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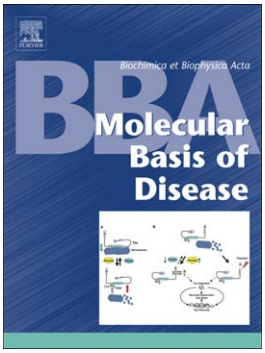

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Hepcidin Induction Limits Mobilisation of Splenic Iron in a Mouse Model of Secondary Iron Overload

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Running title: Iron mobilisation during secondary iron overload

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Abbreviations:CRP (C-Reactive Protein), D0 and D22 (Day 0 and Day 22 of phlebotomy treatment), Fpn (Ferroportin), HAMP (Hepcidin), HFE (Haemochromatosis gene), HIC (Hepatic Iron Content), HJV(Hemojuvelin), SIC (Splenic Iron Content), TFR2 (Transferrin Receptor 2).

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ABSTRACT

Venesection has been proposed as a treatment for hepatic iron overload in a number of chronic liver disorders that are not primarily linked to mutations in iron metabolism genes. Our **aim** was to analyse the impact of venesection on iron mobilisation in a mouse model of secondary iron overload.

C57Bl/6 mice were given oral iron supplementation with or without phlebotomy between day 0 (D0) and D22, and the results were compared to controls without iron overload. We studied serum and tissue iron parameters, mRNA levels of hepcidin1, ferroportin, and transferrin receptor 1, and protein levels of ferroportin in the liver and spleen.

On D0, animals with iron overload displayed elevations in iron parameters and hepatic hepcidin1 mRNA. By D22, in the absence of phlebotomies, splenic iron had increased, but transferrin saturation had decreased. This was associated with high hepatic hepcidin1 mRNA, suggesting that iron bioavailability decreased due to splenic iron sequestration through ferroportin protein downregulation. After 22 days with phlebotomy treatments, control mice displayed splenic iron mobilisation that compensated for the iron lost due to phlebotomy. In contrast, phlebotomy treatments in mice with iron overload caused anaemia due to inadequate iron mobilisation.

In conclusion, our model of secondary iron overload led to decreased plasma iron associated with an increase in hepcidin expression and subsequent restriction of iron export from the spleen. Our data support the importance of managing hepcidin levels before starting venesection therapy in patients with secondary iron overload that are eligible for phlebotomy.

Key words: liver, iron overload, hepcidin, ferroportin.

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1. INTRODUCTION

Iron is essential for life [1, 2]. A balance exists between iron use, absorption, storage, and recycling to fulfil the metabolic demand for iron and maintain physiological concentrations in the plasma compartment and in the different organs.

Hepcidin (produced from the *Hamp1* gene) is a key regulator of iron metabolism [3], and it is synthesised primarily by the liver [4]. Secreted in the plasma, hepcidin acts as a negative regulator of the membrane protein ferroportin (Fpn; produced from the *Slc40a1* gene). Ferroportin is a membrane iron exporter expressed on macrophages and enterocytes. Plasma iron bioavailability is highly dependent on iron recycling by macrophages and digestive iron absorption by enterocytes; therefore, ferroportin plays a major role in iron bioavailability [5-7]. An interaction between hepcidin and ferroportin leads to internalisation and degradation of the ferroportin protein [8]. Excessive hepcidin production has been associated with reductions in iron release from macrophages and intestinal epithelial cells [9]. Conversely, a reduction of hepcidin expression favours iron release and plasma availability [10]. Within the plasma compartment, iron is bound to transferrin, which permits iron delivery to cells through the transferrin receptor 1 (produced from the *TfRc* gene) [11]. An imbalance in iron metabolism leads to abnormal clinical situations, like anaemia [12] or iron overload [13].

Most genetic conditions associated with an iron overload result from mutations in iron metabolism genes (*HFE, HJV, HAMP, TFR2*) that are responsible for defects in hepcidin production or activity. These defects lead to polyvisceral iron deposition, particularly in the liver [14-17]. Other genetic disorders associated with iron overload include mutations of the ferroportin gene, *SLC40A1*. Mutations in *SLC40A1* that result in a hepcidin-insensitive ferroportin protein may lead to an iron overload phenotype similar to that observed with

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hepcidin deficiency. Conversely, mutations that inhibit ferroportin protein expression on the cell membrane may lead to a predominantly macrophage-mediated iron sequestration and thus, decreased plasma iron bioavailability [18].

Secondary iron overload involving the liver may also occur outside the context of haematological diseases [19], in some chronic liver diseases, including viral hepatitis [20] and dysmetabolic hepatosiderosis [21].

Usually, when hepatic iron overload is diagnosed in the absence of anaemia, venesection therapy is undertaken. For example, in genetic haemochromatosis, repeated blood withdrawals induce the mobilisation of iron from storage compartments; this limits iron toxicity and prevents complications associated with iron overload, particularly in the liver [22]. In cases of ferroportin mutations that result in a deficiency of ferroportin at the cell membrane, venesections are sometimes poorly tolerated and may exacerbate anaemia despite the presence of excessive iron stores [23].

Venesections are also frequently proposed as a treatment for limiting hepatic iron excess and its deleterious consequences in the chronic liver diseases mentioned above [24, 25]. In these cases of secondary iron overload, the tolerance and efficacy of venesection have not been fully explored. Moreover, we lack knowledge of the mechanisms and kinetics of iron sequestration and mobilisation. A better understanding of the mechanisms implicated in iron release from the liver would enable improvements in therapeutic strategies for hepatic iron mobilisation in cases of iron overload.

