Université de Montréal

Study of *Rgmc* regulation by iron levels, anemia, inflammation and hypoxia

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Ce mémoire intitulé

Study of *Rgmc* regulation by iron levels, anemia, inflammation and hypoxia

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Résumé

La surcharge génétique en fer, ou l'hémochromatose, peut être causé par des mutations au niveau du gène HFE, hémojuveline (HJV) et hepcidine. Des patients portant des mutations HFE ou HJV ont de faibles niveaux d'hepcidine, indiquant que ces protéines sont impliquées dans la régulation des niveaux basals d'hepcidine.

OBJECTIFS : Nous tentons d'élucider les conditions dans lesquelles l'orthologue de HJV chez la souris, soit la molécule Rgmc (repulsive guidance molecule c) pourraient influencer le métabolisme du fer. Nous investiguerons la réponse de Rgmc aux divers modificateurs du métabolisme du fer : niveaux de fer, anémie, hypoxie et inflammation.

RÉSULTATS : Nous avons trouvé que Rgmc, chez la souris, a un patron d'expression similaire à celui retrouvé chez l'humain étant exprimé dans le muscle squelettique, le coeur et les hepatocytes au niveau du foie. Ceci indique que le contrôle de la fonction de Rgmc, dans cet organe, est probablement médié par les hépatocytes. De plus, nous avons trouvé que l'expression hépatique de l'ARNm de Rgmc est régulée par l'inflammation et l'hypoxie, mais non par les niveaux de fer, la perturbation d'HFE ou l'anémie. Finalement, nous avons étudié les voies de régulation de Rgmc en réponse à l'inflammation et nous avons démontré que Rgmc se retrouve dans la voie de Tlr4, ceci étant similaire à ce qui a été trouvé pour hepcidine. Cependant, les voies de régulation de Rgmc et hepcidine dévient et alors que Il-6 régule hepcidine, nous avons trouvé que Rgmc est régulé par Tnf-α.

CONCLUSION : La regulation de l'ARNm de Rgmc en réponse à l'hypoxie et l'inflammation induite par le LPS pourrait contribuer au contrôle de l'homéostasie du fer dans ces conditions. Des voies signalitiques differéntes régulent Rgmc et hepcidine en réponse au LPS suivant l'activation de Tlr4.

SIGNIFICATION: Ces résultats nous donne une idée considérable des voies régulatrices ayant un impact sur les changements du métabolisme du fer. Ces changements pourrait être pertinent pour expliquer l'origine de plusieurs pathologies affectant l'homéostasie du fer.

Mots Clés

Inflammation, fer, LPS, Tlr4,Tnf-α, Il-6, Rgmc, hepcidine, hémojuveline, anémie des maladies chroniques, hémochromatose héréditaire, hémochromatose juvénile.

Abstract

Genetic iron overload, or hemochromatosis, can be caused by mutations in *HFE*, *hemojuvelin* (*HJV*) and *hepcidin* genes. Patients with mutations either on HFE or HJV have low hepcidin levels, indicating that these proteins are implicated in the regulation of *hepcidin* basal levels.

OBJECTIVES: We intend to elucidate in which conditions the mouse ortholog of HJV, the repulsive guidance molecule c (Rgmc) may influence iron metabolism by investigating its response to the modifiers of iron homeostasis: iron levels, anemia, hypoxia and inflammation.

RESULTS: We found that mouse *Rgmc* has the same pattern of expression as found in humans, being expressed in skeletal muscle, heart and liver, where it was expressed in hepatocytes. Additionally, we found that hepatic *Rgmc* mRNA expression was regulated by systemic inflammation and hypoxia, but not by iron levels, disruption of Hfe or β2m, or anemia. Finally, we studied the *Rgmc* regulatory pathway in response to inflammation and found that *Rgmc* is on the Tlr4 pathway, similar to what is found for hepcidin. However, *Rgmc* and *hepcidin* regulatory pathways deviate and while Il-6 regulates *hepcidin*, we found that *Rgmc* is regulated by Tnf-α.

CONCLUSIONS: *Rgmc* mRNA regulation in response to hypoxia and LPS-induced inflammation may contribute to the control of iron homeostasis in these conditions. *Hepcidin* and *Rgmc* are regulated by different pathways in response to LPS after the common element of Tlr4.

SIGNIFICANCE: These findings provide important insights into the regulatory pathways impacting iron metabolism change, which may be relevant to disease settings affecting iron homeostasis.

Keywords

Inflammation, iron, LPS, Tlr4, Tnf-α, Il-6, Rgmc, hepcidin, hemojuvelin, anemia of chronic disease, hereditary hemochromatosis, juvenile hemochromatosis

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Abbreviations

ACD – Anemia of chronic disease

ATP – Adenosine triphosphate

DCYTB – Duodenal cytochrome b

DMT – Divalent metal transporter

EPO – Erythropoietin

FBS – Fetal bovine serum

FP1 – Ferroportin 1

FTH – Ferritin heavy polypeptide

FTL – Ferritin light polypeptide

GPI – Glycosyl phosphatidylinositol

Hb – Hemoglobin

HCP – Heme carrier protein

HCT – Hematocrit

HH – Hereditary hemochromatosis

HIF – Hypoxia inducible factor

HJV – Hemojuvelin

HO – Heme oxygenase

IL – Interleukin

IRE – Iron responsive element

IRP – Iron regulatory protein

LPS – Lipopolysaccharide

MCV – Mean corpuscular volume

MHC – Major histocompatibility complex

mRNA – Messenger ribonucleic acid

NF-κB – Nuclear factor kappa B

NK – Natural killer

NO – Nitric oxide

NTBI – Non-transferrin bound iron

OMIM – Online endelian inheritance in man

PCR – Polymerase chain reaction

PHL – Phlebotomy

PHZ – Phenylhydrazine

PS – Penicillin-Streptavidin

qRT-PCR – Quantitative real-time PCR

RBC – Red blood cell

RGM – Repulsive guidance molecule

RT-PCR – Reverse transcription-polymerase chain reaction

Tf – Transferrin

TfR – Transferrin receptor

TLR – Toll-like receptor

TNF – Tumor necrosis factor

Note: Gene and protein symbols were written in accordance to international nomenclature conventions (1-3). Briefly, human gene and protein symbols are written in capitals and mouse equivalents are written with the first letter capital and remaining letters in underscore; genes are written in italic while proteins are normal case (*e.g.* RGMa refers to a human protein whereas *Rgma* refers to a mouse gene). Some exceptions exist (e.g. DMT1, which is always written in capitals).

INTRODUCTION

Chemical properties and biological functions of iron

Iron is involved in a wide array of metabolic functions in all organisms and is thus essential for normal cell growth and proliferation. This is namely due to its capacity to form a variety of coordination complexes with organic ligands in a dynamic and flexible mode which, along with its ability to switch between the ferrous (Fe^{2+}) and ferric (Fe^{3+}) states (of +772 mV at neutral pH (4)), allowed for the evolution of several, functionally diverse, iron proteins.

Many of the iron proteins accommodate iron in the form of heme and are generally designated as hemoproteins. The most abundant of the hemoproteins are the oxygen binding proteins hemoglobin and myoglobin, present in great quantities in red blood cells (RBCs) and muscle, respectively (5). Other hemoproteins include cytochromes and enzymes, such as oxygenases, peroxidases, or nitric oxide synthases (4).

Most non-heme iron proteins contain iron-sulfur clusters (6) and play diverse roles ranging from electron transfer, transcriptional regulation and structural stabilization to catalysis (4).

The iron feature of which the above proteins make use, i.e. the capacity of iron to easily participate in oxidation-reduction reactions in conditions compatible with the constrains of the cellular environment, also account for the spontaneous reaction by which iron catalyses the formation of hydroxyl radicals ($^{\bullet}OH$) from hydrogen peroxide (H_2O_2), a process known as Fenton chemistry (7) (figure 1). $^{\bullet}OH$ readily reacts with DNA, proteins and lipids inducing mutations and cellular stress (8).

The reactive essential for the Fenton reaction, H_2O_2, originates as a byproduct of enzymatic reactions or from the detoxification of products such as superoxide ($O_2^{\bullet-}$) by superoxide dismutases and is usually detoxified by catalase or glutathione peroxidase to H_2O (9). H_2O_2 reacts with Fe^{2+}, which is weakly chelated by a variety of ligands such as citrate, phosphate, carbohydrates, carboxylates, nucleotides, nucleosides, polypeptides and phospholipids (10, 11). The impact of iron toxicity through Fenton chemistry is evidenced by the reduction of cellular damage by the use of iron chelators which prevents iron from reacting with H_2O_2 (8).

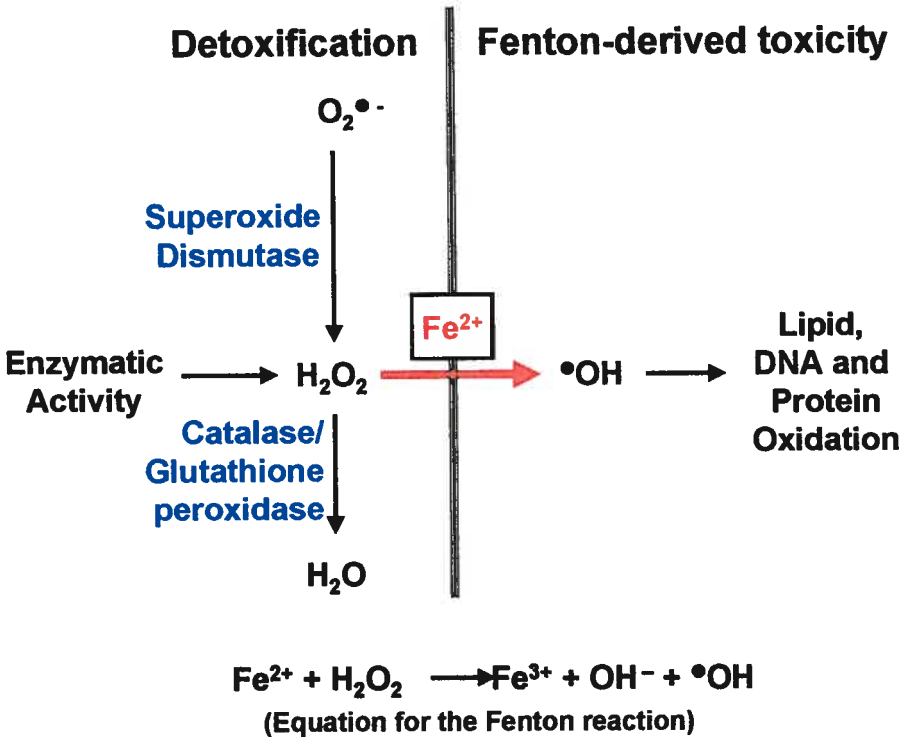

Figure 1 – Iron catalyzed generation of the hydroxyl radical via the Fenton reaction. H_2O_2 arising from the detoxification of superoxide by superoxide dismutase or from other enzymatic activities may be detoxified by catalase or glutathione peroxidase to H_2O or participate in the generation of hydroxyl radicals, a reaction catalyzed by iron as may be seen in the equation for the Fenton reaction.

The duality of iron as an essential micronutrient and as a cellular toxic imposed the evolution of highly regulated systems for the cellular iron uptake and storage in a non-toxic form, which will be discussed in the next section.

Cellular iron balance

Iron transport, uptake and storage

The main source of iron for mammalian cells is from transferrin (Tf), a 79 kD protein divided into two evolutionary related lobes, the N-lobe (336 amino acids) and C-lobe (343 amino acids), which are linked by a short spacer sequence (12). Each lobe contains two domains comprising a series of α-helices, which overlay a central β-sheet backbone (12). The domains interact to form a deep, hydrophilic iron-binding site which has a 'closed' form with one Fe^{3+} atom stabilized at neutral pH and an 'open' form when the pH is reduced to around 5.5 (5, 12).

Tf is synthesized mainly by hepatocytes (13) and is found in various body fluids including plasma, bile, amniotic, cerebrospinal, lymph and breast milk (14). It has an half-life of eight days and plasma concentration is relatively stable from birth, ranging from 2 to 3 g/L in humans (12).

From the 2-3g/L of Tf, that which has bound-iron (holo)-Tf has a higher affinity to the Tf receptors (TfRs) than non-iron-biding (apo)-Tf (12). There are two TfRs. TfR1 is expressed in RBCs, erythroid cells, hepatocytes, monocytes and the blood-brain barrier (12). TfR2 is expressed as two transcripts (α-TfR2 and β-TfR2), with α-TfR2

expressed predominantly on liver cells and β-TfR2 expressed at low levels on a variety of cell types (15).

Figure 2 schematizes the process of Tf-bound iron uptake. Upon interaction of Tf with TfR the Tf-TfR complex is internalized and adenosine triphosphate- (ATP) dependent proton pumps acidify the endosomes to pH 5.5, inducing Tf to release the bound iron (5). The recently identified ferrireductase Steap3 then reduces Fe^{3+} to Fe^{2+} (16), which is transported into the cytoplasm by the divalent metal transporter 1 (DMT1) (17). The apo-Tf-TfR complex remains stable until the endosome is recycled and transported back to the membrane, where apo-Tf liberates from the TfR returning to the extracellular environment (18-20).

It should be noted that not all iron enters the cells through the Tf/TfR system. A non-Tf bound iron (NTBI) uptake system also exists. NTBI is normally found in low levels in healthy individuals. However, in pathological conditions such as primary or secondary iron overload, NTBI levels are augmented.

To maintain cellular iron reserves in a non-toxic form, NTBI and TfR acquired iron is loaded into ferritin, an iron storage protein.

