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Short title: Co-regulation of spleen Fe, Mo and Mn concentrations

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Abbreviations

Cu: copper

Fe: iron

ICP-MS: Inductively Coupled Plasma Mass Spectrometry

Mn: manganese

Mo: molybdenum

Zn: zinc

Abstract

Iron excess increases the hepatic expression of hepcidin, the systemic iron metabolism regulator, that favors the iron sequestration in the spleen. Genetic iron overload related to hepcidin insufficiency decreases the spleen iron concentration, and increases hepatic iron concentrations, whereas during secondary iron overload the hepcidin expression increases together with spleen iron concentration, in addition to hepatic iron concentrations increase. Links between iron metabolism and other metals being suggested, our aim was to investigate, during iron overload, the relationships between the hepatic hepcidin expression level and the hepatic and splenic concentrations of iron, manganese, copper, zinc and molybdenum, determined using ICP-MS. Hepcidin-deficient mice, secondary iron overload mice models and their respective controls were studied. Spleen molybdenum and manganese concentrations paralleled the modulation of: i) spleen iron concentrations, increasing in secondary iron overload, and decreasing in hepcidin deficiency related iron overload, ii) hepatic hepcidin mRNA expression. Our data suggest that iron, manganese and molybdenum metabolisms could share mechanisms controlling their distribution that are associated to hepcidin modulation. In diseases with abnormal hepcidin levels, including chronic inflammation, special attention should be paid to those metals that could participate to the phenotype.

Key words

Metals; Metabolism; Hemochromatosis; Disease

Introduction

Systemic iron metabolism is tightly controlled through the hepcidin/ferroportin axis. Hepcidin, a small peptide secreted into plasma by hepatocytes, interacts with the iron exporter ferroportin, a protein located on the cell membrane of enterocytes and macrophages, which are the main providers of iron for plasma (1). Hepcidin limits the expression and activity of ferroportin on cell membrane and thus reduces iron egress from these cells toward plasma (2). Therefore, modulation of the expression and secretion of hepcidin controls the distribution of iron within the body. Main signals regulating hepcidin are iron status, inflammation and anemia/erythropoiesis (1, 3).

During secondary iron overload, iron excess induces an increase in hepatic hepcidin expression, which favors iron sequestration in macrophages (3, 4), especially in the spleen, in order to limit the toxic effect of iron. Conversely, iron overload following genetic hemochromatosis, related to p.Cys282Tyr mutation in the *HFE* gene, is characterized by a loss of this adaptive mechanism, due to an abnormally low level of hepcidin despite a state of iron excess (5-7). Similar findings have been described in the genesis of rare hemochromatosis linked to mutations in the hemojuvelin (*HJV*) (8), transferrin receptor 2 (*TFR2*) (9), or hepcidin (*HAMP*) genes (10).

Many links between iron metabolism and non-iron metals have been reported (11). On the one hand, there are genes encoding iron metabolism proteins that may also be involved in the metabolism of non-iron metals, including: i) DMT1, involved in the uptake of non-heme iron by enterocytes and its transfer from endocytic vesicles toward cytoplasm, especially in erythroblasts, can transport copper (Cu), zinc (Zn) and manganese (Mn) (12); ii) ferroportin, involved in the egress of iron from macrophages and enterocytes, can also export Zn and Mn (13, 14). Other proteins, such as transferrin, which delivers iron toward cells, can also interact with other metals (see review in (11)). Ceruloplasmin, synthesized by hepatocytes, is reported to facilitate cell iron egress by interacting with ferroportin (15, 16) and oxidizing ferrous to ferric iron through its copper-dependent ferroxidase activity, thus allowing iron association with transferrin and its transport toward every cell of the organism.

On the other hand, there are metals other than iron that are reported to regulate the expression of iron metabolism genes (17). Notably, it has been reported that divalent metals such as Zn and Cu may modulate the expression of hepcidin, through MTF-1 (MRE-binding

transcription factor-1), and that some metals, especially Zn, may modulate the expression of DMT1.

Moreover, genetic or environmental alterations of Cu or Zn metabolism may strongly affect iron homeostasis and favor the occurrence of iron overload such as during hereditary or acquired aceruloplasminemia (15, 18).

Taken together, these elements underline that potential iron - other metal metabolism inter-connections could play a role in the modulation of diseases associated with iron overload, by influencing either the phenotype intensity or its harmful consequences. In addition, the delineation of these interactions could provide new insights that would lead to a better understanding of metal-associated diseases.

Therefore, our aim was to investigate the impact of the physio-pathological mechanisms of iron overload conditions, related to hepcidin deficiency or secondary iron overload, on the liver and spleen content of other metals, including Cu, Zn, Mn and molybdenum (Mo), which have important metabolic functions as cofactors of many enzymes. For this purpose, we investigated mice models of secondary and genetic iron overload diseases.

Materials and Methods

Animals

Animals were maintained in the UMS Biosit animal facilities in Rennes for the *Hfe*-related and secondary iron overload models, and in Toulouse for the *Hjv* and *Bmp6* knockout models. They were maintained in compliance with French law and regulations. Mice were anesthetized and sacrificed. Livers and spleens were dissected and weighed. Liver and spleen samples were quickly frozen in liquid nitrogen, and then stored at -80°C. They were also fixed in 4% buffered-formaldehyde for histological studies.

Experimentally iron overloaded C57BL/6 male mice (Janvier Labs, Le Genest-Saint-Isle, France) were also included in this study by using liver and spleen samples obtained from previous studies and stored at -80°C in the Rennes Experimental Iron Biobank (REIB). Thus, C57BL/6 male mice were iron-loaded using carbonyl iron or iron dextran as previously reported (4, 19). The normal diet contained 160mg iron per Kg. Briefly: i) carbonyl iron was added to the normal diet at different concentrations (0.5% (n = 6), 1.5% (n = 6), 3% (n = 6)) for 4 months, starting at the age of 5 weeks, and controls (n = 5) received the normal diet, or ii) six-week-old males received one single subcutaneous injection of an iron dextran solution containing 50mg/ml iron and 43 mg/ml dextran. Three doses of iron were tested (0.25 g/kg iron (n = 5); 0.5 g/kg (n = 6); 1 g/kg (n = 6)), whereas controls (n = 6) received a single subcutaneous injection of dextran corresponding to the higher dose of dectran used in the 1g/kg iron group. Animals were studied 2 months later.