Therefore, our aims were to analyse the following in a mouse model of secondary iron overload : i) the impact of venesection on serum iron parameters and iron stores and ii) the kinetics of iron mobilisation, with special emphasis on the expression of genes involved in the control of iron metabolism.

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2. MATERIALS AND METHODS

2.1 Animals

Eight week old male C57Bl/6 mice were obtained from Janvier (St Genest, France). They were maintained under standard conditions of temperature, atmosphere, and light, and experimental procedures were performed in agreement with French laws and regulations. All mice had free access to tap water and food.

Mice were divided into two groups (n=30 mice each) that were fed, *ad libitum*, either an iron-rich or normal RM3 (E) diet (SDS, France) for 4 weeks (Figure 1). The iron-rich diet was supplemented, as previously described [4, 26]. with 3% (3g iron/100g RM3 [E]) carbonyl-iron (Sigma, France). Six mice of each group were then sacrificed (D0). Thereafter, all mice were switched to a normal diet until day 22. Both the iron-rich and normal diet groups were then divided in two subgroups (n=12 in each) that received or did not receive phlebotomies. Phlebotomies were performed at days 0, 7, and 14 and consisted of a withdrawal of 300µl of blood from the retro-orbital sinus. Six mice of each subgroup were sacrificed at days 14 and 22. Figure 1 shows an overall view of the different treatment conditions. At sacrifice, the blood, liver, and spleen were collected. The liver and spleen were weighed and fixed in 4% formaldehyde or immediately frozen in liquid nitrogen. Part of the blood was used immediately for haemoglobin measurements and the remaining blood was centrifuged to isolate the plasma.

2.2 Blood analysis

Haemoglobin levels were determined with an ABL725 (Radiometer; Copenhagen, Denmark). The plasma isolated by centrifugation was used to measure serum iron concentrations by spectrophotometry with the Ferene-S method (Ferentest, Biomerieux) on a

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Cobas Mira analyzer (Roche). The total iron binding capacity (TIBC) was determined with the Ramsey protocol (TIBC kit, Biomerieux). The transferrin saturation was then calculated as (plasma iron / TIBC) × 100. The non-transferrin bound iron was measured according to a previously reported method [27].

2.3 Tissue iron determination

Iron concentration was measured in the liver and spleen with the Barry and Sherlock method [28]. Perls' staining [29] was performed on paraffin embedded, 5µm slices of liver and spleen to localise cellular iron stores.

2.4 RNA extraction and quantitative RT-PCR

Total RNA was extracted with the SV Total RNA Isolation System (Promega®, Madison, WI). Quality-checked RNA (1 µg) was used for reverse transcription according to the manufacturer's protocol (M-MLV-RT, Promega®, Madison, WI). We performed real time polymerase chain reactions (PCRs) in triplicate to evaluate the hepcidin (*Hamp1*), ferroportin (*Slc40a1*), transferrin receptor 1 (*TfRc*), and C-reactive protein (*CRP*) gene expression levels in each sample; levels were compared to the levels of 18s RNA as an internal standard. PCR was performed with a qPCR MasterMix Plus for SYBR®Green I according to the manufacturer's instructions (Eurogentec®, Seraing, Belgium). Primer sequences used for the amplification are described in Table 1. The PCR was run on an ABI PRISM 7000 sequence detection system (Applied Bioscience, London, United Kingdom) with the following protocol: 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The PCR products were checked by sequencing.

For each sample, *Hamp1*, *Slc40a1*, *Tfrc*, and *CRP* mRNA cycle threshold (Ct) values were normalized with 18s RNA Ct values. The normalised Ct values of the samples treated

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with iron overload and/or phlebotomies were compared to the normalised Ct values obtained from corresponding untreated control tissues from mice that were sacrificed at day 0 (standards). Results were expressed as the ratio of the sample versus the standard in arbitrary units (AU) for convenience.

2.5 Immunoblotting

Membrane extracts were prepared from nitrogen frozen spleens and livers [30]. Protein concentrations were determined with a BC Assay (Uptima, Interchim); then, 25µg (spleens) or 50µg (livers) samples were loaded onto NuPAGE® Bis-Tris 4-12% gels bathed in MOPS buffer, following the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). The separated proteins were transferred onto nitrocellulose membrane and blocked with 7% milk in TBST (NaCl 0.9%; Tris-HCl 10mM, pH7.5; 0.15% Tween20). Membranes were then incubated with rabbit anti-mouse ferroportin polyclonal antibodies diluted at 1/100 in TBST with 7% milk [30]. After further washing in TBST, membranes were incubated in the presence of a goat anti-rabbit IgG peroxidase-labelled antibody (1/150000, Jackson) and signals were visualised with chemiluminescence (Super signal® west dura, Pierce). As a control, membranes were stripped with Restore Western Blot Buffer (Pierce), incubated for 2 h with mouse anti-Hsc70 antibody (B-6; SC-7298; Santa Cruz) diluted at 1/10000 in TBST with 7% milk, and then washed in TBST. Finally, these membranes were incubated with a goat anti-mouse IgG peroxidase-labelled antibody (1/5000, Dako) and washed with TBST before signal visualisation with chemiluminescence (Super signal® west pico, Pierce).

2.6 Statistical analysis

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The statistical analysis was performed with Statview software (SAS institute, Cary, NC). Non parametric tests were used, including the Mann-Whitney and Kruskall-Wallis tests. A p-value of less than 0.05 was considered statistically significant.