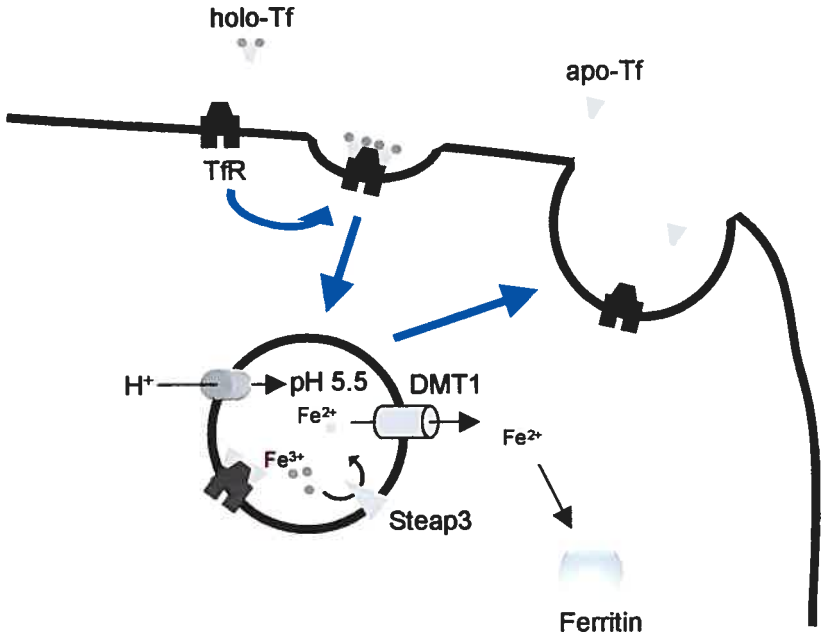

Figure 2 – Tf/TfR-mediated iron uptake. Holo-Transferrin (Tf) interacts with Tf receptor, inducing its endocytosis. A proton pump acidifies the endocytic vesicle to pH under 5.5, which induces the release of Fe^{3+} from Tf. Fe^{3+} is reduced by the ferrireductase Steap3 to Fe^{2+} and is transported to the cytoplasm by the divalent metal transporter 1 (DMT1). Iron may then be incorporated into ferritin for storage. The Tf receptor is then recycled back to the membrane and apo-Tf is released to the extracellular space.

Ferritin is a spherical shell composed of 24 units of the ferritin light (FTL) or heavy (FTH) polypeptides capable of storing around 4500 atoms of iron in a ferrihydrite core (21). The polypeptides are very similar in structure. However, they have been shown to have different physiological properties. FTH is capable of iron binding, and has been associated with ferroxidase activity, whereas the iron binding site in FTL has been replaced by a salt bridge (22). FTL is more stable than FTH and has been shown to promote the ferrihydrite nucleation (23). It is not clear yet how ferritin regulates iron incorporation and release or how each polypeptide participates in this regulation. Generally, ferritins with high FTL percentage are associated with stable ferrihydrite containment and are thus found in tissues associated with iron storage, such as the liver. FTH-rich ferritins are believed to be implicated in iron detoxification through ferrous iron oxidation (24) and are found in energy demanding tissues, such as the heart and brain.

Cellular iron balance is therefore maintained by equilibrium between iron uptake and storage. Although several factors may be implicated in this balance, cellular iron levels are central to the balance modulating the activity of iron regulatory proteins (IRPs).

Iron regulatory proteins and elements

Iron regulatory proteins (IRPs) are messenger ribonucleic acid (mRNA) binding proteins with high affinity for hairpin secondary structures known as "iron responsive elements" (IREs) (25). IREs are present on the untranslated regions of several genes coding for proteins implicated in iron metabolism, including the TfR and ferritin polypeptides (25). The nucleotidic sequence of the IREs is phylogenetically conserved in vertebrates and some insects and bacteria. A typical IRE is composed of around 30 nucleotides and form a 5'-CAGUGN-3' loop (the underlined C and G interact by hydrogen bonding) and a stem with moderate stability, interrupted by an unpaired C residue (25) (figure 3).

Two different IRPs bind IREs, IRP1 and IRP2 (25). Although structurally similar, the two IRPs are very differently regulated. IRP1 contains an iron-sulfur 4Fe-4S cluster that in low LIP levels loses one iron. This leads to a change in the conformation of the protein into the apo-IRP1 IRE-binding form, which has a 3Fe-4S cluster (26). IRP2, on the other hand, is regulated by iron-induced degradation and the amount of bound IRP2 to IREs directly correlates with the amount of protein (27). In summary, IREs will predominantly have IRPs bound (both IRP1 and IRP2) when cellular iron levels are low.

The IRE/IRP system therefore allows for an intracellular iron-sensing mechanism, relaying its signaling by the control of translation and mRNA degradation. The presence of IREs on the 5'UTR inhibits translation when IRPs are bound and IREs on the 3'UTR augment mRNA levels by blocking degradation by RNases.

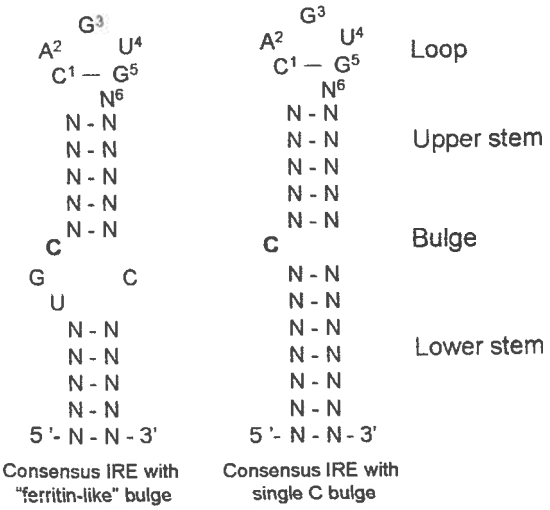

Figure 3 – Consensus IRE motif. Two forms of iron responsive elements (IREs) are shown. On the left is represented an IRE similar to the ferritin IRE, with a UGC on the 5' end of the hairpin, with a single C on the 3' end of the bulge. On the right we see an IRE with a bulge with a single C at 5' end of the hairpin, as found on TfR IREs.

A classical example of gene control by IRPs pertains to the iron storage and uptake equilibrium. FTL and FTH have a 5' IRE and TfR1 has five 3' IREs (25). When LIP levels are low TfR1 mRNA will be protected from degradation and translation of the ferritin polypeptides will be inhibited. Conversely, high LIP levels will release IRPs from the IREs, TfR1 mRNA will be degraded and ferritin translation will be initiated.

As a result, high cellular iron levels elevate ferritin levels, providing a safe storage place for iron and diminish TfR levels, reducing iron uptake. The inverse will be found in conditions of low LIP levels (see figure 4).

The IRE/IRP system allows for the control of intracellular iron levels. However, in mammals, cellular iron levels depend not only on the regulation of iron uptake at the cell membrane, but also on the availability of iron in the body. Mechanisms for systemic iron regulation are therefore required.

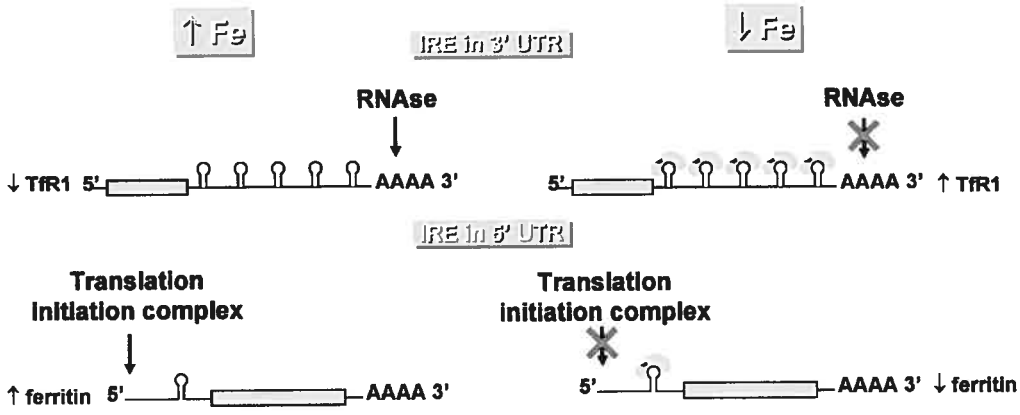

Figure 4 – Responses to iron supply mediated by IRE-IRP interactions. Iron regulatory proteins (IRPs) bind to iron responsive elements (IREs) in low iron conditions and leave IREs free in the presence of iron. IRPs bound to IREs on the 3' UTR (as found in the Tf receptor 1 (TfR1) mRNA transcript), will block mRNA degradation by RNases. IRPs bound to 5' IREs (as found in ferritin) inhibit protein synthesis by perturbing the binding of the translation initiation complex.

The systemic iron recycling

In mammals the major amount of iron is heme-bound, of which iron found in hemoglobin is the most common. It is therefore not surprising that most of the iron in humans is found in erythrocytes (5). From the 3-5 g of iron present in the human body, approximately 1.8 g is found in hemoglobin in the erythrocytes, i.e. roughly 40% of the total body iron. Erythrocytes typically have a life span of 120 days (28). To compensate for senescent erythrocytes, over 200 billion new erythrocytes are produced daily (29), making erythropoiesis the major iron-demanding process in the body.

To provide for erythropoiesis demand, iron is recycled. Macrophage phagocyte senescent RBCs and heme oxygenase (HO) releases the iron from heme. The free iron may then be exported from the macrophages onto the bloodstream to be bound to Tf (29). Subsequently the bone marrow uptakes the Tf-bound iron to fulfill its daily requirements (figure 5).

Proportionally, only a very slight amount of iron is taken up by the remaining cells for physiological use, of which muscle cells are the major iron containing cells, in the form of myoglobin (17). The sole exception is that of the liver. Hepatocytes have very high amounts of ferritin-associated iron and the liver is therefore regarded as an iron

storage compartment, supplying iron in times of dietary iron deficiency (17).

Since most of the body iron is recycled, the requirement of dietary iron is usually due to iron losses. These include iron lost through desquamation of gastrointestinal cells, bleeding and other minor losses (17).

In summary, cells may be regarded as acceptors or donors. Virtually all the cells in the body are iron acceptors, with erythropoietic precursor cells and hepatocytes as the main iron-demanding cells. The cell types that are considered to be iron donors are 1) macrophages, after scavenging of iron from hemoglobin; 2) hepatocytes, in conditions of iron sparseness; and 3) enterocytes, responsible for absorbing iron in the duodenum (see figure 5 for a schematic representation).

The recycling of iron implies that dietary iron requirements are only needed to compensate for iron losses and to support body growth. Given the nonexistence of a controlled iron excretion system, body iron levels must be regulated at the level of iron absorption.

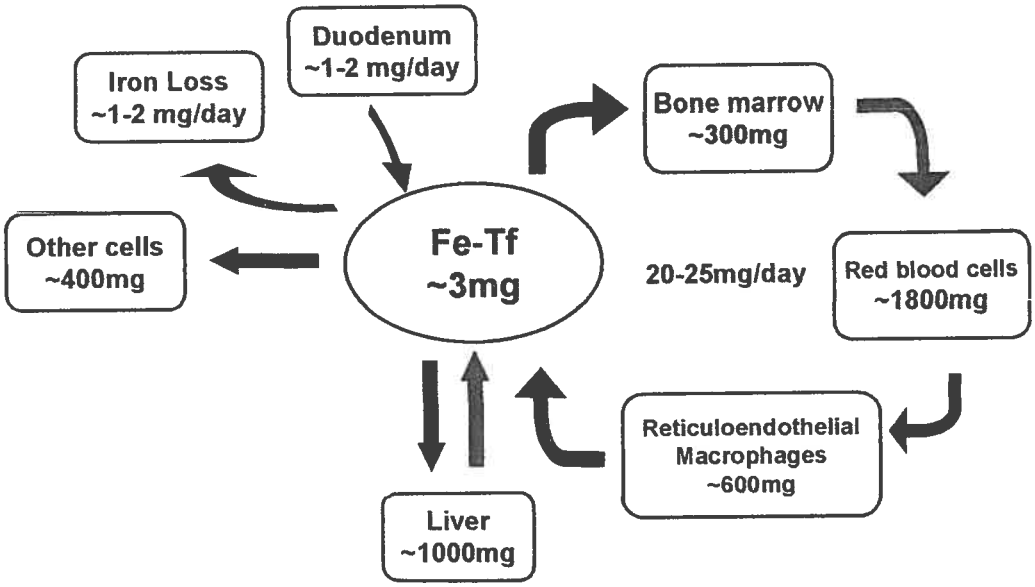

Figure 5 – Systemic iron re-cycling. Iron losses have to be compensated by iron absorption at the duodenum. Absorbed iron is released into the bloodstream where it is bound to Transferrin (Tf). Erythroid precursors uptake the Tf-iron to produce RBCs. Senescent RBCs are phagosized by the reticuloendothelial macrophages and the iron is recycled back into the bloodstream. Hepatocytes store excess iron and supply it when body iron levels are reduced.

Iron absorption

As discussed, normally only a small amount of iron is absorbed daily from the diet. A tight regulation of this absorption is required since excessive iron absorption can ultimately lead to iron overload.

Iron is absorbed mainly at the level of the duodenum, where it arrives in two forms from the diet: heme and non-heme iron. Non-heme iron is present as iron hydroxides and salts and in iron-containing proteins such as ferritin. Of the two forms, heme iron is the more bioavailable, with levels of absorption from a meal containing heme of about 25%.

Heme-bound iron is uptaken by the recently identified heme carrier protein 1 (HCP1) expressed in the apical region of epithelial cells in the duodenum (30), and is not affected by other dietary components, with the possible exception of calcium (31).

Non-heme iron is much less bioavailable, with absorption rates from the diet of 1-15% (32). The absorption efficiency varies with other components of the diet than may enhance or inhibit it. Examples of promoters of iron absorption are ascorbic acid and citric acid (33). Among inhibitors are components such as phytic acid, polyphenols (e.g. tannins) and calcium (32).

Promoters of iron absorption mainly have the effect of converting ferric iron to ferrous iron. In addition, Fe^{3+} can be reduced by the ferrireductase duodenal cytochrome b (Dcytb), present at the brush border membrane of duodenal enterocytes, enriching the amount of luminal Fe^{2+} (34). Fe^{2+} is then uptaken by enterocytes by DMT1 directly from the lumen, which efficiently transports Fe^{2+}, but not Fe^{3+}(35). Inhibitors of iron absorption typically work by chelating iron or compete with iron for DMT1 transport (e.g. calcium) (32, 35).