Hfe$^{-/-}$ (n = 8) and *Hfe*$^{+/+}$ (n = 6) C57BL/6 male mice 12 months old were studied (20). *Hjv*$^{-/-}$ mice on a 129S6/SvEvTac background (21) were bred to *Bmp6*^{tm1Rob} mice (*Bmp6*$^{-/-}$) on an outbred CD1 background (22). Experiments were carried out on males: 10 wild-type (WT), 10 *Bmp6*$^{-/-}$, and 10 *Hjv*$^{-/-}$ littermates of the F2 progeny.

Trace elements quantification

All samples were handled with care in order to avoid environmental contamination. Trace elements quantification was performed on the ÆM2 platform (University of Rennes1, University Hospital).

Liver and spleen samples, stored at -80°C, were desiccated at 120°C for 15 hours in an oven. Dried samples were then weighed and mineralized according to the following protocol: in teflon tubes, nitric acid solution (Fisher Chemical – Optima Grade®) was added to dried

samples and then the tubes were placed in a MARS6® (CEM) microwave with a temperature maintained at 180°C. Solutions were preserved at 4°C until metals quantification.

Iron (^{56}Fe), manganese (^{55}Mn), copper (^{63}Cu), zinc (^{66}Zn), and molybdenum (^{95}Mo) were quantified by ICP-MS (Inductively Coupled Plasma Mass Spectrometry), on an X-Series II from Thermo Scientific® equipped with collision cell technology (ÆM2 platform, University of Rennes 1/Rennes Hospital). The plasma source was argon (Messer®) with a purity >99.999%. The collision/reaction cell used was pressurized with a mixture of helium (93%) and hydrogen (7%) (Messer®). Ultrapure water was obtained from the Millipore Direct-Q® 3 water station. The nitric acid solution was suprapur, at 69% (Fisher Chemical – Optima Grade®). The internal standard was rhodium (Fisher Scientific®). Calibration ranges were prepared using a multi-element calibrator solution (SCP Science® Plasma Cal). Instrument performance was calibrated using multi-element solutions, tune F and tune A, respectively (Thermo®). Certified reference materials were obtained from NCS (bovine liver ZC71001).

Quantification of hepatic hepcidin 1 mRNA

The expression level of hepcidin 1 mRNA transcripts was determined in the liver of carbonyl iron, iron dextran, *Hfe*$^{-/-}$ mice models and their controls by real time quantitative polymerase chain reaction (RT-PCR). Total liver RNAs were isolated using the Nucleospin® 8 RNA (Macherey-Nagel). The mRNAs were reverse transcribed with the M-MLV reverse transcriptase (Promega®). The following primers were used to amplify hepcidin 1 (forward: 5'-TTCCCAGTGTGGTATCTGTTGC-3' and reverse: 5'-GGTCAGGATGTGGCTCTAG GC-3'), Mfsd5 (forward 5'- tgttgggtgtcatacaagc-3' and Reverse 5'- ggtctagcacaggtgtcc-3') and Tbp 'TATA-binding protein) (forward: 5'-AAACTCTGACCACTGCACCG-3' and reverse: 5'-GTGTGGCAGGAGTGATAGGG-3') as references. Real-time quantitative PCR assays were performed using the qPCR MasterMix Plus for SYBR® Green I (Eurogentec®) and the system StepOne Plus (Real-Time PCR System – Applied Biosystems®). All results were analyzed by StepOne Software v2.1 (Applied Biosystems®). For each cDNA sample, the difference between the threshold cycle for hepcidin 1 amplification and the threshold cycle for TBP was calculated. This enabled normalization of the amount of target to the endogenous reference, TBP.

For *Hjv*$^{-/-}$ and *Bmp6*$^{-/-}$ mice and their controls, total liver RNAs were extracted using Tri-zol (Invitrogen, Carlsbad, CA). Complementary DNA (cDNA) was synthesized using MMLV-RT

(Promega, Madison, WI). Quantitative PCR reactions for hepcidin 1 and HPRT (hypoxanthine phosphoribosyltransferase) (as reference) were prepared with the LightCycler 480 DNA SYBR Green I Master reaction mix (Roche, Mannheim, Germany) and run in duplicate on a LightCycler 480 Instrument (Roche) as previously reported (23).

Statistics

A non-parametric Kruskall-Wallis test, followed when appropriate by a pair-wise comparison using a non-parametric Mann-Whitney test, was performed. Correlations were studied using the Spearman test. A $p<0.05$ was considered significant.

Study approval

Experimental protocols were approved by the Rennes Animal Ethics Committees (b-2007-OL-02 and 03) and the Midi-Pyrénées Animal Ethics Committee (6557-20170218011487), respectively. Animals were given free access to tap water and a standard laboratory mouse chow diet.

Results

1- Impact of secondary iron overload on liver and spleen metal concentrations

Carbonyl iron overloaded mice exhibited, as expected (4, 19), a dose-dependent and strong increase in liver and spleen iron concentrations (Fig 1) reaching 10.5- and 2.9-fold, respectively, in 3% carbonyl iron overloaded animals, compared to controls. Whereas no significant modulation of the concentration of other studied metals was found in the liver, it is noteworthy that a dose-dependent increase in Mo was found in the spleen, reaching 4 times the control value in 3% carbonyl iron overloaded animals. There was also a slight increase (35%) in the spleen Mn concentration (Fig 1).

In order to avoid a potential impact of iron content in the diet on the digestive absorption of other metals, we investigated the effect of an iron overload secondary to parenteral iron dextran administration. As expected, in this model (4, 19), we also found a dose-dependent increase in liver and spleen iron concentrations reaching 35.5- and 3.4-fold, respectively, in animals injected with 1 g/kg iron dextran, compared to controls (Fig 2). Interestingly, in the spleen, a dose-dependent increase in both Mo and Mn (3.5- and 2.6-fold, respectively) was observed, as previously shown in the carbonyl iron overload model (Fig 2).

We next analyzed the relationships between the spleen iron concentration and spleen Mo or Mn concentrations. A strong correlation was found: between Mo and iron levels in the carbonyl iron supplemented model and in the iron dextran model (Fig 3A and 3C, Supplemental data S1); between Mn and iron levels in the iron dextran model (Fig 3D and Supplemental data S1). There was only a moderate correlation between spleen Mn and iron levels in carbonyl iron overloaded mice (Fig 3B and Supplemental data S1).