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3. RESULTS

3.1 Impacts of iron loading and phlebotomy on blood iron parameters

At day 0, transferrin saturation was significantly increased in animals fed an iron-rich diet compared to those fed a normal diet (Figure 2A). However, the iron overload did not significantly modulate haemoglobin levels (Figure 2B). At days 14 and 22, the control animals displayed no changes in transferrin saturation or haemoglobin levels. In contrast, iron-loaded mice displayed a significant decrease in transferrin saturation compared to that observed at D0 and compared to control animals at D14 and D22.

In control mice fed a normal diet, phlebotomies had no impact on transferrin saturation or haemoglobin levels until D22, compared to D0 (Figure 2). Conversely, in mice fed an iron-rich diet, phlebotomies induced significant decreases in transferrin saturation and haemoglobin levels compared to control animals, with or without phlebotomies, and compared to non-phlebotomized iron-loaded animals.

3.2 Impacts of iron loading and phlebotomies on iron stores

Mice fed an iron-rich diet presented significantly higher hepatic iron concentrations (HIC) at day 0 compared to mice fed a normal diet (Figure 3A). Iron was mainly localised within hepatocytes with a gradient decreasing towards the centres of the lobes (Figure 4C).

Whether mice were fed iron-rich or normal diets, phlebotomies did not induce modulations of HIC compared to non-phlebotomized animals on D0 and D22 (Figure 3A). The lack of change in hepatic iron deposits was judged both by the intensity of the stain and by the nature of the cell population stained (hepatocytic but not macrophagic) (Figure 4 A-D).

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Splenic iron concentrations (SIC) were also elevated at day 0 in mice fed the iron-enriched diet compared to those fed the normal diet (Figures 3B, 4E, and 4G). Despite the return to a normal diet from D0 to D22, splenic iron accumulated in iron-loaded mice until it reached a plateau at D14.

Phlebotomies in control mice progressively induced a decrease in SIC. The difference in SIC compared to control mice without phlebotomies became significant at D22 (Figure 3B). In contrast, phlebotomies in iron-loaded mice did not cause SIC increases or decreases between day 0 and day 22. Thus, the phlebotomies counteracted the accumulation of splenic iron after iron-loading (Figure 3B). In all conditions, iron was mainly localised within the red pulp cells of the spleen (Figure 4E-H).

3.3 Impacts of iron-loading and phlebotomies on mRNA levels of iron metabolism genes

In the liver, *Tfrc* mRNA levels were significantly decreased from D0 to D22 in iron-loaded mice compared to control mice (Figure 5A). Phlebotomies did not significantly modulate the expression of this gene in controls or iron-loaded mice. Furthermore, the hepatic mRNA levels of *Slc40a1* were not significantly affected by iron enrichment or phlebotomies (Figure 5B). As expected, the *Hamp1* mRNA levels at D0 were increased in the livers of mice fed the iron-rich diet compared to mice fed the normal diet (Figure 5C). Between day 0 and day 22, when mice on iron-rich diet were switched to a normal diet, this expression level decreased slightly, but remained higher than the *Hamp1* mRNA levels found in control mice. Phlebotomies had no impact on hepatic *Hamp1* mRNA levels in control mice. In contrast, phlebotomies induced a significant decrease in *Hamp1* mRNA levels of iron-loaded mice; nevertheless, the levels remained higher than in control mice at D22 (Figure 5C). Neither iron enrichment nor phlebotomies had any impact on the hepatic mRNA levels of *CRP* at D0 and D22 (data not shown).

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In the spleen, mice fed an iron-rich diet displayed higher levels of *Tfrc* mRNA at D0 compared to control mice, despite the increase in SIC (Figure 6A). At D22, *Tfrc* mRNA expression decreased to basal levels in mice that were not phlebotomised. Phlebotomies had no significant effect on splenic *Tfrc* mRNA levels in control mice fed a normal diet (Figure 6A). Conversely, in iron-loaded mice with phlebotomies, the levels of *Tfrc* mRNA in the spleen remained high on day 22 compared to mice fed a normal diet without phlebotomies. At D0, mice fed an iron-rich diet showed increased splenic *Slc40A1* mRNA levels compared to control mice (Figure 6B). However, at day 22, the expression decreased in both diet groups compared to day 0. Phlebotomies induced no significant changes in splenic *Slc40A1* mRNA expression compared to mice without phlebotomies in both diet groups. *Hamp1* mRNA levels in the spleen were weak with large standard deviations; thus, no statistical differences could be detected among the different treatments (data not shown).

Dmt1 and *Dcytb* mRNA levels were also studied in the duodenum; but these did not show any modulations with diet or phlebotomy treatments (data not shown).

3.4 Impacts of iron-loading and phlebotomies on ferroportin protein expression

In the liver, membrane protein extracts obtained from the different mice groups at D0 and D22 were examined for ferroportin protein expression levels (data not shown). Under our assay conditions, all detectable signals were too faint for interpretation.

In the spleen, we were able to detect high levels of ferroportin expression at D0. Compared to mice fed the normal diet, mice fed the iron-rich diet displayed a decrease in ferroportin protein expression (Figure 7A), despite increased mRNA levels (Figure 6B). The difference was most pronounced at D22 (Figure 7B). Phlebotomies induced a slight decrease of ferroportin expression in control animals; in contrast, phlebotomies induced an increase in ferroportin protein expression in iron-loaded mice (Figure 7B, lower panel).