In the enterocyte iron may either be stored in ferritin or is exported by ferroportin 1 (Fp1) to the bloodstream to complete the process of iron absorption. Fp1 is most likely the only iron exporter in mammals, since *Fp1* knockout mice are embryonic lethal (36). After transport of the Fe^{2+} across the enterocytic membrane by Fp1, the ferroxidase hephaestin oxidizes iron to Fe^{3+}, which can then bind to Tf (37).

The scheme in figure 6 illustrates the mechanisms involved in iron absorption.

The observation that in mice, *Fp1* disruption in the duodenum abrogates iron export from the enterocytes suggests that iron absorption is mainly controlled by Fp1 regulation (36). Recently it has been shown that Fp1 can be regulated both at transcriptional and post-translational levels by a newly identified hormone called hepcidin (38).

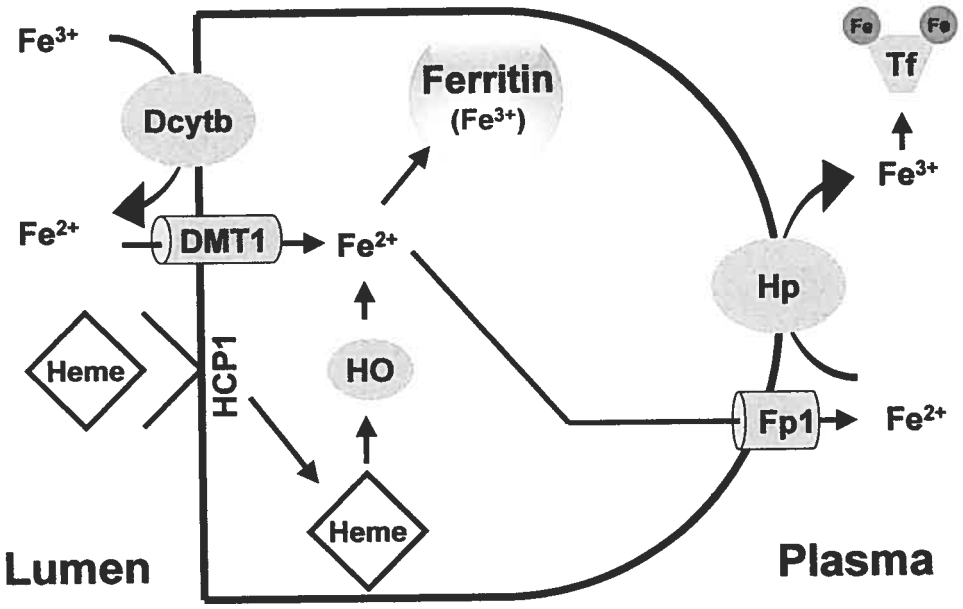

Figure 6 – Intestinal iron absorption. Iron in the diet may be oxidized (Fe^{3+}), reduced (Fe^{2+}) or bound to heme. Fe^{3+} must be reduced by the duodenal cytochrome b (Dcytb) to be transported across the plasma membrane by the divalent metal transporter 1 (DMT1) into the enterocyte. Heme is uptaken by a specific carrier, the heme carrier protein 1 (HCP1) and its iron is scavenged by heme oxygenase (HO). Iron is then incorporated into ferritin for storage (the stored iron is eventually lost by desquamation of the epithelial cells) or exported out of the enterocyte by ferroportin 1 (Fp1), oxidized by hephaestin (Hp) and bound to Tf for transport in the blood.

Hepcidin

Hepcidin was recently discovered independently by three groups as a mouse peptide expressed in response to iron levels and lipopolysaccharide (LPS) (39) and as a human antimicrobial peptide of the β-defensin family found in urine (40) and blood – where it was designated LEAP-1 (liver expressed antimicrobial peptide) (34). In all reports the authors show that the peptide is highly expressed in the liver.

Mice deficient for hepcidin are iron overloaded (41) while conversely, transgenic mice overexpressing hepcidin are severely anemic (42) further stressing the important role of hepcidin in iron metabolism.

A clue onto the mechanism of action for hepcidin comes from the observation that hepcidin interacts with the iron exporter Fp1 inducing its internalization and degradation (38), and reducing *Fp1* mRNA expression (43). Lower protein Fp1 levels would ultimately lead to reduced amounts of iron being exported from the enterocytes into the circulation, therefore reducing iron absorption.

Collectively, these observations strongly support the notion that hepcidin is a negative regulator of iron absorption. In fact, *hepcidin* mRNA levels can be regulated by iron, anemia, inflammation and hypoxia (44), four pathways regulating iron homeostasis.

Regulation of iron homeostasis

Iron absorption is adjusted depending on the amount of stored iron, the erythropoietic demand, oxygen levels and inflammation (43). It was initially hypothesized that several different regulators were implicated in the regulation of iron absorption in those conditions. The identification of hepcidin as a negative regulator of iron absorption, and the observation that its expression is regulated in all the above conditions (44) suggests that hepcidin is the common regulator in all conditions. Figure 7 schematizes the hypothesized pathways regulating hepcidin in response to iron levels, erythropoietic demand, oxygen levels, and immune system activation.

Regulation of iron homeostasis by iron stores

It is logical that the iron stores, or iron levels, should be one of the factors regulating iron absorption, and therefore hepcidin, in order to avoid iron overload or deficiency.

The liver serves as an iron storage organ by uptaking excess iron and later distributing it when body iron levels are low. Due to this function of iron storing and since the liver is the main site of hepcidin production, body iron stores sensing is believed to take place in this organ.

Serum ferritin, Tf and soluble TfR have all been proposed as candidate signaling molecules for the regulation of hepcidin in response to iron levels (45-47), however which molecule, if not all mentioned, and the pathways leading to hepcidin regulation have still to be elucidated.

Regulation of iron homeostasis by erythropoietic demand

Erythropoietin (EPO), which regulates RBC production is a glycoprotein mainly produced in the kidneys, but also synthesized in the liver and to a lesser extent in the spleen, lung, testis and brain (48). EPO inhibits the apoptosis of erythrocytic progenitors in the bone marrow (49), allowing for an increased number of matured hematopoietic cells in the presence of high levels of EPO.

Tissue hypoxia is the main stimulus of EPO production and its levels exponentially increase with decreasing blood hemoglobin (Hb) concentration (50). *EPO* gene expression is not only stimulated when the O_2 capacity of the blood decreases (usually correlating with the Hb concentration), but also when the arterial pO_2 decreases (as found in high altitudes) (51).

There are several regulatory DNA sequences in the neighborhood of the *EPO* gene, but the key element is located within the so-called hypoxia

response element, which is bound by the hypoxia-inducible transcription factors (HIFs). HIFs are dimers composed of one α and one β subunit. There are at least three subtypes of the HIF-α subunit (1α, 2α and 3α). HIF-1α/β is generally considered the primary mediator of hypoxia-induced gene expression (48). HIF-1α is regulated by oxygen-dependent degradation. Only in hypoxia is HIF-1α enabled to enter the nucleus and to heterodimerize with HIF-1β, inducing HIF-1-regulated genes, such as *EPO*.

In summary, erythropoietic demand, regulated by EPO, arises in two main situations: decreased Hb levels as found in anemia, or decreased pO_2 levels, as found in hypoxia due to environmental low oxygen levels, such as found in high altitudes. The first is described below and the second is described in following section.

Anemia

Anemia is defined as a deficiency in hemoglobin and/or RBC levels linked to poor tissue oxygenation. Patients commonly report a feeling of weakness and fatigue and in cases of severe anemia, shortness of breath. Etiologically anemia is caused by 1) decreased RBC production; 2) increased RBC destruction and/or 3) blood loss.

Decreased RBC production may arise, for instance, due to low body iron levels. The condition termed iron deficiency anemia arises when

erythroid progenitors have insufficient hemoglobin synthesis due to the low iron availability. RBCs produced in conditions of iron deficiency typically have a smaller volume, as measured by the mean corpuscular volume (MCV), and is therefore morphologically classified as a microcytic anemia (52).

Another form of anemia that is associated with impaired iron distribution to erythroid precursors is the anemia of chronic disease (ACD). However, in contrast to iron deficiency anemia, patients with ACD do not have decreased body iron levels. In addition, in contrast to iron deficiency anemia, the pathogenesis of ACD is not only associated with impaired iron distribution to erythroid precursors, but also with increased RBC destruction and impaired EPO production.

ACD may arise in individuals with chronic infections and cancer. The underlying pathogenesis induce secretion of high levels of pro-inflammatory cytokines (53), which contribute to the development of the anemia by inhibiting EPO production (54), increasing the rate of RBC phagocytosis by macrophages (55) and inhibiting the release of iron from hepatocytes and macrophages through the upregulation of hepcidin (56). The hepcidin-mediated decrease in iron release to the serum is believed to be the main causative factor of the underlying chronic hypoferremia in ACD, which leads to deficient iron delivery to the erythroid precursors.

Regulation of iron homeostasis by Hypoxia

Subjecting mice to hypoxia by housing in a hypobaric chamber results in *hepcidin* downregulation, as found in anemia and consistent with the iron demand for erythropoiesis (44), and hepatocytes cultured *in vitro* subjected to hypoxia decrease *hepcidin* expression (44).

However, other stimuli seem to be implicated in the increased iron absorption in response to hypoxia. An increase in erythropoietic demand induced by EPO is known to decrease hepcidin levels (57), but seems to produce little or no increase in iron absorption (58, 59). The increase in iron absorption observed in hypoxia, although it is likely regulated by hepcidin to a certain level, seems to require other factors, such as Il-6, since administration of Il-6 antibody to hypoxic mice halves the hypoxic response of iron absorption (60). These additional factors support for an hypoxia pathway separate from the erythropoietic pathway (61).

Regulation of iron homeostasis by the immune system

Iron is important in several aspects of immunosurveillance because of its growth-promoting role for immune cells and its interference with cell-mediated immune effector pathways and cytokine activities (62-64).

Iron homeostasis and the adaptive immune system

It has been demonstrated that iron deficiency as well as iron overload can exert subtle effects on immune status by altering the proliferation and activation of T-, B- or natural killer (NK)-cells (53). Conversely, indirect evidence (Hfe, a nonclassical major histocompatibility complex class I that has been implicated in iron sensing is directly recognized by cytolytic CD8+ T cells (65); higher iron levels are found in Hfe-/- recombinase-activating gene (Rag)1-/- double knockouts than in either of the single knockouts (66); and low CD8+ peripheral T cell numbers increases the risk of severe hemochromatosis (67)) suggests that the immune system could participate in iron homeostasis.

Iron homeostasis and the innate immune system

Pathogens require iron for growth and replication. Since the amount of iron that is not protein-bound in serum and tissues is diminute (68), bacteria have evolved several mechanisms for seizure and uptake of protein-bound iron, ranging from production of iron sequestering molecules (siderophores), to importers of Tf and lactoferrin (69).

Conversely, the host has developed mechanisms to hinder iron availability for bacteria. One of these mechanisms is the reduction of seric iron levels (hypoferremia) (70).

Hepcidin expression is increased in response to inflammation by Il-6 and Il-1β (71, 72) and, since it is has been shown that hepcidin inhibits Fp1 (38), the sole iron exporter in mammals, and it is capable of inducing hypoferremia, it has been hypothesized that the development of hypoferremia is dependent on *hepcidin* upregulation (73).

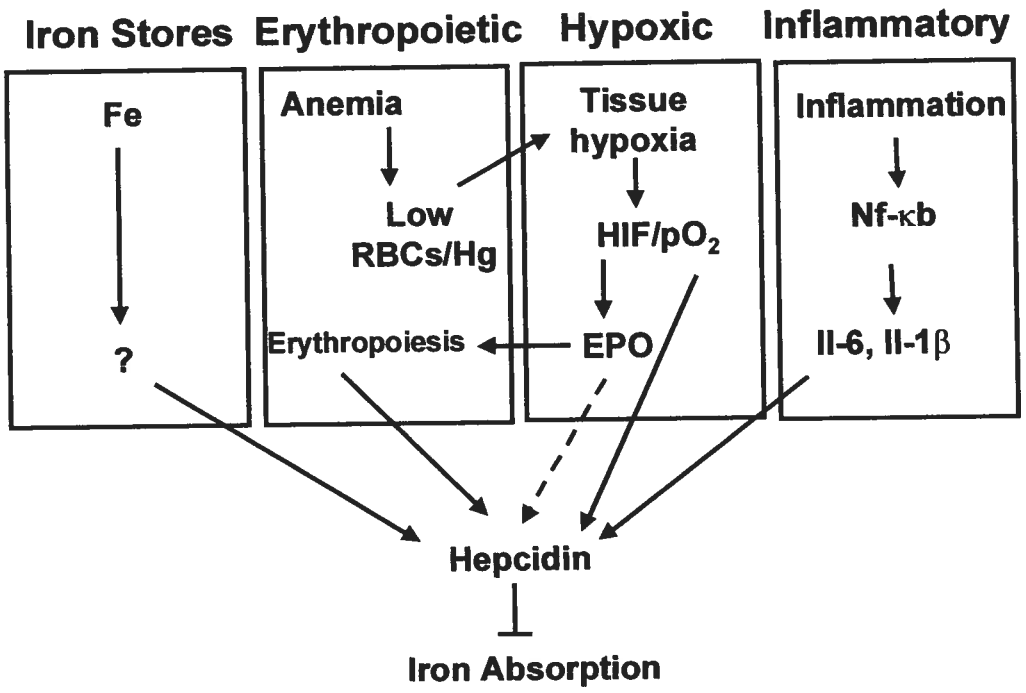

Figure 7 – Pathways for hepcidin regulation. Signaling of iron stores level leads to the regulation of *hepcidin* levels. The erythropoietic and hypoxic pathways are intertwined since low red blood cells (RBCs) or low hemoglobin levels (Hb) in anemia induce tissue hypoxia, activating HIFs and augmenting EPO production. Il-6 and Il-1β are produced in response to inflammation and these regulate *hepcidin.* Dashed lines – hypothetical pathways.