In line with the mechanisms regulating the expression of hepatic hepcidin mRNA during secondary iron overload, the hepatic iron excess was responsible for a significant increase in hepcidin mRNA levels in both models (Fig 4 A and B).

2- Impact of iron overload related to hepcidin deficiency on liver and spleen metal concentrations in mouse genetic hemochromatosis models

As spleen Mo and Mn levels increased during iron overload inducing increased hepcidin expression, we analyzed the impact of iron overload related to hepcidin deficiency. Mild (*Hfe*$^{-/-}$) and severe (*Hjv*$^{-/-}$ or *Bmp6*$^{-/-}$) mouse genetic hemochromatosis models characterized by hepcidin deficiency and iron overload were investigated.

As expected, *Hfe*$^{-/-}$ mice presented (Supplemental data S2; Fig 4C) a slight increase (3.9-fold) in the liver iron concentration compared with wild-type animals and a small but not significant decrease in hepatic hepcidin. In addition, there was no significant difference between *Hfe*$^{-/-}$ mice and wild-type mice for hepatic Cu, Zn, Mn and Mo concentrations. Spleen iron concentrations did not differ between *Hfe*$^{-/-}$ and control mice and Mn was the only metal that decreased slightly (36%).

In *Hjv*$^{-/-}$ or *Bmp6*$^{-/-}$ mice, which had a much stronger hepcidin deficiency (Fig 4D), there was a strong increase in the hepatic iron concentration compared with control mice (20.1-fold in *Bmp6*$^{-/-}$ and 14.3-fold in *Hjv*$^{-/-}$ mice) (Fig 5). In addition, the spleen iron concentration was significantly decreased in *Bmp6*$^{-/-}$ (65%) and *Hjv*$^{-/-}$ (56%) compared with control mice (Fig 5). Moreover, the spleen Mo concentration was significantly decreased in the two knockout mice models (52% and 56% in *Bmp6*$^{-/-}$ and *Hjv*$^{-/-}$ mice, respectively). There was also a decrease in the spleen Mn concentration in *Bmp6*$^{-/-}$ mice (26%) but not in *Hjv*$^{-/-}$ mice. In addition, in the two knockout models, the spleen Cu level was significantly higher whereas the liver Cu concentration was lower compared to control mice in *Hjv*$^{-/-}$ only.

It is noteworthy that the spleen iron and Mo concentrations were strongly correlated in the three genetic iron overload models (Fig 6A and 6C) whereas the Mn and iron concentrations in the spleen were correlated in *Bmp6*$^{-/-}$ and *Hjv*$^{-/-}$ mice but not in *Hfe*$^{-/-}$ mice (Fig 6B and 6D).

3- Relationships between hepcidin expression and iron and metals concentrations in liver and spleen

As the spleen iron concentration is strongly regulated by hepcidin levels, we hypothesized that the Mo concentration in the spleen could also be correlated with hepatic hepcidin expression levels. Indeed, we found that spleen Mo levels were correlated to hepatic mRNA hepcidin levels in all our models except *Hfe*$^{-/-}$ (Table 1). In addition, the spleen Mn level was correlated to hepatic hepcidin mRNA in *Bmp6*$^{-/-}$ mice and those that received iron dextran. In the liver (Supplemental data S3), Cu and Zn concentrations were inversely correlated with iron levels in *Hjv*$^{-/-}$ and iron dextran mice, respectively.

Table 1. Relationships between hepatic hepcidin 1 mRNA level and liver and spleen metals concentrations – iron (Fe), copper (Cu), manganese (Mn), molybdenum (Mo) and zinc (Zn) – in primary and secondary iron overloads.

			Liver metals					Spleen metals				
	Condition		Fe	Cu	Mn	Mo	Zn	Fe	Cu	Mn	Mo	Zn
Hepatic hepcidin 1 mRNA level	*Primary iron overload*	*Hfe*	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
		Bmp 6	Rho = - 0,62 p = 0,003	ns	ns	ns	ns	Rho = 0,66 p = 0,002	Rho = - 0,49 p = 0,03	Rho = 0,69 p = 0,001	Rho = 0,55 p = 0,01	ns
		Hjv	Rho = - 0,63 p = 0,003	Rho = 0,66 p = 0,001	ns	Rho = 0,55 p = 0,01	ns	Rho = 0,74 p < 0,001	Rho = - 0,58 p = 0,007	ns	Rho = 0,76 p < 0,001	ns
	Secondary iron overload	*Carbonyl*	Rho = 0,67 p < 0,001	ns	ns	ns	ns	Rho = 0,51 p = 0,02	ns	ns	Rho = 0,61 p = 0,003	ns
		Dextran	Rho = 0,87 p < 0,001	ns	ns	ns	Rho = - 0,57 p = 0,009	Rho = 0,82 p < 0,001	ns	Rho = 0,75 p < 0,001	Rho = 0,83 p < 0,001	ns

Correlations between hepatic hepcidin 1 quantitative PCR values (-ΔCt) and metals concentrations were studied using the Spearman test. Statistically significant correlations are represented by correlation coefficient *(Rho)* and p-value *(p)*. *ns*: Non statistically significant correlations are represented.

Discussion

Iron metabolism is tightly regulated in order, in one hand to supply a sufficient amount of iron to cells and, in the other hand to avoid the development of iron overload disease. Our data demonstrate that both spleen Mo and Mn concentrations parallel the modulation of spleen iron stores in iron overload mice models, suggesting that the three metabolisms share regulatory mechanisms. Moreover, they suggest that hepcidin/ferroportin axis could be directly or indirectly associated to this process.