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For an overall view of the different parameters studied, the results are schematically summarised in Table 2.

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4. DISCUSSION

During chronic liver disease with secondary hepatic iron deposition, phlebotomies have been proposed as a treatment to prevent iron toxicity (Reviewed in: [31]). Our goal was to evaluate, in a model of secondary iron overload, the kinetics of iron mobilisation produced by phlebotomies in connection with modulated iron metabolism gene expression. First, we induced secondary carbonyl iron overload in a murine model [26]. Conversely to the secondary iron overload observed in Beta-thalassemic mice and humans which present with a strong decrease of hepcidin expression and cannot be phlebotomized, due to the anaemia related to this haematological disease [32, 33], carbonyl iron loaded animals are not known to have haematological abnormalities and have an hepcidin expression increase [4]. We then mobilised iron stores with mild phlebotomies that mimicked those performed in human patients, especially during dysmetabolic hepatosiderosis in which hepcidin expression is increased [34]. The 0.3 ml of blood removed from a 30g mouse was equivalent to about 13% of the total blood volume. This was similar to the 10% of total blood volume removed with a 500ml phlebotomy in a human being.

On the day dietary iron supplementation was withdrawn (D0), iron deposits were found mainly within hepatocytes in the liver and in cells of the splenic red pulp, which are known to comprise primarily macrophages [35].

In the liver, we observed an absence of iron deposits within resident macrophages; this contrasted with findings in another mouse strain treated with carbonyl iron overload [26]. This discrepancy could be explained by the discovery of the C57Bl/6-specific gain of function mutation in the *Mon1a* gene. The *Mon1a* gene product is a protein implicated in vesicular trafficking of the ferroportin protein to the cell membrane [36]. However, that report

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emphasised that the major phenotype of the *Mon1a* mutation was an alteration in the spleen. In our model, we did observe some hepatic consequences of iron overload, including an increase of hepcidin1 unrelated to inflammation and a decrease of transferrin receptor 1 mRNA levels. Both these changes were expected with iron overloading [37]. In addition, there were no modulations of hepatic ferroportin mRNA levels; the ferroportin protein was not clearly detectable.

In the spleen, we found that ferroportin protein expression was decreased with iron overloading. This could be a consequence of inducing hepatic hepcidin expression. Indeed, it has been reported that increased hepcidin expression may inhibit iron egress from splenic macrophages and thus contribute to the development of splenic iron overload [38]. In addition, in the spleens of iron-loaded mice, we found increased *TfRc* mRNA levels, which were maintained at D22 after phlebotomies. This observation could point to another, undefined, regulatory mechanism for *TfRc* mRNA production that may overcome regulation due to the binding of iron regulatory protein 1 to an iron-responsive element in the spleen. This undefined regulation system could be related to that responsible for the induction of *TfRc* mRNA found, despite high ambient iron levels, during erythropoiesis [39, 40].

The switch to a normal diet from the iron-rich diet led to a decrease in transferrin saturation at D22. Nevertheless, hepatic iron concentrations remained steady and splenic iron increased. These results demonstrated that a secondary iron overload could unexpectedly induce a decrease in plasma iron bioavailability through splenic iron sequestration, even when animals were fed a normal diet. This was possibly related to the expression levels of hepatic hepcidin, which remained higher in iron-loaded mice than in control animals, despite a slight decrease compared to the levels at D0.

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Our results support the hypothesis that hepatic hepcidin overexpression has a major effect on splenic macrophage iron export function. This was reinforced by the fact that, despite the increase in splenic ferroportin mRNA levels in iron-loaded mice, there was a strong decrease in ferroportin protein expression. Our results in iron-loaded mice indicated that the hepcidin inhibition of ferroportin expression [8] overcame transcriptional and posttranscriptional signals related to increasing the cellular iron stores within the spleen [41, 42].

Interestingly, the effects of hepcidin were not detected in the livers of iron-loaded mice. Indeed, the switch to a normal diet did not induce iron accumulation within Kupffer cells, as it did in spleen cells, and did not modulate the liver iron content or distribution. Taken together, our data suggest that hepatic cells, including hepatocytes and Kupffer cells, may be less sensitive to the regulatory effects of hepcidin on iron metabolism. This hypothesis was reinforced by the fact that we could not clearly detect an induction of ferroportin protein expression in iron-loaded livers compared to control livers.

The phlebotomy treatments provided an opportunity for analysing the kinetics of iron mobilisation in parallel with hepcidin and ferroportin expression.

In animals fed a normal diet, phlebotomies had no impact on blood iron parameters. The maintenance of sufficient plasma iron bioavailability was partly related to a decrease in SIC, which attested to an increase of iron egress from macrophages. However, relative to splenic weight, the SIC decrease was apparently insufficient to adequately compensate for the iron loss due to venesection. Thus, iron levels were also likely maintained through: i) iron mobilisation from other macrophage compartments and ii) digestive iron absorption [9]. The latter pathway was considered due to previous reports that described the fulfilment of iron needs via intestinal iron absorption and ferroportin protein induction at the basal membranes

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of enterocytes [5, 43, 44]; this pathway was also described under conditions of hepcidin deficiency [45]. It must be noted that control animals did not display disturbances of plasma iron parameters or anaemia; therefore no hypoxia and no decrease in hepatic hepcidin1 mRNA expression occurred; this suggested that animals could strictly compensate for iron losses despite the erythroid activity induced by phlebotomies. This result was surprising, in light of recent results that suggested erythropoietic activity was a determinant in the inhibition of hepcidin mRNA expression [46, 47]. In this study, a decrease of ferroportin protein levels was observed in the absence of hepcidin modulation. This could indicate that partial compensation for iron loss was accomplished by using most of the iron provided in the normal diet; thus, the subsequent storage in splenic cells was limited and led to the global decrease in splenic iron. These conditions gave rise to the prominent role of transcriptional and post-transcriptional regulation of ferroportin gene expression.