Hereditary Hemochromatosis

Hereditary hemochromatosis (HH) is an iron overloading disease caused by mutations in Hfe (type I), hepcidin or hemojuvelin (type II), transferrin receptor 2 (type III) and ferroportin 1 (type IV). In this study we will give emphasis to type I and II HH.

Hereditary hemochromatosis - Type I

Type I hemochromatosis is an autosomal recessive disorder of iron metabolism (online mendelian inheritance in man (OMIM) 235200). Patients absorb excessive amounts of dietary iron, which leads to iron overload. This excess iron accumulates mainly in the liver, pancreas and heart.

If left untreated, patients may develop iron-induced complications such as cirrhosis, diabetes or cardiomyopathy typically occurring around the 5^{th} decade of life.

Treatment usually consists of bleeding (phlebotomy) and patients rarely develop significant clinical symptomatology, having a life expectancy similar to healthy individuals (74).

Mutations on *HFE*, a nonclassical major histocompatibility complex class 1 (MHC-I) molecule is the causative factor of type I HH.

The HFE ancestral peptide binding groove is too narrow to allow classic antigen presentation (75) and the function of HFE has not yet been established. However, the observation that HH type I patients and mice deficient in Hfe have low basal hepcidin levels (76) indicates that Hfe plays a role in the setting of adequate basal hepcidin expression levels. The low basal hepcidin levels are consistent with the excessive iron absorption found in the patients, which leads to tissue iron overload.

Two main mechanisms of action of HFE that may impact on hepcidin expression levels have been proposed: the inhibition by HFE of the Tf/TfR iron import system, and the free HFE signaling to extra- or intra-cellular partners.

Inhibition of Tf/TfR import

HFE interacts with TfR (75) and competes with differric Tf for TfR binding (figure 8A), suggesting that some of the effects of HFE on iron homeostasis result from the inhibition of Tf-mediated iron import by HFE (77, 78) (79) (figure 8B).

Free HFE signaling

Accumulating evidence suggests that the effect of HFE on iron homeostasis may not be solely due to an inhibition of the Tf-mediated iron import. Evidence supporting this hypothesis includes the facts that 1) Tf is capable of effectively compete with HFE for TfR binding even at

concentrations of Tf well below those found in the blood (80), and therefore HFE would have very limited capacity to block Tf-mediated iron uptake; 2) the W81A HFE mutant, which has 5000-fold lower affinity for TfR1 binding than wildtype HFE (81), regulates Tf-mediated iron uptake to the same extent as wildtype HFE *in vitro* (82) and; 3) HFE lowers intracellular iron levels even in cells that lack TfR1, providing evidence for another mechanism of intracellular iron level regulation, probably through control of NTBI iron uptake (80) (figure 8C).

In addition, the recent observation that T cells directly recognize HFE (83) indicates that HFE may have a role as a signaling molecule for the adaptive immune system, which has the capacity to regulate iron homeostasis through the release of cytokines (figure 8C) .

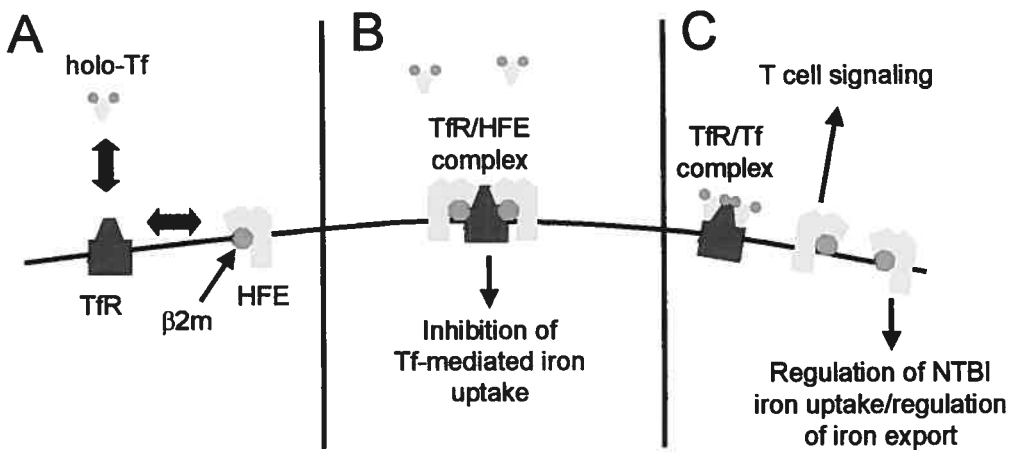

Figure 8 – Mechanistic models for HFE function. (A) HFE competes with Tf for transferrin receptor (TfR) binding. It is believed that this competition results in (B) an inhibition of Tf-mediated iron uptake by HFE or (C), the release of HFE from TfR, allowing T cell recognition of HFE or intracellular signaling leading to the regulation of NTBI iron uptake or iron export.

Hereditary hemochromatosis - Type II

Type II HH is characterized by an earlier onset than type I HH and was therefore also designated Juvenile Hemochromatosis (JH). It is associated with a high lethality rate caused mainly by cardiomyopathy by the 3rd decade of life (84). JH has been found to have two causative factors: loss of function mutations in hepcidin (OMIM 606464) (85) and hemojuvelin (HJV) (OMIM 608374) (86).

Interestingly, JH patients with mutations in *HJV* have very low hepcidin levels (87). The low hepcidin levels in classical HH and JH provide for a common mechanism of pathology and implicate HFE and HJV in hepcidin regulation, however, the pathway leading to this regulation still has to be discerned.

HJV is a soluble or glycosyl phosphatidylinositol (GPI)-anchored protein of unknown function that has an Arg-Gly-Asp (RGD) domain and a partial von Willebrand factor type D domain and shows high similarity with human repulsive guidance molecules (RGM) a and b, two proteins involved in axonal guidance (88). Due to this high similarity, the mouse ortholog of HJV is termed Rgmc (see figure 9 for an alignment of HJV and Rgmc proteins). However, Rgmc, in contrast to Rgma and Rgmb, is not expressed in the nervous system and does not seem to be implicated in axonal guidance (89).

HJV has been shown to be expressed in the heart, skeletal muscle, liver, and fetal liver and absent from brain, colon, thymus, spleen, kidney, duodenum, placenta, lung, lymph, lymphocytes and bone marrow (86).

It is not clear how HJV functions. As mentioned before, loss of function mutations leads to very low hepcidin levels, which seems to be the causative factor for the iron loading, but it is not known how HJV is implicated in hepcidin regulation.

This thesis centers on the control of *Rgmc* expression in mouse models of altered iron homeostasis to elucidate in which conditions Rgmc may influence iron metabolism.

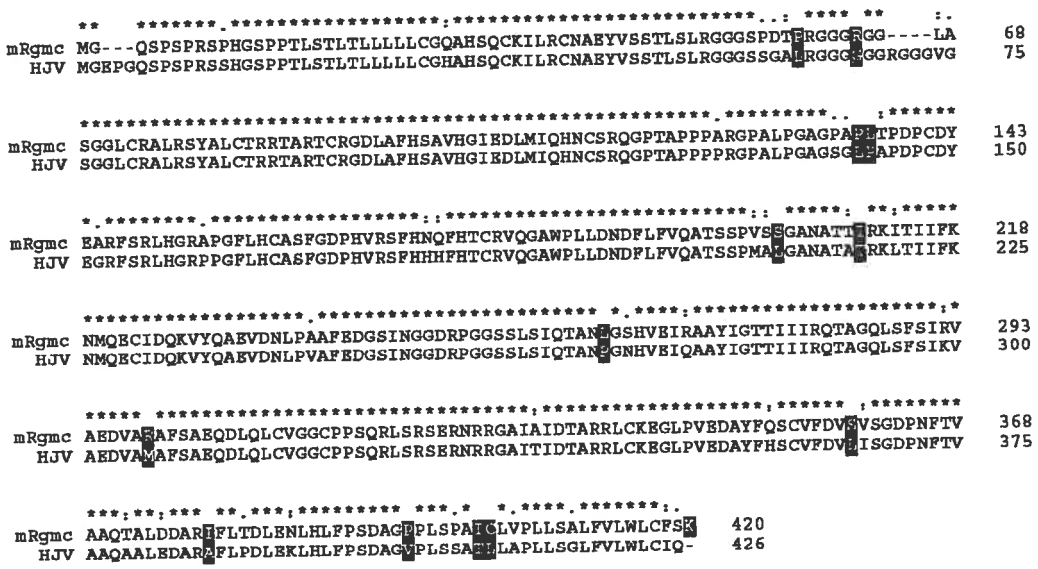

Figure 9 – Hemojuvelin and the mouse ortholog Rgmc protein alignment. ClustalX was used for the alignment. Low scoring aminoacids are marked with black background.

Objectives and specific aims

Objective:

We intend to elucidate in which conditions HJV/Rgmc may influence iron metabolism by investigating its pattern of expression in response to the modifiers of iron homeostasis: iron levels, anemia, hypoxia and inflammation.

Aim 1 – Characterization of *Rgmc* tissue and cell mRNA expression by quantitative real-time polymerase chain reaction.

In humans, the *Rgmc* ortholog, *HJV*, has been shown to be expressed in the heart, skeletal muscle, liver, and fetal liver and absent from brain, colon, thymus, spleen, kidney, duodenum, placenta, lung, lymph, lymphocytes and bone marrow (86). To elucidate whether Rgmc has a similar pattern of expression in mice, we assessed *Rgmc* mRNA expression by quantitative real-time polymerase chain reaction (qRT-PCR) in heart, skeletal muscle, liver, brain, thymus, spleen, kidney, duodenum, lung and bone marrow. We also investigated whether *Rgmc* is expressed in hepatocytes or liver mononuclear cells.

Aim 2 – Profiling of *Rgmc* liver mRNA expression in mouse models of altered iron metabolism.

We decided to study *Rgmc* mRNA expression at the level of the liver, given the importance of this organ on iron homeostasis and as the main site of synthesis of hepcidin. Expression levels were measured by qRT-PCR using mouse models for the four modifiers of iron homeostasis: iron levels, anemia, hypoxia and inflammation. To study *Rgmc* mRNA expression in response to iron levels, we used mouse models of nutritional iron loading and deficiency. Additionally we used the genetic iron overloaded mouse models of HH: *Hfe* and *β2m* knockout mice. For the analysis of the effect of erythropoiesis on iron homeostasis, we used the models of phenylhydrazine (PHZ)-induced hemolytic anemia and phlebotomy-derived anemia. To study the effect of increased erythropoiesis without anemia, as found in hypoxia, we subjected mice to 10% oxygen levels for 1, 3 and 5 days. Finally, we used LPS to modify iron homeostasis in response to inflammation.

Aim 3 – Study *Rgmc* regulatory pathway downstream of LPS.

We found that *Rgmc* is markedly downregulated in inflammation. We therefore performed a time-course analysis of *Rgmc* regulation and

studied the regulatory pathway in response to LPS treatment. We studied the capacity of Toll-like receptor (Tlr)4 deficient mice and *Hfe* and *β2m* knockout mice to regulate *Rgmc* mRNA expression in response LPS. In addition, we investigated the regulation of Rgmc in response to the inflammatory cytokines Il-6 and tumor necrosis factor (Tnf-)α *in vivo* and *in vitro*.

Materials and Methods

Animals

All animal manipulations were carried out under Canadian Council on Animal Care regulations and approved by the Animal Care Committee of the Centre Hospitalier de l'Universite de Montreal (CHUM). *Hfe*$^{-/-}$ mice have been previously described (90). *β2m*$^{-/-}$, *Il-6*$^{-/-}$, C3H/HeJ and C3H/OuJ were purchased from Jackson Laboratories (Ben Harbor, Maine, USA). Except for the C3H mouse strains, all animals were backcrossed over 10 generations with C57Bl/6. Animals were tended for in light- and temperature-controlled environment and were given free access to tap water and food – the commercial diet TD 2018 (Harlan Teklad, Madison, Wisconsin, USA) except otherwise stated.

Animal Treatments

For the inflammatory stimulus, 10 weeks-old mice were injected intra-peritoneally with 5 mg/Kg *E. coli* LPS serotype 055:B5 (Sigma-Aldrich, St. Louis, Missouri, USA) or 1 µg recombinant mouse Il-6 (3 hours before sacrifice) or Tnf-α (6 hours before sacrifice) (Cederlane Laboratories Ltd, Hornby, Ontario).

To analyze iron homeostasis in response to iron levels, 8 weeks-old mice were subjected to an iron-deficiency diet (TD 80396; Harlan Teklad) or the standard commercial diet supplemented with 2.5% (wt/wt) carbonyl iron (Sigma-Aldrich) for two weeks.

To induce anemia through phlebotomy (PHL), 0.25 ml of blood was extracted by retro-orbital puncture from anesthetized mice. The procedure was repeated 24 hours later, and the animals were sacrificed 16 hours after the last PHL.

Hemolytic anemia was produced by intra peritoneal administration of 40 mg/kg body weight of PHZ (Sigma-Aldrich), once daily for 4 days and the mice were sacrificed at day 5.

Hypoxia was elicited by housing of mice in a closed chamber with controlled injection of N_2 and O_2 and with outward ventilation for removal of excess CO_2 and humidity. Mice were subjected to a O_2 gradual reduction O_2 from 20.8% to 10% O_2 over 6 hours and were subsequently housed at 10% O_2 for 1, 3 or 5 days.

Primary hepatocytes isolation and cultures

Mice were anesthetized and the livers were perfused with HEPES buffer (Sigma-Aldrich) at 37 °C for 4 minutes at 10 ml/min followed by a

perfusion with HEPES buffer supplemented with 0,14 U/ml Collagenase D (Roche, Sainte-Foy, Quebec) at 37 °C for 7-8 minutes at 10 ml/min.

Dissociated cells were isolated by gentle rubbing of the liver with a rubber policeman. Hepatocytes were pelleted at 100 x *g* for 4 min at 4°C and washed twice with Williams E incomplete medium (Invitrogen). The washed hepatocytes were then resuspended in 25 ml of Williams incomplete media (Invitrogen) and layered onto a 50-ml conical tube containing 20 ml of Percoll (Sigma-Aldrich) (1 part 10x Hank's balanced salt solution and 9 parts Percoll). The hepatocytes were mixed with Percoll by inverting five times and pelleted by centrifugation at 300 x *g* for 10 min at 4°C.