We used two different physio-pathological types of iron overload model. The first one is an experimentally-induced iron overload that provokes iron deposits in hepatocytes and macrophages, especially in the spleen. In these mice, iron excess induces increased hepatic hepcidin expression in order to limit digestive iron absorption and decrease iron leakage from macrophages toward plasma (4). The second one results from the invalidation of *HFE*, *HJV* or *BMP6* genes, which in mice leads to a moderate to strong hepcidin deficiency relative to body iron stores (21, 24-26), similarly to what is found in Human genetic hemochromatosis. This is due to a defect in the BMP/SMAD signal transduction pathway, which is the major inducer of hepcidin expression in the case of iron overload. Our data shows that hepatic iron overload is not associated with a significant modulation in the spleen iron concentration in *Hfe*$^{-/-}$ mice, but with a decrease in the spleen iron concentration in *Hjv*$^{-/-}$ and *Bmp6*$^{-/-}$ mice. This is in accordance with the fact that an abnormally low level of hepcidin favors excessive iron leakage from the spleen (5). As expected, the phenomenon was very strong in *Hjv*$^{-/-}$ mice, mimicking severe juvenile hemochromatosis in humans, as well as in *Bmp6*$^{-/-}$ mice. In *Hfe*$^{-/-}$ mice, the phenotype is less severe, as reported in the classic form of genetic hemochromatosis related to C282Y mutation in humans (1).

In these mice models, we first found that both spleen Mo and Mn concentrations paralleled modulations of the spleen iron concentration, i.e. a simultaneous increase in the secondary iron overload and a decrease in the genetic iron overload. This was found whatever the cause of iron overload for Mo, whereas for Mn, this correlation was found in secondary iron overload models and in *Bmp6*$^{-/-}$ mice. Secondarily, the spleen Mo concentration was correlated to hepatic hepcidin mRNA levels in the severe iron overload models. Moreover, in *Hfe*$^{-/-}$ mice model, which exhibits a lighter hepatic iron overload phenotype than *Hjv*$^{-/-}$ and *Bmp6*$^{-/-}$ mice without a spleen iron concentration decrease, there was no modulation of the

spleen Mo concentration. For Mn, spleen concentrations were correlated to hepatic hepcidin mRNA in the iron dextran mice and *Bmp6*$^{-/-}$ mice models.

Taken together, these data suggest that, as for the spleen iron concentration (2, 5), hepcidin could participate in the control of spleen Mo and Mn concentrations. Given the known impact of hepcidin on ferroportin internalization and degradation (2) and our previous data showing an inverse relationship between hepcidin mRNA expression in the liver and ferroportin protein expression in the spleen (23, 27), and given the parallel modulation of iron, Mo and Mn observed in the spleen, it is well possible that Mo and Mn, similarly to iron, are transported out of the splenic macrophages by ferroportin. It is noteworthy that, in contrast to iron, there was no major increase in Mo or Mn in the liver in our models. This absence of a major increase in Mo and Mn in the liver, despite the fact that hepatocytes have been also reported to express ferroportin (28), contrasts with hepatic accumulation of iron. This can be explained by the fact that, whereas hepatocytes play a major role in the storage of excess iron that cannot be actively excreted by the organism, both Mo and Mn can be actively excreted in urine and/or bile (29-33).

Molybdenum is an indispensable trace element for eukaryotes, as the activities of some enzymes, including sulfite oxidase, mitochondrial amidoxime reducing component, xanthine oxidoreductase and aldehyde oxidase (34) are Mo-dependent. The association of Mo with these enzymes is carried out via the MoCo (Molybdenum Cofactor), synthesized by cells that incorporate Mo.

Some links between Mo and iron metabolisms have been suggested. The main interrelation is the role that xanthine oxidase could play in iron release from ferritin (35). In addition, patients with iron deficiency anemia have lower levels of Mo in their blood, and co-administration of Mo with iron was reported to improve the efficacy of iron supplementation on anemia during pregnancy (36). More recently, it has been demonstrated that (see review in (37): i) MOCS1A, involved in MoCo synthesis, requires two Fe-S clusters; ii) Atm3, playing a role in Fe-S cluster metabolism, is also involved in the synthesis of MoCo; and iii) xanthine oxidoreductase and aldehyde oxidase activities are dependent on Fe-S clusters, in addition to MoCo. It is noteworthy that we did not find, in liver and spleen, a significant modulation of the mRNA level of Mfsd5 (Supplemental data S4), the only identified Mo transporter. Our data provide new insights into the relationships between the hepatic hepcidin mRNA levels, iron and Mo concentrations in the spleen.

The consequences of such findings for patients should be carefully evaluated. Mo deficiencies are mostly rare genetic diseases linked to mutations in the genes involved in the synthesis of MoCo and leading to fatal neurological diseases (34). Excess Mo is mainly related to occupational activities or contaminated nutrients, whose consequences are not well characterized. It has been reported in duck that spleen Mo accumulation decreases antioxidant capacity, favors cell apoptosis and increases heat shock proteins and TNFα in spleen (38) (39). Similar data have been found in chickens fed with a high Mo diet (40). In humans, excess Mo has been associated with purine metabolism modulation, joint symptoms and/or the development of gout suggesting the potential involvement of Mo in this disease (31, 41, 42), xanthine oxidase being the Mo-dependent enzyme involved in uric acid production. Moreover, it is noteworthy that, during *HFE*-hereditary hemochromatosis, hyperferritinemia level, which reflect iron stores, was recently found to be associated with the risk of hyperuricemia (43). Lastly, it has been suggested that Mo accumulation is correlated with C-parathormone and calcium levels and could contribute to dialysis-related arthritis (44). Taken together, these elements suggest that a change in the spleen Mo concentration could modulate the immune function and/or favor metabolic alterations during iron overload diseases, as well as in other conditions with a high level of hepcidin, including chronic diseases and/or metabolic syndromes. Whether a disturbance of Mo flow plays a role in the occurrence of microcrystalline arthropathy should be considered. In addition, the potential impact of abnormal Mo metabolism during inflammatory states, with high hepcidin levels, such inflammatory systemic diseases, must be evaluated.

Manganese is an essential component of metalloenzymes such as Mn superoxide dismutase, which is involved in the control of oxidative stress.

Links between iron and Mn metabolisms have already been suggested in humans (45). Mn is potentially taken up by DMT1 (12), the transmembrane iron importer expressed in enterocytes, which also plays a role in the egress of iron from endocytic vesicles toward the cytosol in other cells. Mn is probably also exported from cells by ferroportin protein, the only known iron exporter, expressed on enterocytes and macrophages and controlled by hepcidin (14). Moreover, relationships between genetic iron overload related to *Hfe* deficiency and alteration of Mn metabolism have been reported, suggesting relationships between iron and Mn metabolisms (46, 47). Our results demonstrating that both genetic and experimentally-induced iron overloads modulate Mn and iron concentrations in the spleen reinforce these

findings. Moreover, the correlation between the spleen Mn concentration and hepatic hepcidin level is in accordance with the idea that the hepcidin/ferroportin duo plays a role in the maintenance of Mn homeostasis and corresponds to recent studies (47, 48, 49,50) showing Mn metabolism alteration, as well as accumulation in the bones of flatiron mice, a model for the ferroportin disease.