In animals fed an iron-rich diet, the effect of switching to a normal diet was emphasised with the phlebotomies. The presence of plasma iron deficiency was indicated by anaemia. Inadequate iron mobilisation from the spleen may have, at least partly, accounted for the maintenance of splenic iron between D0 and D22. This inertia in the splenic response to maintain plasma iron bioavailability was most likely related to an abnormally high level of hepcidin expression. Indeed, in this anaemic group, the hepcidin mRNA levels remained higher than those found in mice fed a normal diet with or without phlebotomies, but were lower than those found in mice fed an iron-rich diet without phlebotomies. The decrease in hepcidin expression permitted higher ferroportin expression on cell membranes; hence, this explains the increase in ferroportin protein expression found in iron-loaded mice with phlebotomies compared to those without phlebotomies. However, the poor phlebotomy tolerance in iron-loaded mice indicated that the decrease in hepcidin was insufficient for the

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normalisation of plasma iron, despite the enhanced ability of the C57Bl/6 strain to express ferroportin protein at the cellular membrane due to the mutation in *Mon1a* gene [36].

The results of this study raised questions concerning the biological impact of ferroportin in the liver, due to the absence of hepatic iron mobilisation in iron-loaded phlebotomized mice and the putative low sensitivity of hepatic cells to hepcidin, as previously discussed. A previous study also raised doubts about the responsiveness of hepatocytes to hepcidin through potential ferroportin protein regulation [48]. We did not further investigate this point in this study, because it would have been risky to force higher levels of iron mobilisation from hepatic cells by increasing the amount of blood withdrawn; the iron enriched mice were already in an anaemic state after the mild phlebotomies. However, future investigations could be performed in mice that do not adequately modulate hepcidin levels in response to iron store levels (i.e., $HFE^{-/-}$ mice) .

In conclusion, our results demonstrated that in our model of secondary iron overload: i) a decrease of plasma iron bioavailability was present, ii) iron mobilisation by venesection primarily originated from the spleen and probably partly from enterocytes and iii) hepatic iron stores were not mobilised at this stage. This suggested that, during secondary iron overload with an increase in hepcidin expression, phlebotomies might be not entirely appropriate for mobilising excessive hepatic iron. Taken together, these data suggest that: i) a plasma hepcidin assay could be useful for monitoring hepcidin levels before and during venesections in patients with secondary iron overload that are eligible for phlebotomy, and ii) it may be appropriate to consider the use of iron chelators in these circumstances.

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Figure legends

<u>Figure 1:</u> Flow chart of the experimental design

After 28 days of an iron-rich (3% iron) or normal (0% iron) diet, all the mice (n=30 for each condition) were put on a normal diet (D0). Then, for each group, 12 mice were subjected to a series of three phlebotomies (¶) and 12 mice served as controls. Six animals were sacrificed (culled) at each indicated time point and condition (†).

<u>Figure 2:</u> Transferrin saturation and haemoglobin levels

Transferrin saturation (A) and haemoglobin levels (B) were measured in mice fed a diet supplemented with 0% (squares) or 3% (triangles) carbonyl-iron. In each group, phlebotomies were given to half the mice (grey symbols) and not to the other half (black symbols). Bars represent mean +/- standard deviation (n=5 to7). * Statistically different from D0; [&] statistically different from the corresponding control (0%) condition; [#] statistically different from the corresponding non-phlebotomised condition.

<u>Figure 3:</u> Hepatic and splenic iron concentrations.

Hepatic (A) and splenic (B) iron concentrations (HIC and SIC, respectively) of mice fed a diet supplemented with 0% (squares) or 3% (triangles) carbonyl-iron. In each group, phlebotomies were given to half the mice (grey symbols) and not to the other half (black symbols). Each bar represents mean +/- standard deviation (n=5 to7). * Statistically different from D0; [&] statistically different from the corresponding control (0%) condition; [#] statistically different from the corresponding non-phlebotomised condition. Note that the scales on the Y-axes are different in (A) and (B) due to a higher basal level of iron in the spleen compared to the liver.

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Figure 4: Hepatic and splenic iron distributions

Perls' staining of representative livers (A-D) and spleens (E-H) show locations of iron stores in mice fed a diet supplemented with 0% (upper panels) or 3% (lower panels) carbonyl-iron. Iron stores in livers of mice that received phlebotomies (left panels) or did not receive phlebotomies (right panels) are compared. Magnification was 100 times.

Figure 5: Hepatic mRNA expression of iron metabolism genes

Hepatic mRNA levels of (A) *Tfrc*, (B) *Slc40a1*, and (C) *Hamp1* were determined by quantitative RT-PCR in groups of mice fed a diet supplemented with 0% (squares) or 3% (triangles) carbonyl-iron. In each group, phlebotomies were given to half the mice (grey symbols) and not to the other half (black symbols). Note that the Y-scales are different in each panel, and reflect the units for each measured parameter. Each bar represents the mean +/- standard deviation (n=5 to 7). * Statistically different from D0; [&] statistically different from the corresponding control (0%) condition; [#] statistically different from the corresponding non-phlebotomized condition.