Finally, hepatocytes were washed once and were plated at 2.5 x 10^4 per cm^2 onto type I collagen in Williams E medium with 10% fetal bovine serum (FBS) and 1% penicillin and streptavidin (PS). Two hours after plating, medium was changed and replaced with fresh medium. 20 hours after plating cells were treated with 20 ng/ml Il-6 or Tnf-α or 100 ng/ml LPS for 24 hours.

Liver mononuclear cells (LMCs) isolation

LMCs were separated by centrifugation using the lympholyte density medium (Cederlane, Hornby, ON, Canada), following the suppliers' instructions.

RNA quantification

Tissue samples were stored in RNALater (Ambion, Austin, Texas, USA) following the suppliers' instructions. Total RNA was subsequently extracted with TRIZOL reagent (Invitrogen). Reverse transcription (RT) was performed with the Omniscript RT Kit (Qiagen, Mississauga, Ontario) using random hexamers (Invitrogen) and RNase inhibitor (Invitrogen) in the concentrations suggested by the supplier.

mRNA levels of *Rgmc*, *β-actin* and *hepcidin* were measured by real-time PCR in a Rotor Gene 3000 Real Time DNA Detection System (Montreal Biotech Inc, Kirkland, Quebec) with the QuantiTect SYBR+Green I PCR kit (Qiagen). All primers were designed using the Primer3 algorithm (91) so that they would flank at least one intron. The following primers were used in this study: *β-actin* 5'-TGTTACCAACTGGGACGACA-3' and 5'-GGTGTTGAAGGTCTCAAA-3'; *Rgmc* 5'-AATTTCACACATGCCGTGTC-3' and TCAAAGGCTGCAGGAAGATT-

3'; *hepcidin* 5'-AGAGCTGCAGCCTTTGCAC-3' and 5'-GAAGATGCAGATGGGGAAGT-3'.

Relative quantitation was performed using standard curves constructed from serial dilutions of PCR products and were attributed arbitrary concentrations. All standard curves generated were found to have excellent PCR amplification efficiency (90%-96%; 100% indicates that after each cycle the amount of template is doubled), as determined by their slopes. mRNA expression for each gene was determined by direct comparison with the standard curve of the specific target generated in each PCR run. Expression levels of the genes of interest were normalized to the housekeeping gene *β-actin*.

Serum iron and transferrin saturation measurements

Serum iron and total iron binding capacity (TIBC), and transferrin saturation were assessed by colorimetric assay with the Kodak Ektachem DT60 system (Johnson & Johnson, Ortho Clinical Diagnostics, Mississauga, Ontario).

Measurements of tissue iron concentration

Tissue samples were dried by incubating at 106 °C overnight and weighed to determine the tissue dry weight. Tissues were then ashed by incubating at 500 °C for 17h and iron was dissolved in 6N HCl. Iron levels were quantified by atomic absorption spectroscopy at the Geochemistry laboratory of the École Polytechnique de l'Université de Montréal.

Statistical analysis

Student t test (unpaired, 2-tailed) was used for comparison between 2 groups. Multiple comparisons were statistically evaluated by one-way analysis of variance (ANOVA) followed by the Benferroni test.

Results

Aim 1 - Tissue Expression

Rgmc tissue and cell expression

The understanding of the role of Rgmc encompasses the study of its tissue expression. We investigated the pattern of mRNA expression of *Rgmc* by qRT-PCR and found it expressed in the liver, heart and skeletal muscle (figure 10A) and absent from spleen, duodenum, kidney, lung, thymus, brain and bone marrow. This is in accordance to what has been previously described for mouse (89) and the human ortholog *HJV* (86). Therefore, as in human *HJV* mRNA expression, mouse *Rgmc* mRNA expression is found in the organs that are most affected in JH.

Taking into account the importance of the liver in iron homeostasis, we concentrated our studies in this organ and evaluated mRNA production in hepatocytes and mononuclear cells, separated using differential centrifugation in order to understand in which cell population further *in vitro* experimentation on *Rgmc* regulation should be performed. We found *Rgmc* mRNA expression only in the hepatocyte fraction (figure 10B) indicating that Rgmc signaling is being mediated by the hepatocytes.

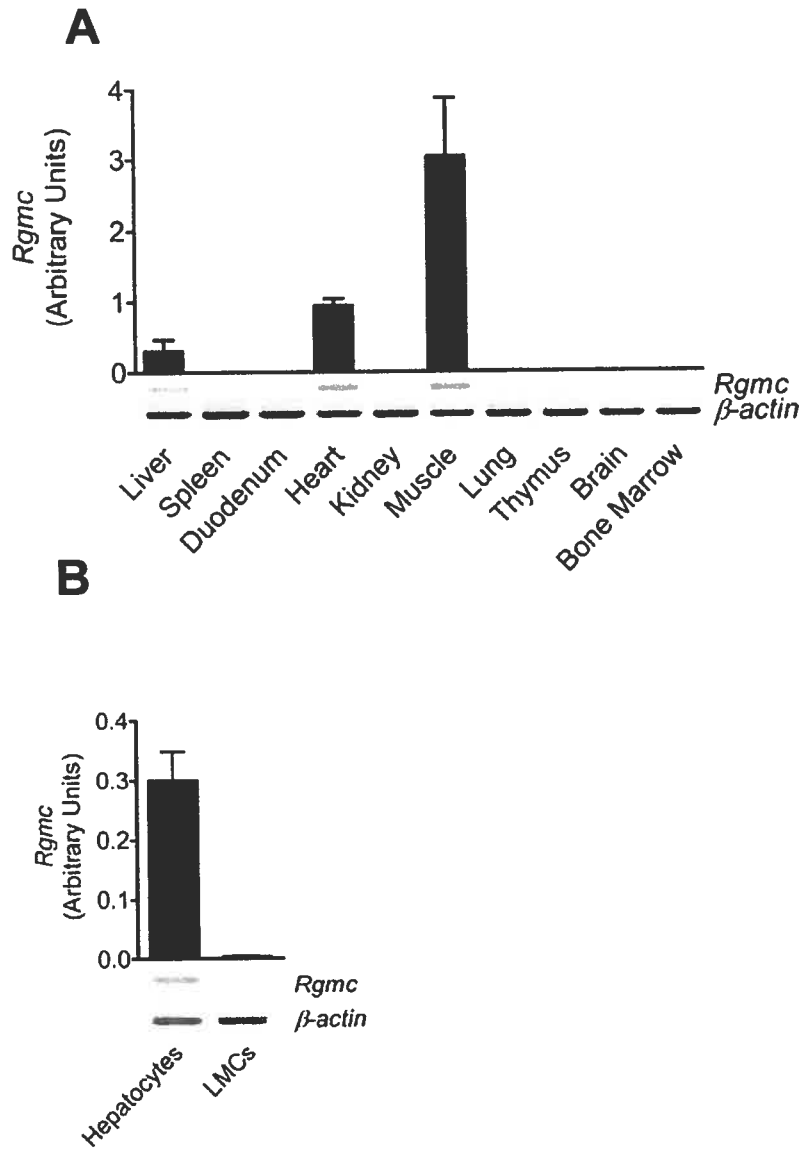

Figure 10 – Murine *Rgmc* tissue and cell expression. *Rgmc* mRNA expression was assessed using qRT-PCR (bargraphs) and classical RT-PCR (gel images) in several tissues (A), and in two liver cell fractions obtained by differential centrifugation, separating the hepatocytes from the remaining liver mononuclear cells (LMC) (B). Results are presented as means ± SEM (n=2-3).

Aim 2 – Rgmc response to modifiers of iron homeostasis

Iron Levels

To assess how iron levels regulate *Rgmc* we next quantified *Rgmc* mRNA levels in 10 weeks old C57Bl/6 mice subjected to standard, low, or high iron diets for two weeks.

We observed 2% decrease in Mean Corpuscular Volume (MCV) in the mice fed the low iron diet (table I), indicative that cell division in the bone marrow continues for longer than usual, resulting in smaller cells. However the mice did not developed anemia, as seen by analyzing RBC, Hb and HCT values. Mice fed with the high iron diet had slightly increased RBCs (7%), Hb (6%), HCT (9%) and MCV (4%).

Table I – Hematological indices of mice treated with a low, standard and high iron containing diets. Red blood cells (RBC), hemoglobin (Hb), hematocrit (HCT) and mean corpuscular volume (MCV) of 10 weeks old mice treated for two weeks with a diet deficient in iron (-Fe), a standard laboratory diet (Std) or a diet with additional 2.5% w/w carbonyl iron (+Fe). Results are presented as means ± SD (n=6 mice per group). Statistical analysis was performed by one way ANOVA followed by the Benferroni test. *p<0,05, **p<0,01, ***p<0,001 (compared to Std).

Group	RBC (10^6/ml)	Hb (g/dl)	HCT (%)	MCV (fL)
-Fe	9.5 ± 0.4	13.1 ± 0.5	42.4 ± 1.8	45 ± 0.8**
Std	9.2 ± 0.2	13.2 ± 0.2	42.6 ± 0.8	46 ± 0.4
+Fe	9.8 ± 0.2*	14.0 ± 0.3**	46.5 ± 0.9***	48 ± 0.5**

Mice fed with the low iron diet decreased body stores, as may be observed in the decrease of liver iron levels (43%) in comparison with mice fed a standard iron diet (table II), indicating that mice did indeed reduce the liver iron stores. However, this reduction in the liver iron stores did not influence circulating iron levels, since SI and TS parameters do not change, probably due to the release of liver stored iron into the circulation. Mice fed with the high iron diet became iron overloaded as seen by the over two-fold increase in circulating iron levels as well as liver iron levels, compared to controls (table II).

Rgmc mRNA levels, however, did not differ among the mice subjected to the different diets (figure 11A), indicating that *Rgmc* regulation at the

mRNA level is not implicated in the control of iron homeostasis in response to iron stores.

Table II – Iron measurements in mice treated with a low, standard and high iron containing diets. Serum iron (SI), transferrin saturation (TS) and liver iron levels were quantified in 10 weeks old mice treated for two weeks with a diet deficient in iron (-Fe), a standard laboratory diet (Std) or a diet with additional 2.5% w/w carbonyl iron (+Fe). Results are presented as means ± SD (n=6 mice per group). Statistical analysis was performed by one way ANOVA followed by the Benferroni test. ***p<0,001 (compared to Std).

Group	SI (μM)	TS (%)	Liver Iron (μg iron/g dry weight)
-Fe	21 ± 3	40 ± 2	124 ± 17***
Std	19 ± 2	46 ± 5	221 ± 20
+Fe	39 ± 4***	109 ± 15***	569 ± 177***

Hereditary hemochromatosis

Based on the observation that mice lacking a functional Hfe protein have lower than expected basal *hepcidin* levels (92), *hepcidin* has been proposed to be in the Hfe pathway. In continuation with the hypothesis that *hepcidin* is a downstream target of Rgmc, we investigated whether *Rgmc* levels in the primarily iron-overloaded *β2m* and *Hfe* knockout mice may explain the low basal *hepcidin* levels.

$\beta 2m^{-/-}$ and $Hfe^{-/-}$ mice have around 2-fold more liver and circulating iron than controls (table III), similar to mice fed with a high iron diet

compared with controls (table II). In spite of the high iron levels, these mouse models of HH displayed *Rgmc* levels akin to wildtype mice (figure 11B), suggesting that abnormal *Rgmc* regulation in this mice is not the causative factor for their low basal hepcidin levels in these strains.

Table III – Iron measurements in mice with genetic iron overloading. Serum iron (SI), transferrin saturation (TS) and liver iron levels were quantified in 10 weeks old wildtype (Wt), $\beta 2m^{-/-}$ or $Hfe^{-/-}$ mice. Results are presented as means ± SD (n=6 mice per group). Statistical analysis was performed by one way ANOVA followed by the Benferroni test. ***p<0,001 (compared to Wt).

Group	SI (μM)	TS (%)	Liver Iron (μg iron/g dry weight)
Wt	20 ± 3	41 ± 9	308 ± 40
Hfe$^{-/-}$	45 ± 3***	96 ± 8***	726 ± 148***
β2m$^{-/-}$	34 ± 6***	94 ± 21***	738 ± 227***

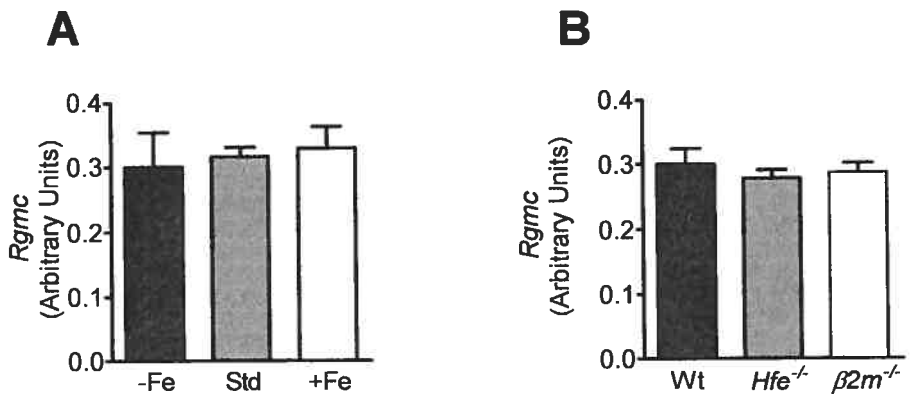

Figure 11 – Hepatic *Rgmc* mRNA expression in response to iron levels and in HH mouse models. *Rgmc* mRNA expression quantified by qRT-PCR and normalized to *β-actin* in (A) 10 weeks old mice treated for two weeks with a diet deficient in iron (■), a standard laboratory diet (▒) or a diet with additional 2.5% w/w carbonyl iron (□); (B) 10 weeks old wildtype (■), *Hfe*$^{-/-}$ (▒) or *β2m*$^{-/-}$ (□) mice. Results are presented as means ± SEM (n=6 per group). Statistical analysis was performed by one way ANOVA.