Thus, the consequences of links between iron metabolism disturbance, hepcidin and Mn metabolism should also be evaluated carefully, especially regarding neurological effects. For example, although Mn deficiency is not well characterized in humans, excess of Mn may induce motor incoordination, memory deficit and psychiatric disorders (51), as found in patients exposed to environmental contamination and those exhibiting liver failure during chronic liver diseases (52). *Hfe*$^{-/-}$ mice exposed to Mn demonstrate an alteration of spatial memory (53) and emotional behavior. These data suggests that hepcidin deficiency could induce vulnerability to Mn toxicity. The potential impact of Mn metabolism alteration when hepcidin is increased during secondary iron overload and chronic inflammatory states should also be evaluated. Lastly, further studies are required to determine whether iron metabolism manipulation and/or hepcidin modulation is potentially interesting to tackle diseases associated with Mn metabolism.

In summary, our data obtained in mice models demonstrate that, during iron overload spleen Mo and Mn concentrations follow the spleen iron concentrations and hepatic hepcidin expression. They suggest that diseases of iron metabolism related to systemic misdistribution could be associated to Mn and Mo metabolism alterations. Knowing the important biological role of both metals, the evaluation of the consequences of Mo and Mn metabolic alterations during iron metabolism diseases, particularly for brain, joints, bones and immunity, requires special attention. Moreover, whether treatments controlling hepcidin and its interaction with ferroportin, that play a major role in systemic iron metabolism control, could act on Mn or Mo homeostasis should be characterized.

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Author contributions

T. Cavey, M. Ropert and O. Loréal designed research, performed research, analysed data and wrote the paper. P. Brissot analyzed data and wrote the paper. MP. Roth performed research and wrote the paper. C. Latour, ML. Island, P. Leroyer and H. Coppin performed research. P. Guggenbuhl and C. Bendavid wrote the paper.

Conflict of interest disclosures

The authors have declared that no conflict of interest exists concerning this work.

Figure legends

Figure 1. Quantification of metals in carbonyl iron overloaded mice.

The iron was added to a standard diet (control mice (C); n=5) at different concentrations: 0.5% (n=6), 1.5% (n=6), 3% (n=6). Iron (Fe), copper (Cu), manganese (Mn), molybdenum (Mo) and zinc (Zn) concentrations were determined in the liver (upper panel) and spleen (lower panel). The median is represented by a horizontal line within the boxes, the 25th and 75th percentiles are represented by the lower and upper lines of boxes, respectively, and the 10th and 90th percentiles are represented by horizontal lines located on either side of boxes. A non-parametric Kruskall-Wallis test, followed by a pair-wise comparison using a Mann-Whitney test, was performed. Statistically significant differences are presented as *$p<0.05$ and **$p<0.01$.

Figure 2. Quantification of metals in iron dextran overloaded mice.

The mice received one subcutaneous injection of iron dextran at different concentrations: 0.25 (n=5), 0.5 (n=6), 1 g/kg (n=6) and were compared to control mice (C) (n=6). Iron (Fe), copper (Cu), manganese (Mn), molybdenum (Mo) and zinc (Zn) concentrations were determined in the liver (upper panel) and spleen (lower panel). The median is represented by a horizontal line within the boxes, the 25th and 75th percentiles are represented by the lower and upper lines of boxes, respectively, and the 10th and 90th percentiles are represented by horizontal lines located on either side of boxes. A non-parametric Kruskall-Wallis test, followed by a pair-wise comparison using a Mann-Whitney test, was performed. Statistically significant differences are presented as *$p<0.05$ and **$p<0.01$.

Figure 3. Relationship between spleen iron and both molybdenum and manganese concentrations in secondary iron overloads.

Relationships between spleen iron and molybdenum concentrations (A and C), and spleen iron and manganese concentrations (B and D) in carbonyl iron overloaded mice (A and B) mice and iron dextran overloaded mice (C and D). The A and B panel include mice with iron added to a normal diet at different concentrations (0.5%, 1.5%, 3%; n=6 in each group) and control mice (C; n=5). The C and D panel include mice that received one subcutaneous injection of iron dextran at 0.25 (n=5), 0.5 (n=6), 1 g/kg (n=6) and control mice (C; n=6). Correlations were studied using the Spearman test. Statistically significant correlations are represented by correlation coefficient (*Rho*) and p-value (*p*).

Figure 4. Liver hepcidin 1 mRNA levels in experimental models.

A: Carbonyl iron overloaded mice receiving iron added to a normal diet at different concentrations: 0.5% (n=6), 1.5% (n=6), 3% (n=6) and control mice (C; n=5). B: Iron dextran overloaded mice receiving one subcutaneous injection of iron dextran at different concentrations: 0.25 (n=5), 0.5 (n=6), 1 g/kg (n=6) and control mice (C; n=6). C: *Hfe*$^{+/+}$ (n=6) and *Hfe*$^{-/-}$ mice (n=8). D: *Bmp6* KO mice (n=10), *Hjv* KO mice (n=10) and control mice (WT; n=10). Hepcidin mRNA level was normalized to the expression level of TBP (A, B, C) or HPRT (D). Values of gene expression are expressed relatively to the control group's mean value (M) : 2^-(DCt-M). M corresponds to the delta Ct mean in the control group. The median is represented by a horizontal line within the boxes, the 25th and 75th percentiles are represented by the lower and upper lines of boxes, respectively, and the 10th and 90th percentiles are represented by horizontal lines located on either side of boxes. A non-parametric Kruskall-Wallis test, followed by a pair-wise comparison using a Mann-Whitney test, was performed. Statistically significant differences are presented as *p<0.05 and **p<0.01 and ***p<0.001.