Figure 6: Splenic mRNA expression of iron metabolism genes

Splenic mRNA levels of (A) *Tfrc* and (B) *Slc40a1* were determined by quantitative RT-PCR in groups of mice fed a diet supplemented with 0% (squares) or 3% (triangles) carbonyl-iron. In each group, phlebotomies were given to half the mice (grey symbols) and not to the other half (black symbols). Each bar represents the mean +/- standard deviation (n=5 to 7). * Statistically different from D0; [&] statistically different from the corresponding control (0%) condition; [#] statistically different from the corresponding non-phlebotomized condition.

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Figure 7: Splenic ferroportin protein detection by immunoblotting

Ferroportin protein (fpn) was analysed by western blot in the spleens of mice. Anti-Hsc70 antibody was used as a loading control. (A) Impact of the iron enriched diet (3%) on ferroportin protein expression levels was analysed at day 0 (D0). (B) Impact of the iron-enriched diet with phlebotomies (3%P) on ferroportin protein expression levels was analysed at day 22 (D22) for mice fed a normal diet (0%) and mice fed an iron-enriched diet (3%; n=3).

Table 1: Primers used in quantitative RT-PCR to measure mRNA levels of *Hamp1*, *Slc40a1*, *Tfrc*, and *CRP* standardised with 18s RNA.

Gene	**Forward primer**	**Reverse primer**
Hamp1	5'-CCTATCTCCATCAACAGATG-3'	5'-AACAGATACCACACTGGGAA-3'
Slc40a1	5'-GCTGCTAGAATCGGTCTTTGGT-3'	5'-CAGCAACTGTGTCACCGTCAA-3'
Tfrc	5'-TCATGAGGGAAATCAATGATCGTA-3'	5'-GCCCCAGAAGATATGTCGGAA-3'
CRP	5'- TTGTCCTTTTCTCAGCAGCCA-3'	5'-AAAGACAGAACCCTATATGAAGAGCTAGAG-3'
18s	5'-AGCTAATACATGCCGACGGG-3'	5'-GGAGCTCACCGGGTTGG-3'

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Table 2: Schematic overview of all the parameter modulations. For a given condition, the effect is compared to control mice fed a normal diet without phlebotomies at the corresponding time point. The number of arrows indicates the degree of the effect, and the darkness of the cells indicates the amplitude of the variation within a parameter; darker=higher amplitude; lighter=lower amplitude

PARAMETERS	**EFFECT OF :**			
	Iron enrichment (D0)	Back to normal diet after iron enrichment (D22)	Phlebotomies (D22)	Iron enrichment + Phlebotomies (D22)
Transferrin saturation	↑	↓	↔	↓↓
Haemoglobin level	↔	↔	↔	↓↓
Hepatic iron concentration	↑	↑	↔	↔
Hepatic Hamp1 mRNA level	↑↑↑	↑↑	↔	↑
Hepatic Tfrc mRNA level	↓	↓	↔	↓
Hepatic Slc40a1 mRNA level	↔	↔	↔	↔
Splenic iron concentration	↑	↑↑	↓	↑
Splenic Tfrc mRNA	↑	↔	↔	↑
Splenic Slc40a1 mRNA	↑	↑	↔	↑
Splenic Fpn protein	↓	↓↓	↓	↔

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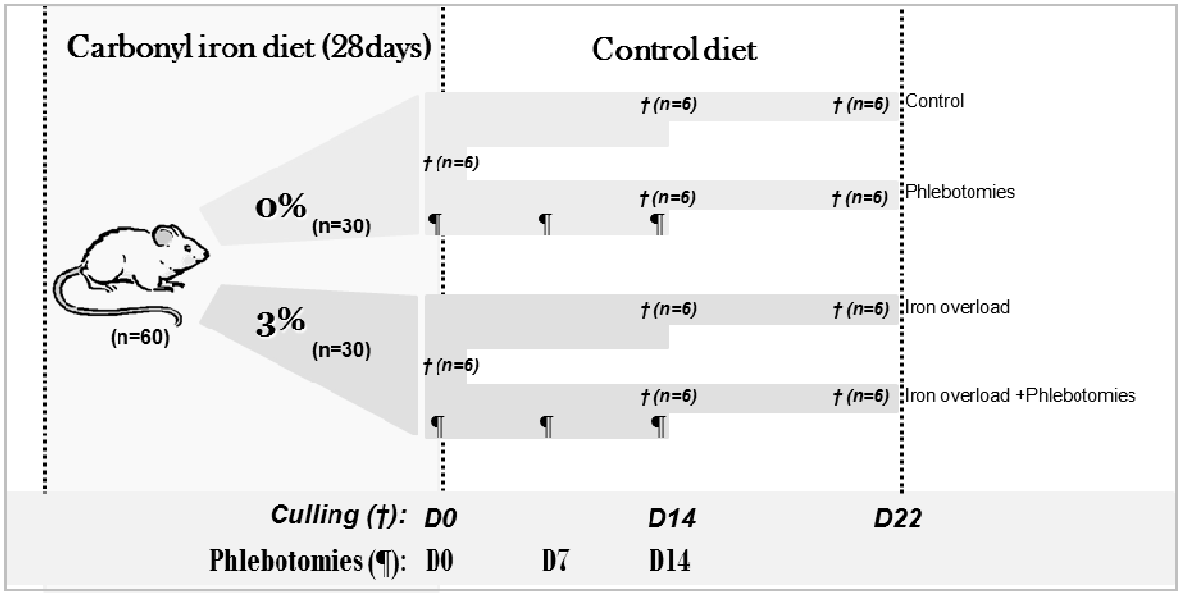