Hypoxia

To investigate whether *Rgmc* mRNA levels are regulated by hypoxia, we subjected 10 weeks-old mice to 10% oxygen levels for 1, 3 or 5 days. Mice subjected to hypoxia elevated RBCs by 15% by day 1 to a maximum of 25% increase by day 5 (figure 12A). HCT values were also elevated by day 1 to 15% and the maximum increase of 27% was attained by day 5 (figure 12B). This increase of RBCs and HCT values in response to hypoxia are in accordance to what has previously been described (93) and indicate that the mice increased the erythropoiesis in response to the hypoxic *stimulus*.

TS increased 44% at day 1 and decreased to 40% of basal levels by day 3, returning to levels similar to normoxic controls by day 5 (figure 12D). SI behaved similarly. However, in contrast to TS, the slight increase by day 1 was not statistically different, there was a marked decrease to 51% of normoxic controls by day 3. At day 5 SI levels were similar to normoxic mice (figure 12C).

The increase of TS at day 1 suggests a mobilization of iron from storage to meet the erythropoietic demand and the TS and SI decrease by day 3 may reflect a reduction of iron in the stores. By day 5 TS and SI start to stabilize and approach the levels found in normoxic control.

Rgmc levels were found upregulated 2-fold by day 1, after which it decreases to 146% of basal levels by day 3 and to levels similar to normoxic controls by day 5 (figure 13) suggesting that Rgmc may play a role in the changes observed in iron metabolism during acute hypoxia.

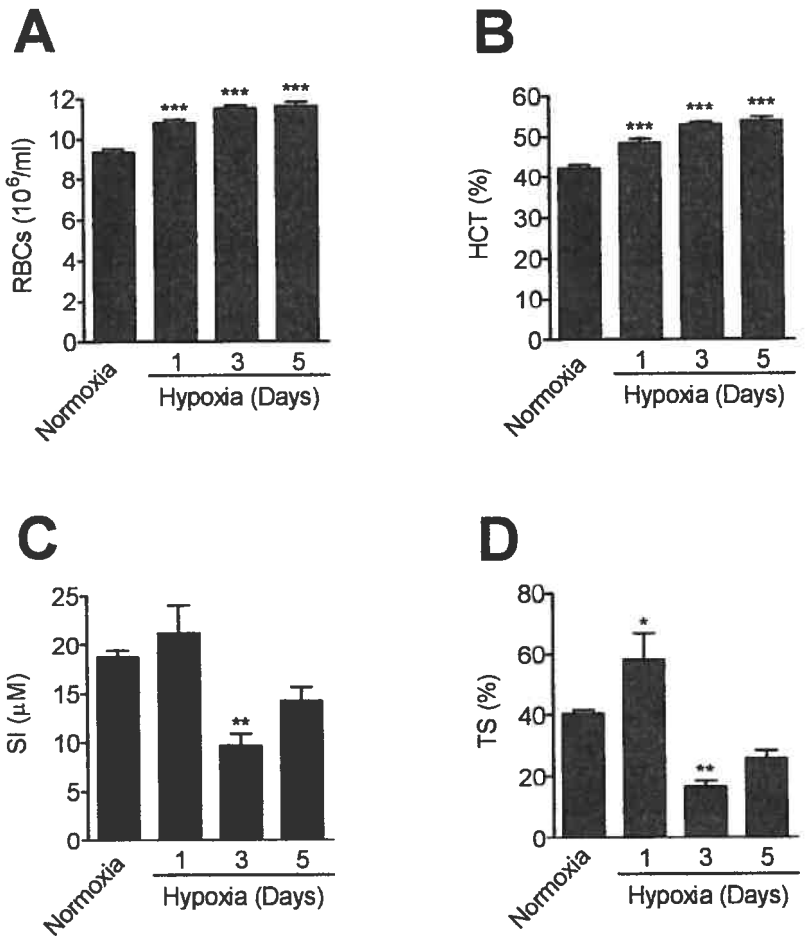

Figure 12 – Hematological indices and iron measurements in mice subjected to hypoxia. 10 weeks old mice were subjected to 10% oxygen levels (hypoxia) for 1, 3 or 5 days. Controls remained in normoxic conditions (21% oxygen). Blood was extracted as described in materials and methods and the hematological indices of red blood cells (A) and hematocrit (B) were measured. Serum iron (C), and transferrin saturation (D) were also quantified. Results are presented as means ± SEM (n=5 per group). Statistical analysis was performed by one way ANOVA followed by the Benferroni test. *p<0,05, **p<0,01, ***p<0,001 (compared to normoxic group).

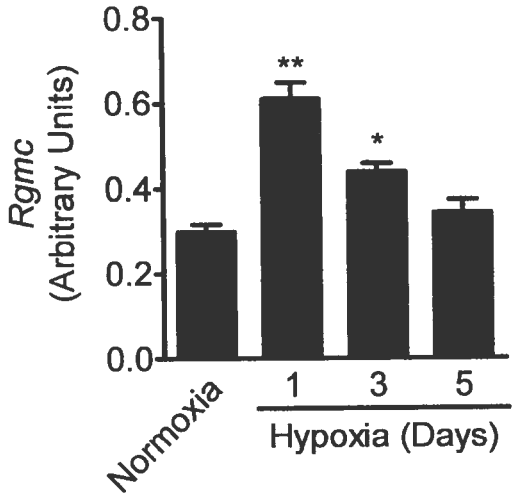

Figure 13 – Hepatic *Rgmc* mRNA expression in response to hypoxia. *Rgmc* mRNA expression quantified by qRT-PCR and normalized to *β-actin* in 10 weeks old mice subjected to 10% oxygen levels (hypoxia) for 1, 3 or 5 days. Controls remained in normoxic conditions (21% oxygen). Results are presented as means ± SEM (n=5 per group). Statistical analysis was performed by one way ANOVA followed by the Benferroni test. *p<0,05, **p<0,01 (compared to normoxic group).

Anemia

To assess how Rgmc may be important for iron homeostasis in anemia, we subjected 10 weeks-old C57Bl/6 mice to phlebotomy- and hemolytic-derived anemias. In the phlebotomy-treated mice, successive bleedings lead to a 3-fold reduction of RBC numbers, Hb levels and HCT (table IV), whereas no significant changes were found at the levels of SI or TS (table V). In hemolytic anemia, the lysis of RBC led to a 3-fold lower RBC count and HCT and a 26% reduction on Hb (table IV). However, in contrast to what was found in the phlebotomy model, a drastic increase in SI (3-fold) and TS (2-fold) is also found, probably due to the release of the iron from the lysed RBCs into the bloodstream (table V). This data indicates that we were able to induce anemia in both of the mouse models.

Rgmc mRNA expression was found not to change in either of the two models (figure 14), suggesting that, in contrast to what was found in the model for hypoxia, changes in *Rgmc* mRNA expression are not implicated in the regulation of iron metabolism in response to anemia.

Table IV – Hematological indices in PHL and PHZ treated mice. Red blood cells (RBC), hemoglobin (Hb), hematocrit (HCT) and mean corpuscular volume (MCV) of 10 weeks old mice phlebotomized (PHL) or with phenylhydrazine(PHZ)-induced anemia, as described in the material and methods section. Results are presented as means ± SD. Statistical analysis was performed by one way ANOVA followed by the Benferroni test. ***p<0,001 (compared to control).

Group	N	RBC (10^6/ml)	Hb (g/dl)	HCT (%)	MCV (fL)
Control	6	9.3 ± 0.3	13.7 ± 0.5	42.3 ± 1.8	46 ± 0.8
PHL	5	3.2 ± 0.3***	5.1 ± 0.6***	14.3 ± 1.3***	45 ± 0.8
PHZ	6	2.9 ± 0.2***	10.2 ± 0.9***	13.0 ± 1.7***	45 ± 2.9

Table V – Iron measurements in mice PHL and PHZ treated mice. Serum iron (SI) and transferrin saturation (TS) in 10 weeks old mice phlebotomized (PHL) or with phenylhydrazine(PHZ)-induced anemia, as described in the methods section. Results are presented as means ± SD. Statistical analysis was performed by one way ANOVA followed by the Benferroni test. ***p<0,001 (compared to control).

Group	N	SI (μM)	TS (%)
Control	6	25 ± 3	58 ± 7
PHL	5	24 ± 7	56 ± 16
PHZ	6	71 ± 10***	120 ± 19***

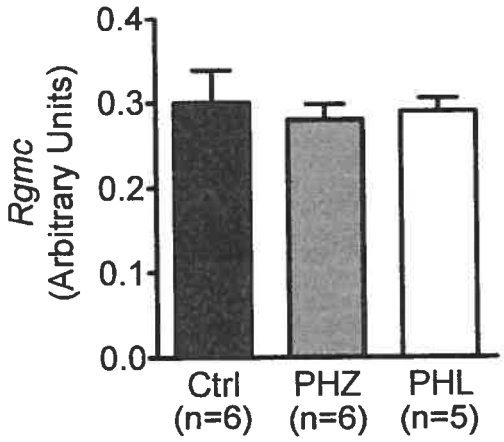

Figure 14 – Hepatic *Rgmc* mRNA expression in response to anemia. *Rgmc* mRNA expression quantified by qRT-PCR and normalized to *β-actin* in 10 weeks old non-anemic control mice (■), with phlebotomy-derived anemia (PHL) (▩) or with phenylhydrazine-induced anemia (PHZ) (□). Results are presented as means ± SEM. Statistical analysis was performed by one way ANOVA.

Inflammation

In inflammation hypoferremia takes place, characterized by a decrease in SI and TS, as seen in table VI (2-fold reduction for both parameters). Hypoferremia implies a change in iron homeostasis. To elucidate whether *Rgmc* regulation may be implicated in iron homeostasis in inflammation we treated mice with lipopolysaccharide (LPS) for 6 hours and quantified *hepcidin* and *Rgmc* mRNA levels. We found that mice treated with LPS downregulate *Rgmc* mRNA expression 6h after injection to 2% of basal levels (figure 15), indicating that regulation of *Rgmc* mRNA levels may be implicated in the changes observed in iron homeostasis in response to inflammation.

Table VI – Iron measurements in response to systemic inflammation. 10 weeks old mice were treated with LPS and sacrificed after 6 hours. Serum iron (SI) and transferrin saturation (TS) was measured. Results are presented as means ± SD. Statistical analysis was performed using the Student *t* test. ***p<0,001.

Group	SI (μM)	TS (%)
CTRL	17 ± 1	30 ± 8
LPS	7 ± 1***	16 ± 2***

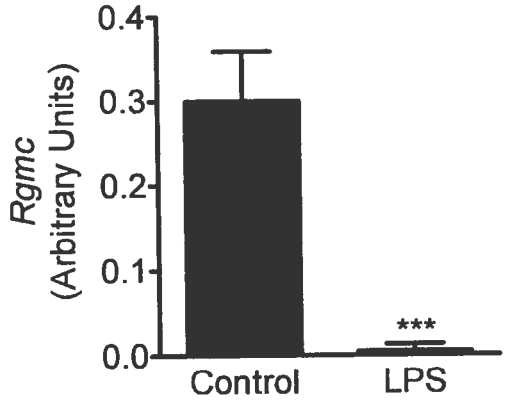

Figure 15 – Hepatic *Rgmc* mRNA expression in response to systemic inflammation. *Rgmc* mRNA expression quantified by qRT-PCR and normalized to *β-actin* in 10 weeks old control mice and in mice treated with LPS for 6 hours. Results are presented as means ± SEM (n=6). Statistical analysis was performed using the Student *t* test. ***p<0,001.

Aim 3 – Inflammatory Pathway

Time Course

The observation that *Rgmc* is regulated by inflammation at 6h led us to further study the kinetic response subjecting 10 weeks-old C57Bl/6 mice to LPS treatment for 6h, 17h, 24h and 48h both in the liver and heart.

Mice injected with LPS developed hypoferremia as seen by a decrease of SI and TS to 30% of basal levels by 6h without significant changes after 17h or 24h. After 48h both parameters rise to 140% (SI) and 160% (TS) of basal levels (figure 16A and B).

We found that after the downregulation observed at 6h, *Rgmc* mRNA levels consistently rose to 21% of basal levels by 17h after treatment and reached 40% of basal levels by 48h after the treatment, still significantly lower than control (figure 16C). Similarly, cardiac *Rgmc* had an approximate 50% reduction in mRNA levels that persisted for 48 h (figure 16D).

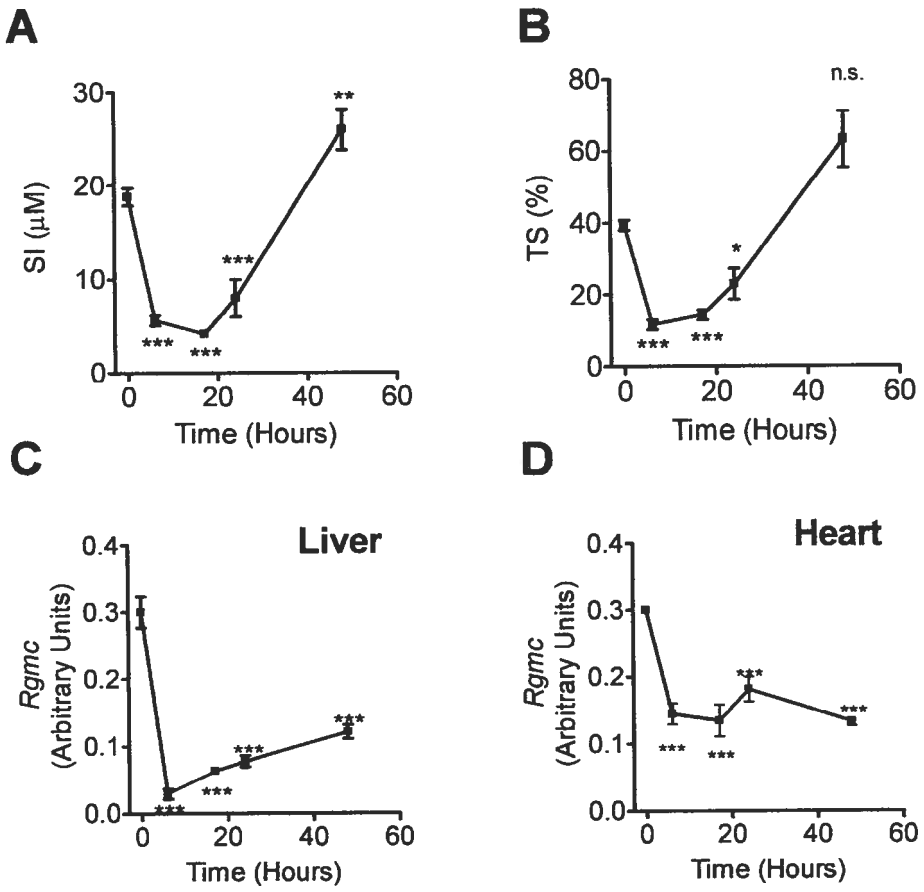

Figure 16 – Iron parameters and gene expression in response to systemic inflammation over time. Mice were treated with LPS and sacrificed 6, 17, 24 and 48 hours after injection (n=4). Serum iron (SI) (A) and transferrin saturation (TS) (B) were measured, as well as hepatic (C) and cardiac (D) *Rgmc* mRNA levels expression, quantified by qRT-PCR and normalized to *β-actin*. Results are presented as means ± SEM (N=4 per group). Statistical analysis was performed by one way ANOVA followed by the Benferroni test. *p<0,05; **p<0,01; ***p<0,001 (compared to controls).

