-Ch M, Leon-Velarde F, Dorrington KL, Mole DR, Robbins PA. 2012. Regulation of hepcidin expression at high altitude. Blood 119(3):857-60.

Tandara L, Salamunic I. 2012. Iron metabolism: current facts and future directions. Biochem Med (Zagreb) 22(3):311-28.

Tanno T, Bhanu NV, Oneal PA, Goh SH, Staker P, Lee YT, Moroney JW, Reed CH, Luban NL, Wang RH and others. 2007. High levels of GDF15 in thalassemia suppress expression of the iron regulatory protein hepcidin. Nat Med 13(9):1096-101.

Tanno T, Porayette P, Sripichai O, Noh SJ, Byrnes C, Bhupatiraju A, Lee YT, Goodnough JB, Harandi O, Ganz T and others. 2009. Identification of TWSG1 as a second novel erythroid regulator of hepcidin expression in murine and human cells. Blood 114(1):181-6.

Torti FM, Torti SV. 2002. Regulation of ferritin genes and protein. Blood 99(10):3505-16.

Vecchi C, Montosi G, Zhang K, Lamberti I, Duncan SA, Kaufman RJ, Pietrangelo A. 2009. ER stress controls iron metabolism through induction of hepcidin. Science 325(5942):877-80.

Verga Falzacappa MV, Casanovas G, Hentze MW, Muckenthaler MU. 2008. A bone morphogenetic protein (BMP)-responsive element in the hepcidin promoter controls HFE2-mediated hepatic hepcidin expression and its response to IL-6 in cultured cells. J Mol Med (Berl) 86(5):531-40.

Verga Falzacappa MV, Vujic Spasic M, Kessler R, Stolte J, Hentze MW, Muckenthaler MU. 2007. STAT3 mediates hepatic hepcidin expression and its inflammatory stimulation. Blood 109(1):353-8.

Veuthey T, Wessling-Resnick M. 2014. Pathophysiology of the Belgrade rat. Front Pharmacol 5:82.

Vokurka M, Krijt J, Sulc K, Necas E. 2006. Hepcidin mRNA levels in mouse liver respond to inhibition of erythropoiesis. Physiol Res 55(6):667-74.

Volke M, Gale DP, Maegdefrau U, Schley G, Klanke B, Bosserhoff AK, Maxwell PH, Eckardt KU, Warnecke C. 2009. Evidence for a lack of a direct transcriptional suppression of the iron regulatory peptide hepcidin by hypoxia-inducible factors. PLoS One 4(11):e7875.

Vulpe CD, Kuo YM, Murphy TL, Cowley L, Askwith C, Libina N, Gitschier J, Anderson GJ. 1999. Hephaestin, a ceruloplasmin homologue implicated in intestinal iron transport, is defective in the sla mouse. Nat Genet 21(2):195-9.

Waheed A, Britton RS, Grubb JH, Sly WS, Fleming RE. 2008. HFE association with transferrin receptor 2 increases cellular uptake of transferrin-bound iron. Arch Biochem Biophys 474(1):193-7.

Wallace DF, Summerville L, Lusby PE, Subramaniam VN. 2005. Prohepcidin localises to the Golgi compartment and secretory pathway in hepatocytes. J Hepatol 43(4):720-8.

Wang RH, Li C, Xu X, Zheng Y, Xiao C, Zerfas P, Cooperman S, Eckhaus M, Rouault T, Mishra L and others. 2005. A role of SMAD4 in iron metabolism through the positive regulation of hepcidin expression. Cell Metab 2(6):399-409.

Ward DM, Kaplan J. 2012. Ferroportin-mediated iron transport: expression and regulation. Biochim Biophys Acta 1823(9):1426-33.

West AP, Jr., Bennett MJ, Sellers VM, Andrews NC, Enns CA, Bjorkman PJ. 2000. Comparison of the interactions of transferrin receptor and transferrin receptor 2 with transferrin and the hereditary hemochromatosis protein HFE. J Biol Chem 275(49):38135-8.

Yun S, Vincelette ND. 2015. Update on iron metabolism and molecular perspective of common genetic and acquired disorder, hemochromatosis. Crit Rev Oncol Hematol 95(1):12-25.