Figure 1

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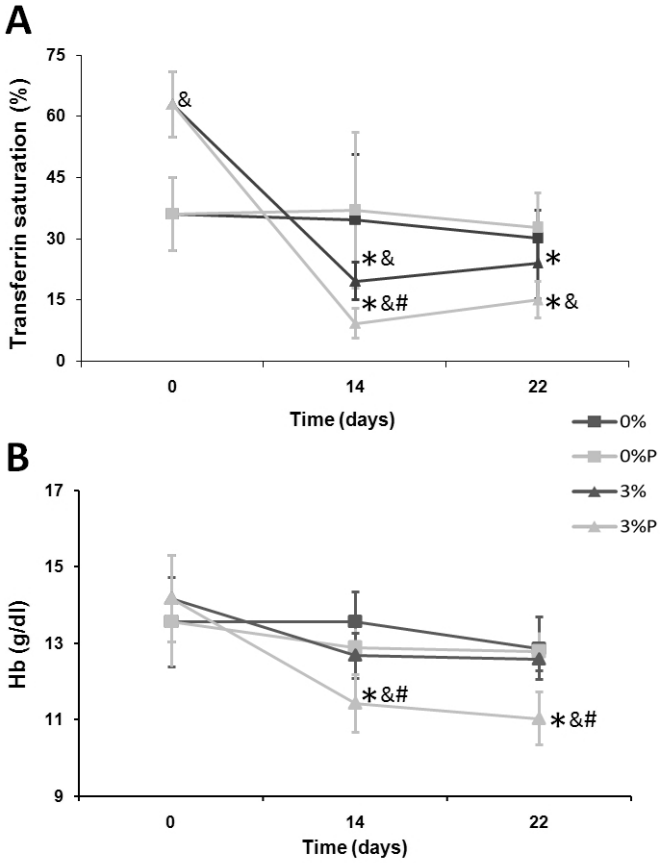

Figure 2

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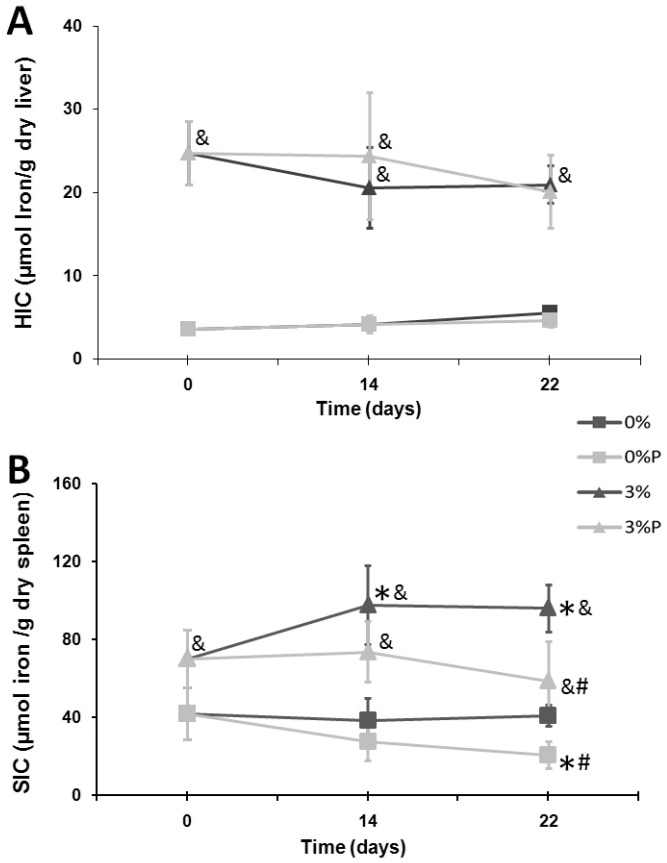

Figure 3

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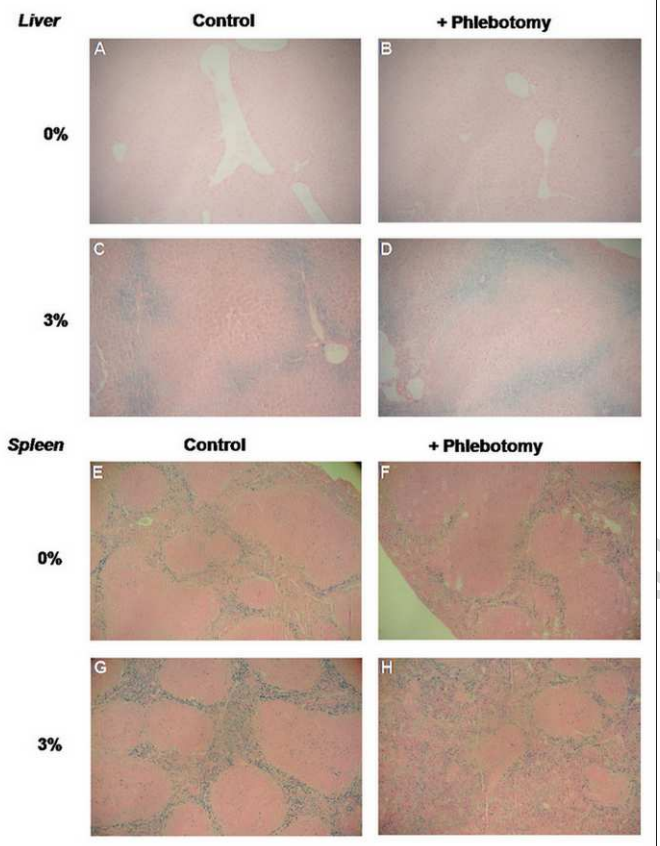