Rgmc repression by LPS is independent of Hfe

Next, to assess whether changes in *Rgmc* mRNA expression are appropriately elicited in Hfe-deficient mice, we measured *Rgmc* mRNA expression in *Hfe*$^{-/-}$ and *β2m*$^{-/-}$ mice after LPS administration. As shown in figure 17, Hfe-deficient mice responded like wildtype mice, by dramatically down-regulating their hepatic *Rgmc* mRNA expression. This demonstrates that the ability to down-regulate *Rgmc* during the acute phase response remains intact in the absence of functional Hfe.

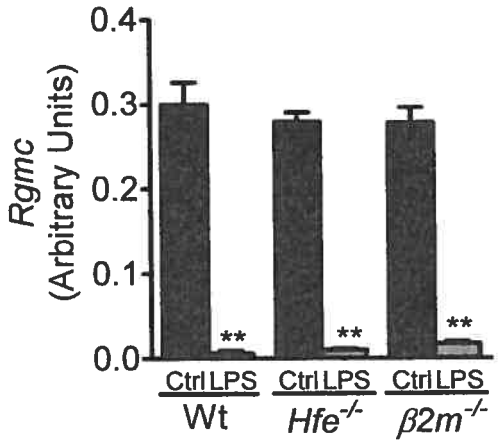

Figure 17 – Hepatic *Rgmc* mRNA expression in response to systemic inflammation in hereditary hemochromatosis mouse models. *Rgmc* mRNA expression quantified by qRT-PCR and normalized to *β-actin* in 10 weeks old wildtype (Wt), *Hfe*$^{-/-}$ and *β2m*$^{-/-}$ control mice and in mice treated with LPS for 6 hours. Results are presented as means ± SEM (n=6). Statistical analysis was performed using the Student *t* test. **p<0,01.

Rgmc regulation is Tlr4-dependent

Pathogen-associated molecular patterns (PAMP), including LPS are recognized by TLRs which, upon activation, lead to the production of pro-inflammatory cytokines. LPS is a PAMP that is recognized by Tlr4 (94). To investigate whether Tlr4 is on the regulatory pathway of *Rgmc* in response to LPS, we injected wildtype and Tlr4-deficient mice with LPS. These mouse strains have more iron, and develop a smaller hypoferremia (reducing SI by 34% and TS by 43% - table VII), compared with C57Bl/6 mice. *Rgmc* mRNA expression levels, however, differed dramatically between both strains after treatment (figure 19). Wildtype mice downregulated *Rgmc* levels to 1% of the basal levels, whereas the Tlr4-deficient mouse strain did not significantly respond to the treatment, placing *Rgmc* regulation in response to inflammation on the Tlr4 pathway.

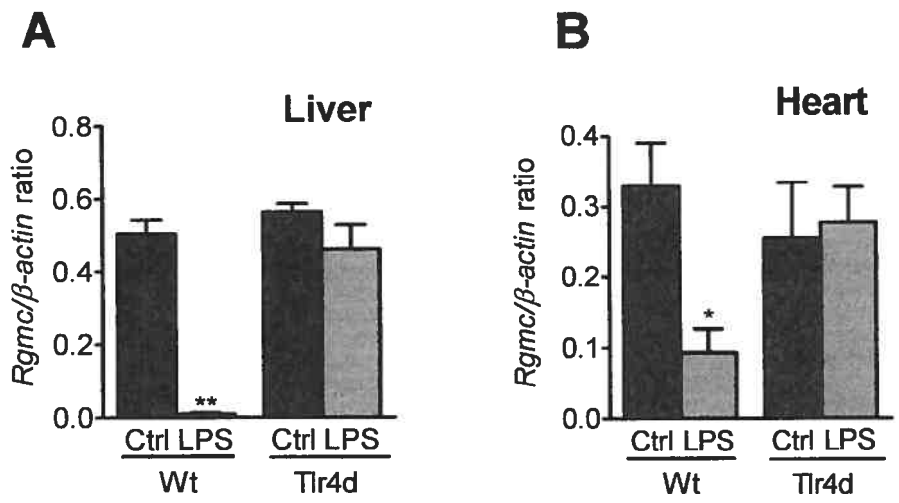

Figure 18 – Hepatic *Rgmc* regulation is Tlr4-dependent. *Rgmc* mRNA expression quantified by qRT-PCR in wildtype or Tlr4-deficient (Tlr4d) mice treated with LPS for 6 hours. Results are presented as means ± SEM (n=5). Statistical analysis was performed by one way ANOVA followed by the Benferroni test. ***p<0,001 (compared to control).

Table VII – Iron measurements in response to systemic inflammation in wildtype and Tlr4-deficient mice. 10 weeks old wildtype or Tlr4-deficient (Tlr4d) mice were treated with LPS and sacrificed after 6 hours. Serum iron (SI) and transferrin saturation (TS) was measured. Results are presented as means ± SD. Statistical analysis was performed using the Student *t* test. *p<0,05 (compared to respective control).

Group	N	SI (μM)	TS (%)
Wt Control	4	35 ± 4	61 ± 14
Wt LPS	4	23 ± 7*	35 ± 10*
Tlr4d Control	5	35 ± 5	70 ± 10
Tlr4d LPS	5	20 ± 8*	39 ± 18*

Tnf-α downregulates Rgmc

The Tlr4 signaling pathway leads to activation of the transcription factor Nf-κB, which initiates the transcription of pro-inflammatory cytokine genes such as Tnf-α and Il-6. To ascertain whether those cytokines are implicated in *Rgmc* regulation, we treated C57Bl/6 mice with recombinant mouse Il-6 or Tnf-α for 3 or 6 h or with LPS for 6 h.

Tnf-α and Il-6 were both able to reduce *Rgmc* mRNA, albeit less than LPS treatment. In fact, Tnf-α induced a 72% decrease at 3h and 45% at 6 h, while Il-6 caused only a 40% decline at 3 h (figure 19A).

To investigate whether Tnf-α regulates *Rgmc* by acting directly on hepatocytes, we isolated primary hepatocytes and incubated them for 24 h with Tnf-α, Il-6 and LPS and quantified *Rgmc* mRNA expression levels. Tnf-α, but not Il-6 or LPS, elicited a significant downregulation of *Rgmc* mRNA levels (38%) (figure 19B). Similar to what has previously described (72), we found that Il-6 upregulates *hepcidin* by 38% in mouse primary hepatocytes, whereas LPS induces a 29% decrease (figure 19C). Finally, *hepcidin* mRNA levels did not significantly change in response to Tnf-α.

This data indicates that Tnf-α is capable of directly regulating *Rgmc* in hepatocytes.

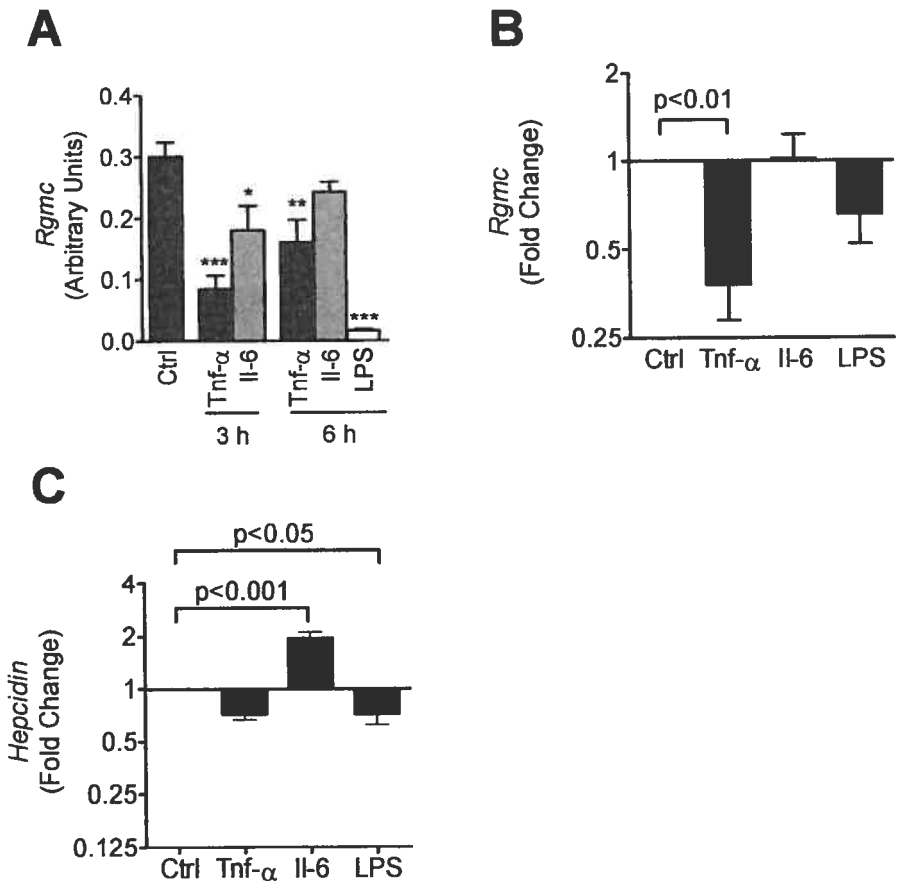

Figure 19 – *Rgmc* mRNA expression is decreased by Tnf-α, but not Il-6. *Rgmc* mRNA expression quantified by qRT-PCR and normalized to *β-actin* in control (Ctrl), Tnf-α, Il-6 and LPS-treated mice (A). *Rgmc* (B) and *hepcidin* (C) mRNA expression quantified by qRT-PCR and normalized to *β-actin* in primary hepatocytes treated with Tnf-α or Il-6 (4 experiments) or LPS (3 experiments) for 24 hours. Results are presented as means ± SEM. Statistical analysis was performed by one way ANOVA followed by the Benferroni test. *p<0,05; **p<0,01; ***p<0,001 (compared to control).

Il-6 is not required for *Rgmc* down-regulation in response to LPS

To further exclude the possible requirement for Il-6 in *Rgmc* down-regulation during inflammation, we tested the response to LPS of Il-6 knockout mice.

As seen in table VIII both mouse strains significantly decreased SI and TS levels in response to the LPS treatment.

There were no differences in basal *Rgmc* levels among the mouse strains, and both were able to regulate *Rgmc* levels in response to LPS (figure 20). Wildtype and *Il-6* knockout mice reduced *Rgmc* mRNA expression to 8% and 11 % of basal levels, respectively.

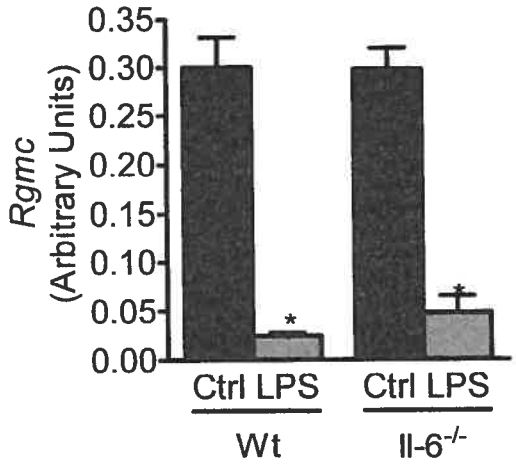

Figure 20 – *Il-6*$^{-/-}$ mice downregulate *Rgmc* mRNA in response to LPS. *Rgmc* mRNA expression quantified by qRT-PCR and normalized to *β-actin* in control (■) and mice treated with LPS for 6 hours (□) in wildtype (Wt) and *Il-6*$^{-/-}$. Results are presented as means ± SEM (n=4). Statistical analysis was performed by one way ANOVA followed by the Benferroni test. * p<0,05 (Compared to respective control)

Table VIII – Iron measurements in mice treated with LPS or a high iron containing diet low. Serum iron (SI), transferrin saturation (TS) and liver iron levels were quantified in 10 weeks old wildtype (Wt), or *Il-6*$^{-/-}$ mice treated with LPS for 6 hours. Results are presented as means ± SD. Statistical analysis was performed by one way ANOVA followed by the Benferroni test. *p<0,05; **p<0,01; ***p<0,001 (compared to respective control).

Group	Treatment	N	SI (μM)	TS (%)
Wt	Ctrl	4	16 ± 1	33 ± 2
	LPS	4	8 ± 2 *	16 ± 2 **
Il-6$^{-/-}$	Ctrl	4	16 ± 1	38 ± 3
	LPS	4	8 ± 1 ***	22 ± 4 *

Discussion

In this study we wished to elucidate where, when and how *Rgmc* is regulated in mice. We therefore investigated the pattern of tissue expression, the regulation in response to modifiers of iron metabolism and the regulatory pathway in response to systemic inflammation.