Figure 5. Quantification of metals in iron overloaded mice related to hepcidin deficiency.
In *Bmp6*$^{-/-}$ mice (Bmp6 KO; n=10), *Hjv*$^{-/-}$ mice (Hjv KO; n=10) and control mice (WT; n=10): iron (Fe), copper (Cu), manganese (Mn), molybdenum (Mo) and zinc (Zn) concentrations were determined in the liver (upper panel) and spleen (lower panel). The median is represented by a horizontal line within the boxes, the 25th and 75th percentiles are represented by the lower and upper lines of boxes, respectively, and the 10th and 90th percentiles are represented by horizontal lines located on either side of boxes. A non-parametric Kruskall-Wallis test, followed by a pair-wise comparison using a Mann-Whitney test, was performed. Statistically significant differences are presented as *$p<0.05$, **$p<0.01$ and ***$p<0.001$.

Figure 6. Relationship between spleen iron and both molybdenum and manganese concentrations in iron overloads related to hepcidin deficiency.
Relationships between spleen iron and molybdenum concentrations (A and C), spleen iron and manganese concentrations (B and D) in *Hfe*$^{+/+}$ (n=6) and *Hfe*$^{-/-}$ (n=8) mice (A and B) and *Bmp6* KO (n=10), *Hjv* KO (n=10) and control mice (WT; n=10) (C and D). Correlations were studied using the Spearman test. Statistically significant correlations are represented by correlation coefficient (*Rho*) and p-value (*p*). Not statistically significant correlations are represented by *ns*.

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Figure 1

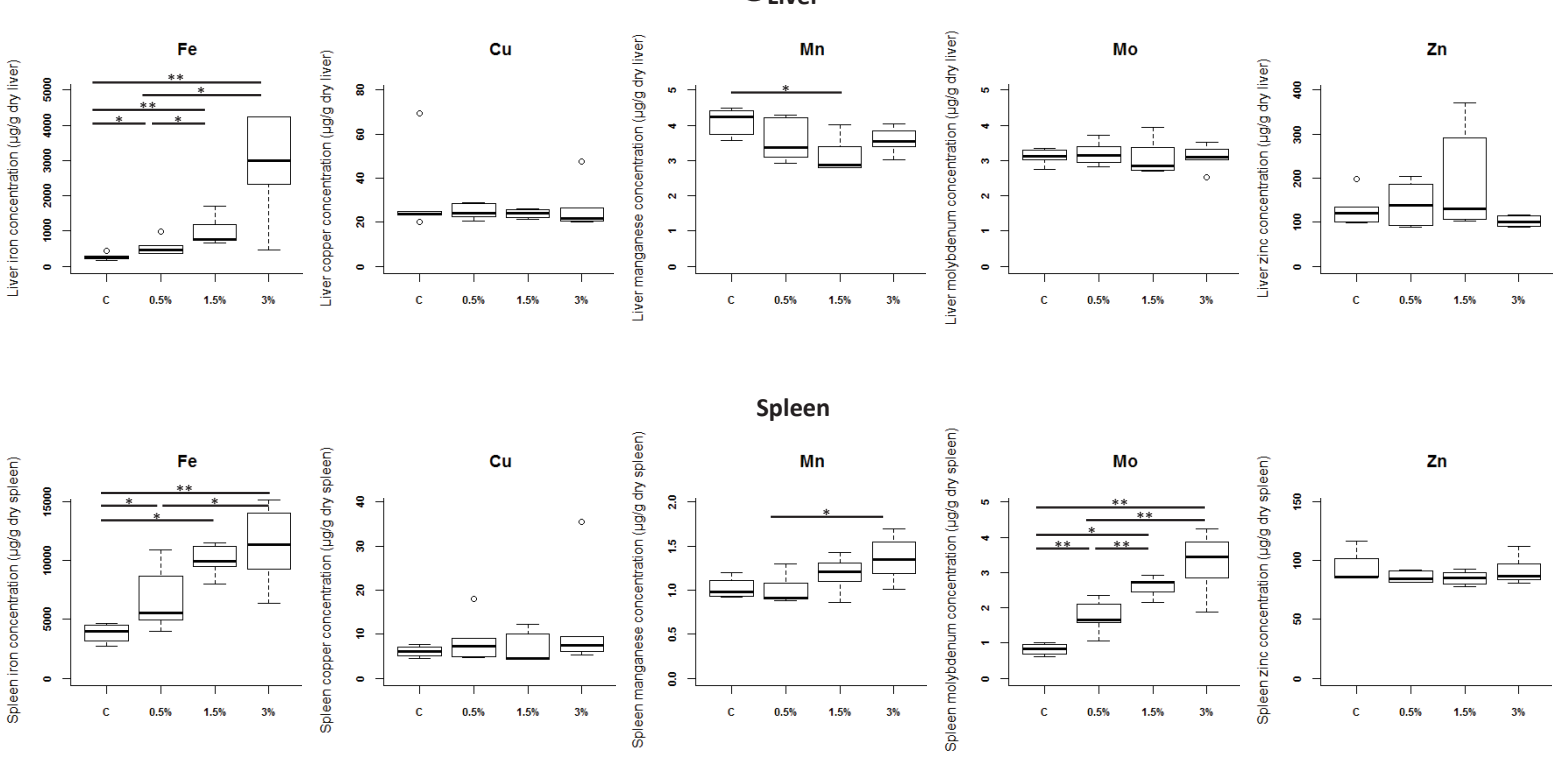

Figure 1.