Figure 4

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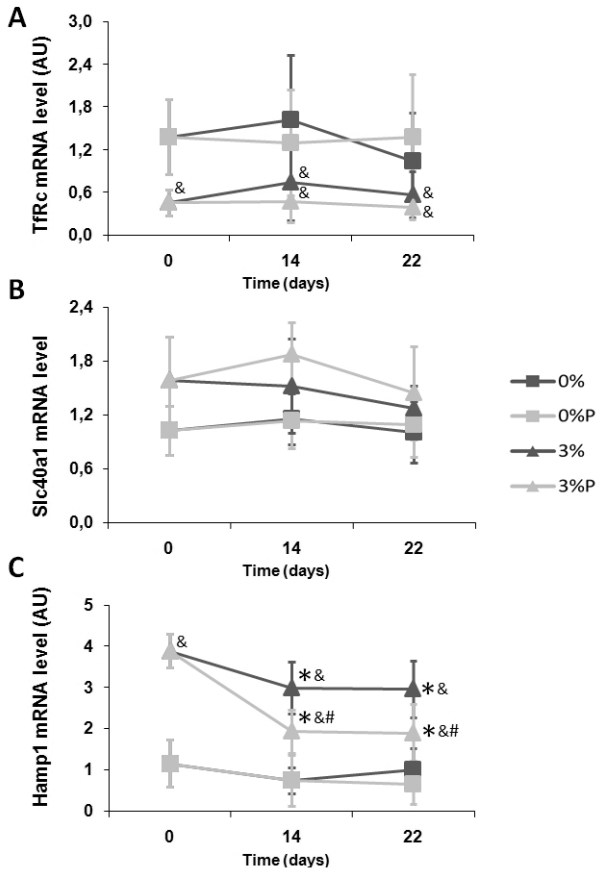

Figure 5

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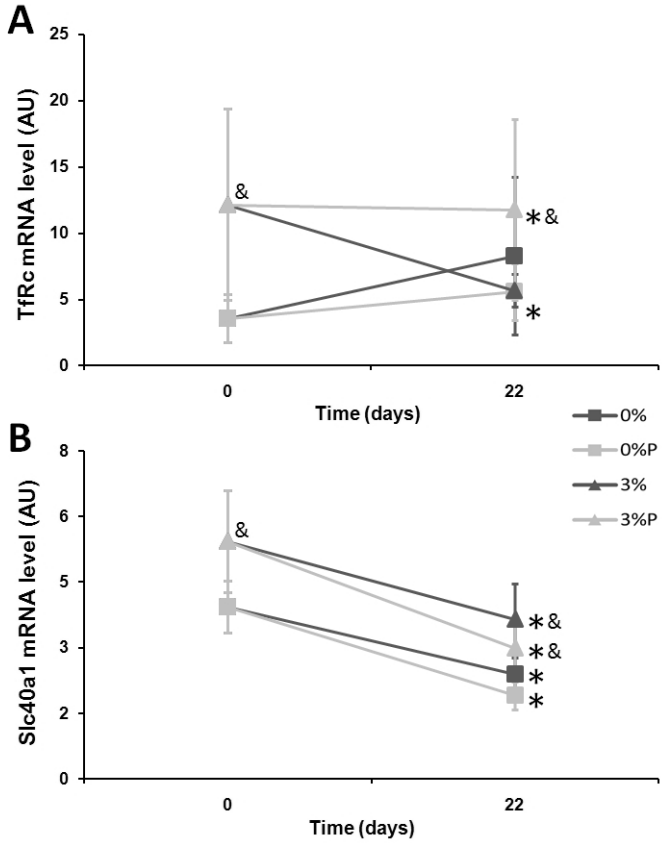

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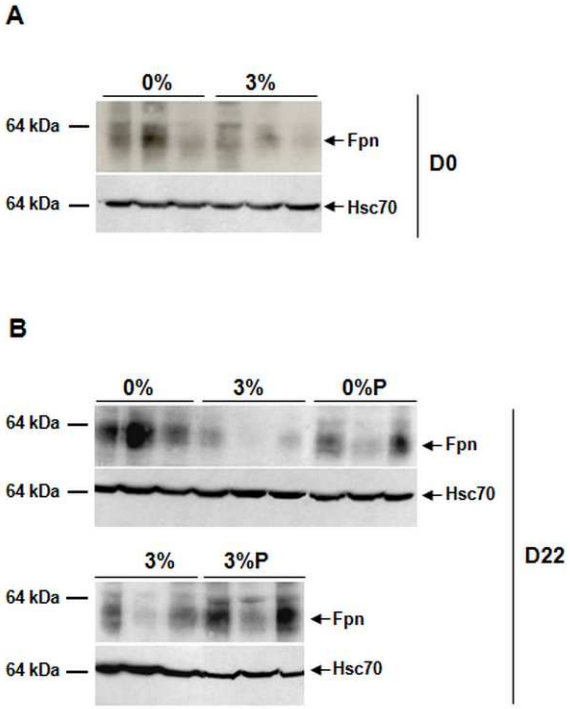

Figure 7

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