Tissue expression

We have found that *Rgmc* is expressed in skeletal muscle, heart and liver – where *Rgmc* is expressed only at the levels of hepatocytes – indicating that human (86) and mice (this study) have the same pattern of expression.

Similarly, others conclude that Rgmc is absent from macrophages and other liver mononuclear cell and that it is exclusively present in periportal hepatocytes (95). In that study the authors knocked out the *Rgmc* gene and inserted the lactosidase reporter gene under the *Rgmc* promoter, allowing for the histological observation of the sites of *Rgmc* expression.

Rgmc/HJV is present in the cell membrane, intracellular compartments and the supernatant of cultured cells (96) and serum (97), suggesting that Rgmc may function both as a GPI anchored protein and as a diffused molecule. Supporting this hypothesis is the fact that soluble

HJV is capable of regulating iron homeostasis in primary human hepatocytes (97).

The possibility that Rgmc may function as a diffused molecule is further supported by the identification of the Rgmc receptor: neogenin (96). Neogenin is also a receptor for Rgma and Rgmb, mediating signaling regarding axonal guidance. *In vitro* transfection in human HEK293 cells with *neogenin* and *HJV* increased iron uptake and ferritin levels, in comparison with control cells or cells transfected with only neogenin or *HJV*, demonstrating that neogenin also has a role in iron homeostasis.

The presence of *Rgmc* transcript only at the level of periportal hepatocytes therefore does not necessarily imply that it mediates the signaling only in these cells. It can be released from the periportal zone and, through neogenin, it may mediate signaling to the rest of the liver.

In addition, the expression of Rgmc in tissues other than the liver, along with the observation that it is found in the serum as a diffused molecule (97), raises the question of how Rgmc may be implicated in local iron metabolism in the heart and muscle and whether its activity is merely local or also systemic.

Rgmc may be capable of providing signals to any cell expressing neogenin. Neogenin expression has been almost exclusively studied in embryonic development in relation to signal mediation for Rgma and

Rgmb. Now it is evident that neogenin tissue expression and regulation may have an important role in iron metabolism in the adult, making it an interesting molecule to study in this field.

Rgmc regulation in response to modifiers of iron homeostasis

Rgmc regulation in response to Iron stores

Human sufferers become iron loaded either through the diet (e.g. HH patients) or through repeated blood transfusions (e.g. treated thalassemics) (98).

We have analyzed *Rgmc* regulation in response to iron stores by varying the amount of iron in the diet and have not found any variations in *Rgmc* mRNA levels between mice fed with low, standard or high iron diets, models of dietary iron loading.

In contrast to our data, it has recently been reported by that repeated daily iron-dextran injection for 5 days in mice increases *Rgmc* expression levels (99). A substantial difference between that model (designated parenteral iron loading, a model of iron loading such as found in treated thalassemics) and ours is that in our model macrophages are not iron loaded, in contrast to what happens in the

iron-dextran model (99, 100). Iron loading at the level of the macrophages may affect cytokine production (101) and ROS levels and induce the differences found between the models.

Despite the fact that *Rgmc* mRNA levels are not regulated by iron, recent evidence emerging from *Rgmc* knockout mouse studies indicate that Rgmc plays an essential role in the iron-sensing pathway at the systemic level (95). Injection of iron-dextran in *Rgmc* knockout mice does not result in the upregulation of *hepcidin*, in contrast to what is observed in the wildtype animals, indicating that Rgmc is implicated in the mediation of iron sensing signaling.

In addition, in both HEK293 and Hep3B cell lines engineered to express HJV, soluble HJV release was progressively inhibited by increasing iron concentrations (97) indicating that iron-mediated release of HJV may be one mechanism by which Rgmc signals iron levels to downstream targets.

Together, this data suggests that the iron signaling mediated by Rgmc is performed by regulation of Rgmc secretion, without the adjustment of expression levels. This implies that Rgmc is constantly being produced and secreted, and that the *Rgmc* transcript levels found in the cells are enough to produce the required amounts of protein for secretion, independently of the level of iron stores.

Rgmc levels in Hereditary hemochromatosis

Liver iron levels in mice with dietary iron overload reach levels similar to those found in the genetically iron overloaded mice $\beta 2m^{-/-}$ and $Hfe^{-/-}$. These mouse models have *Rgmc* levels similar to those found in Wt mice, indicating that perturbation of *Rgmc* mRNA regulation does not participate on the pathogenesis of classical hereditary hemochromatosis. The low basal hepcidin levels found in these mice are therefore not explained by low *Rgmc* transcript levels.

It is possible that Hfe abrogation alters Rgmc function at the protein level. However, if Hfe would alter Rgmc signaling, for instance, by participating on the regulation of Rgmc secretion, $\beta 2m^{-/-}$ and $Hfe^{-/-}$ mice would be expected not to properly regulate hepcidin in response to iron levels. Nevertheless, these mice have been found to have an adequate hepcidin response in response to low or high iron diet treatments as shown by us (102) and others (103).

Rgmc regulation in response to anemia and hypoxia

We have found that acute hypoxia, but not anemia, regulates *Rgmc* mRNA expression. Similarly, it has been recently reported that *Rgmc* mRNA expression is not altered in response to phlebotomy-induced anemia (104) or EPO *in vivo* (57).

It is believed that hepcidin levels directly correlate with *Rgmc* levels, as it has been observed *in vitro* (96). However, the upregulation of *Rgmc* mRNA levels in response to hypoxia does not induce an upregulation in hepcidin transcription (data not shown).

Hepcidin has recently been shown to be regulated by erythropoiesis based on the observations that hepcidin downregulation in anemia is inhibited by blockage of erythropoiesis by irradiation or posttransfusion polycythemia, even in the presence of exogeneous EPO (105). The fact that *Rgmc* mRNA is not regulated by erythropoiesis (as seen in the models for anemia and exogenous EPO administration), in contrast to what was shown for hepcidin (105), suggests that hepcidin and Rgmc are regulated by different pathways in hypoxia.

Anemia is known to induce tissue hypoxia. Therefore, the absence of *Rgmc* mRNA regulation in response to anemia raises the question of which factors are differentially regulated in hypoxia induced by low oxygen levels and anemia-induced hypoxia.

EPO and HIF-1 are activated in both models and are therefore unlikely to be the ones implicated in *Rgmc* mRNA regulation. Additional studies will be required to identify the Rgmc regulatory pathway in response to hypoxia.

Rgmc regulation in response to inflammation

As others (57), we have found that *Rgmc* is downregulated 6 hours after LPS-induced inflammation. In addition, we have found that this downregulation persists for at least 48h after the stimulus.

Hepcidin seems to be regulated by two independent pathways: one in response to inflammation, through Il-6 and Il-1β (71, 72), and another, in response to iron levels, through Rgmc and neogenin (95, 96). Others have suggested that Rgmc downregulation in response to LPS serves to switch off hepcidin regulation by iron levels eliminating interference from this pathway and allowing hepcidin to be regulated only by cytokines (95).

We propose that the low *Rgmc* levels, sustained for at least 48 hours after LPS challenge may desensitize the iron signaling pathway, considering that Rgmc has been shown to be implicated in iron sensing (95). This may facilitate the iron release from macrophages and hepatocytes allowing the replenishment of serum iron levels and the recovery from the hypoferremia.

The response to LPS seems to be tissue-specific, as Rgmc down-regulation is not seen in the skeletal muscle of mice treated with LPS (95), in contrast to what is observed in the liver. We further show that the down-regulation of Rgmc mRNA expression also occurs in the heart,

where hepcidin is as well expressed (100). This indicates that Rgmc regulation by LPS may have an important role in cardiac iron homeostasis in cardiomyopathy. This could be pertinent, since cardiomyopathy is considerably more frequent in JH than in HH.

Rgmc regulatory pathway in inflammation

The molecular signaling mechanism for Rgmc regulation by LPS seems to involve Tlr4, as *Rgmc* down-modulation was abrogated in both the liver and heart of Tlr4-deficient mice treated with LPS. However, Tlr4-deficient mice and mice lacking downstream elements, such as Il-6 knockout mice are still able to respond with hypoferremia, which indicates that iron metabolism changes elicited by LPS may occur through both Tlr4- and non-Tlr4-dependent mechanisms.

Activation of the Tlr4 pathways ultimately results in the production of pro-inflammatory cytokines, including Il-6 and Tnf-α, which are known to directly influence iron metabolism. We found that mice injected with Tnf-α or Il-6 downregulated *Rgmc* mRNA levels. However, of these cytokines, only Tnf-α down-regulated *Rgmc* mRNA levels *in vitro*. Pro-inflammatory cytokines are known to induce each other's expression (106), which may explain why Il-6 had a modest repressive effect on *Rgmc* mRNA expression *in vivo*.

The fact that *Il-6*$^{-/-}$ mice downregulated *Rgmc* mRNA in response to LPS without differences in comparison to wildtype mice indicates that Il-6 is not required for the regulation of *Rgmc* mRNA in response to LPS. This response contrast with hepcidin, which is regulated by Il-6 *in vitro*, and has an impaired regulation in Il-6 knockout mice injected with LPS (107).

Since Tnf-α is capable of *Rgmc* mRNA regulation both *in vitro* and *in vivo*, it is possible that this cytokine, which is produced after LPS stimulation, may be implicated in the regulation of *Rgmc* mRNA levels in LPS treated mice. However, further studies using Tnf-α knockout mice or antibody-mediated Tnf-α blocking *in vivo* will be required to understand whether that cytokine is essential for *Rgmc* mRNA regulation in response to LPS or if other cytokines are also capable of inhibiting *Rgmc* mRNA expression.

In conclusion, we show that mouse *Rgmc* has the same pattern of expression as found in humans, being expressed in skeletal muscle, heart and liver, of which the last two are the main organs affected in JH. In the liver, the *Rgmc* transcript was detected in the hepatocytes, and was absent from macrophages and other liver mononuclear cells, indicating that the control of Rgmc function in this organ is likely mediated by hepatocytes.

Hepatic *Rgmc* mRNA expression was regulated by systemic inflammation and hypoxia, but not by iron levels, disruption of Hfe or β2m, or anemia.

We found that cardiac *Rgmc* is also regulated in inflammation and that both hepatic and cardiac *Rgmc* are on the Tlr4 pathway, similar to what is was found for hepcidin (102). However, *Rgmc* and hepcidin regulatory pathways deviate and while Il-6 regulates hepcidin, *Rgmc* is regulated by Tnf-α.

These findings provide important insights into the regulatory pathways impacting iron metabolism changes that may be relevant to disease settings affecting iron homeostasis.

Although it is important to elucidate how *Rgmc* transcription is regulated, the physiologic relevance of the identified changes found will benefit from studies at the protein level. The recent commercialization of anti-Rgmc antibodies will allow for additional studies concerning how the transcriptional regulation is reflected at the protein level and how Rgmc secretion is controlled.

Future perspectives

Iron is implicated in the regulation of Tnf-α by LPS since iron overloaded mice have a higher Tnf-α response than controls as observed by us (data not shown). Additionally, as shown in this work, Tnf-α is implicated in the regulation of iron homeostasis genes.

These observations suggest a cross-communication mechanism between the inflammatory and iron regulatory pathways. Interestingly, hypoxia may also be implicated in this cross-communication, since it enhances the Tnf-α response to LPS (108).

We believe that this putative cross-communication participates in the regulation of iron homeostasis through the regulation of Tnf-α.

We hypothesize that Rgmc regulates hepcidin and other iron-related genes in response to a cross talk between hypoxia, iron levels and the immune system through a Tnf-α-related pathway. To test this hypothesis, we propose the following experimental strategies:

1. The activation of genes by Tlr4 is mediated by two transcription factors: Nf-κb and interferon regulatory factor (IRF)3 (109).

To investigate the role of these proteins in the differential regulation of *Rgmc* and *hepcidin* by Tlr4 we will study *in vivo* iron homeostasis during

inflammation using knockout mice of downstream targets of Tlr4. In addition, we will investigate how chemical inhibition and dominant-negative forms of the proteins on the Tlr4 pathway alter iron homeostasis *in vitro*.

2. Heat shock protein (HSP)70 and nitric oxide (NO) are modulated in response to the iron regulators: iron levels, hypoxia and inflammation (110, 111) and are implicated in the modulation of Tnf-alpha production (108, 112), filling our criteria for elements implicated in a cross-communication between the iron regulators.

To explore the participation of HSP70 and NO on Tnf-alpha secretion and *Rgmc* regulation, we will study iron homeostasis in response to the activation or inhibition of those factors *in vivo* and *in vitro*.

3. Rgmc seems to be capable of mediating iron-sensing both as a membrane-bound and as a secreted molecule (97). The over-expression of the Rgmc receptor, neogenin is accompanied by an increase in intracellular iron levels (96) and seems to give a growth advantage to cancer cells (113).

To explore the downstream pathway of Rgmc through neogenin and its relevance in cancer, we will investigate how neogenin is regulated in response to the regulators of iron homeostasis by qRT-PCR. In addition we will use microchip analysis and 2D gel electrophoresis to identify

neogenin downstream targets. Finally, we will investigate how neogenin disruption affects growth and survival of neoplasic cells.

Significance

Long periods of inflammation, such as found in chronic infections, alcohol abuse, cancer and autoimmune diseases, causes a sustained sequestration of iron in hepatocytes and macrophages, accompanied by a strong inhibition of iron absorption due to the high hepcidin levels. This sequestration leads to both hyperferremia in hepatocytes and macrophages, and serum hypoferremia. Insufficient hemoglobin production derived from the hypoferremia ultimately leads to anemia of chronic disease (ACD).

In addition, the upregulation of neogenin in breast (113), esophageous (114) and ovarian (Le Page *et al*, unpublished data) cancers, which require large amounts of iron for tumor growth, suggests that the *HJV*/neogenin pathway may be targeted in cancer therapies to deprive the neoplasic masses of iron.

The understanding of the regulation of the HJV pathway and its downstream elements may provide targets for therapies aimed at ameliorating the symptomatology of ACD and reducing the availability of iron for tumor growth as a co-therapy in cancer treatment.

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