Figure 2

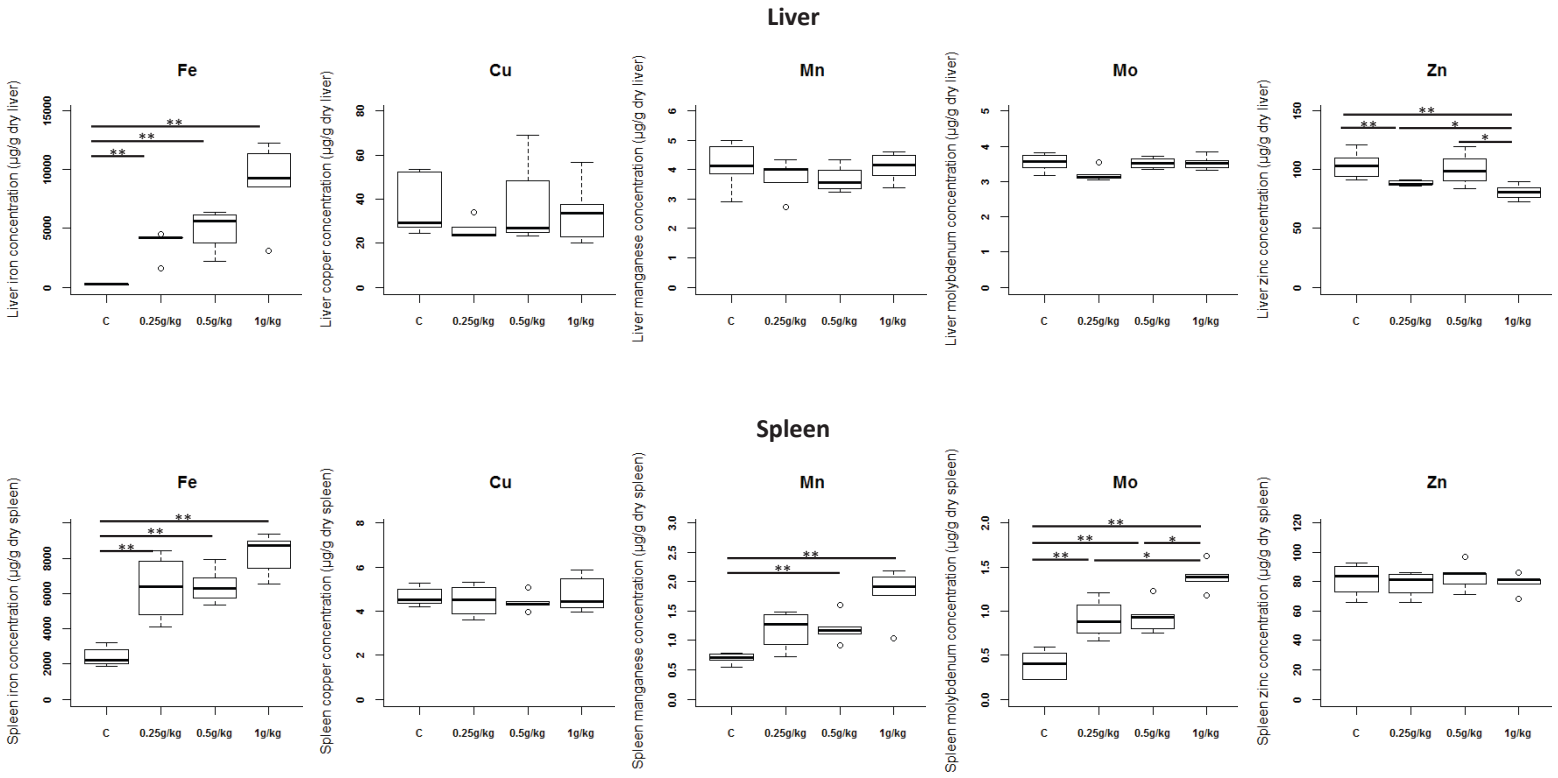

Figure 2.

Figure 3

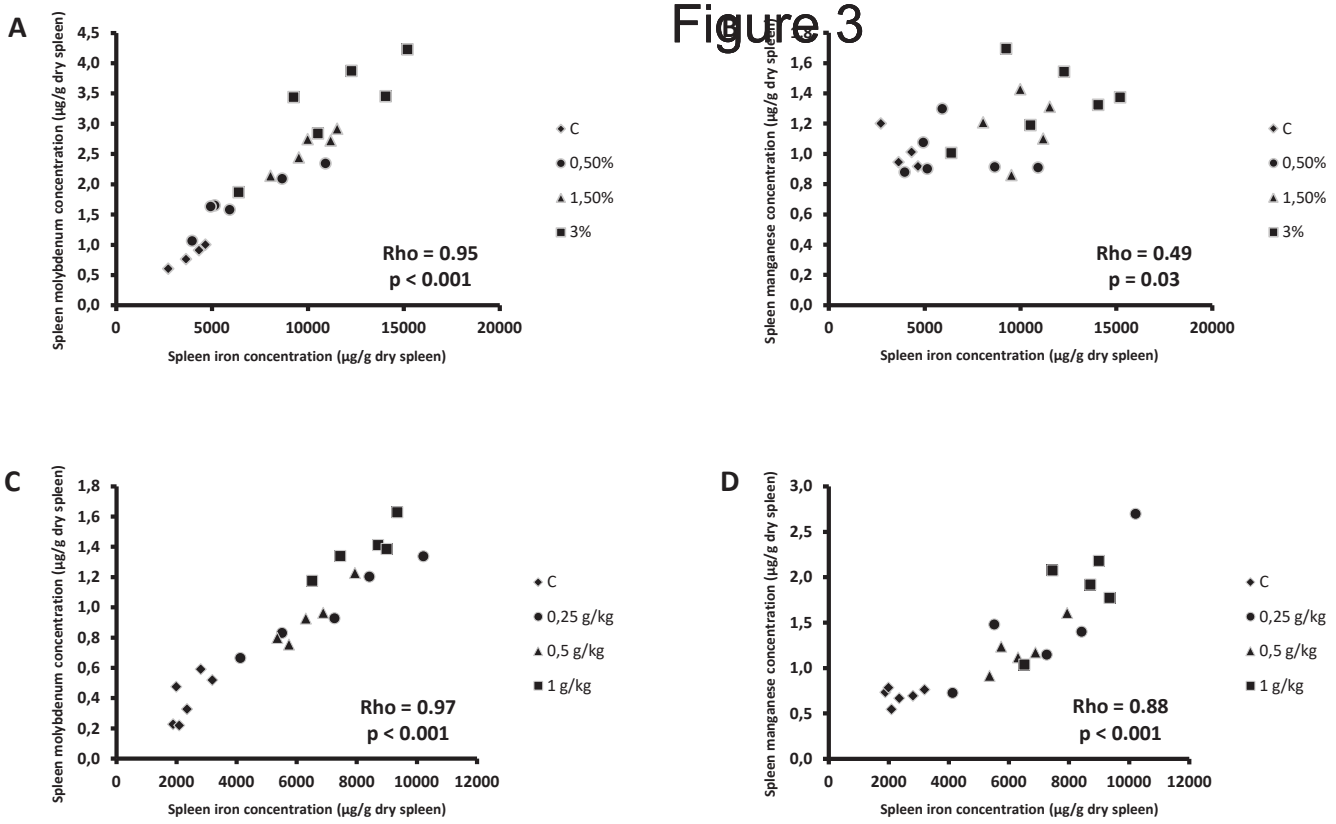

Figure 3.

Figure 4

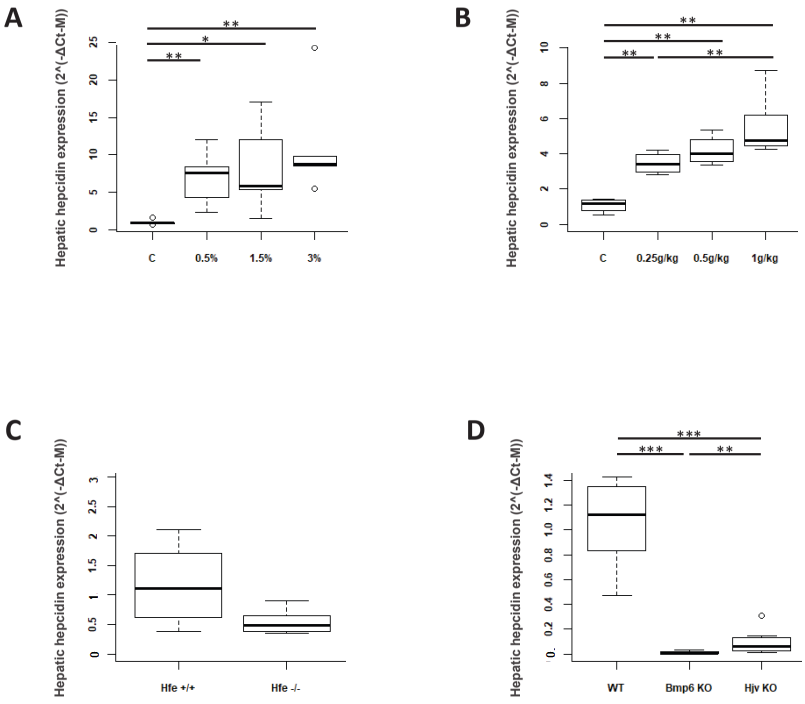

Figure 4.

Figure 5

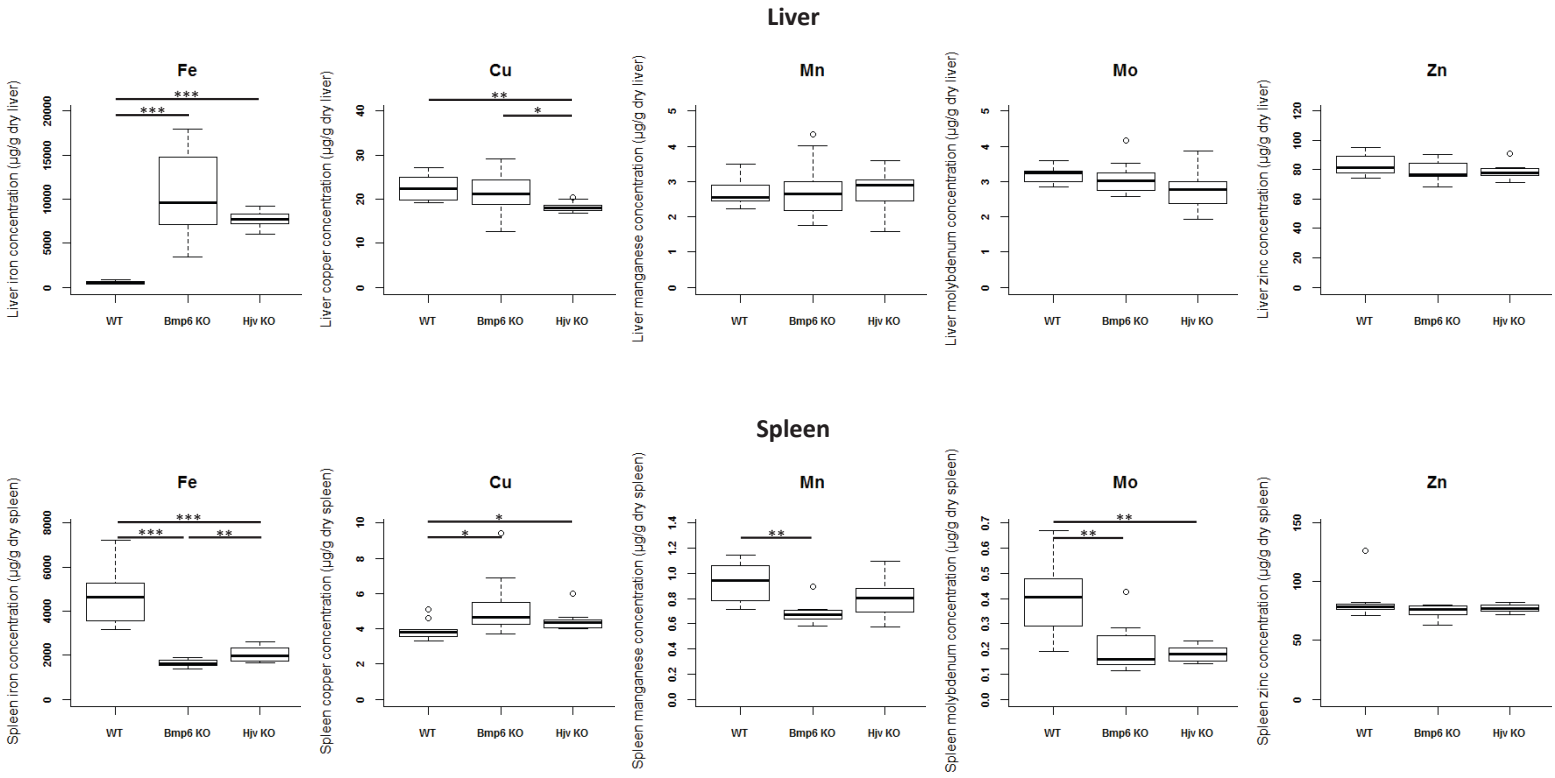

Figure 5.

Figure 6

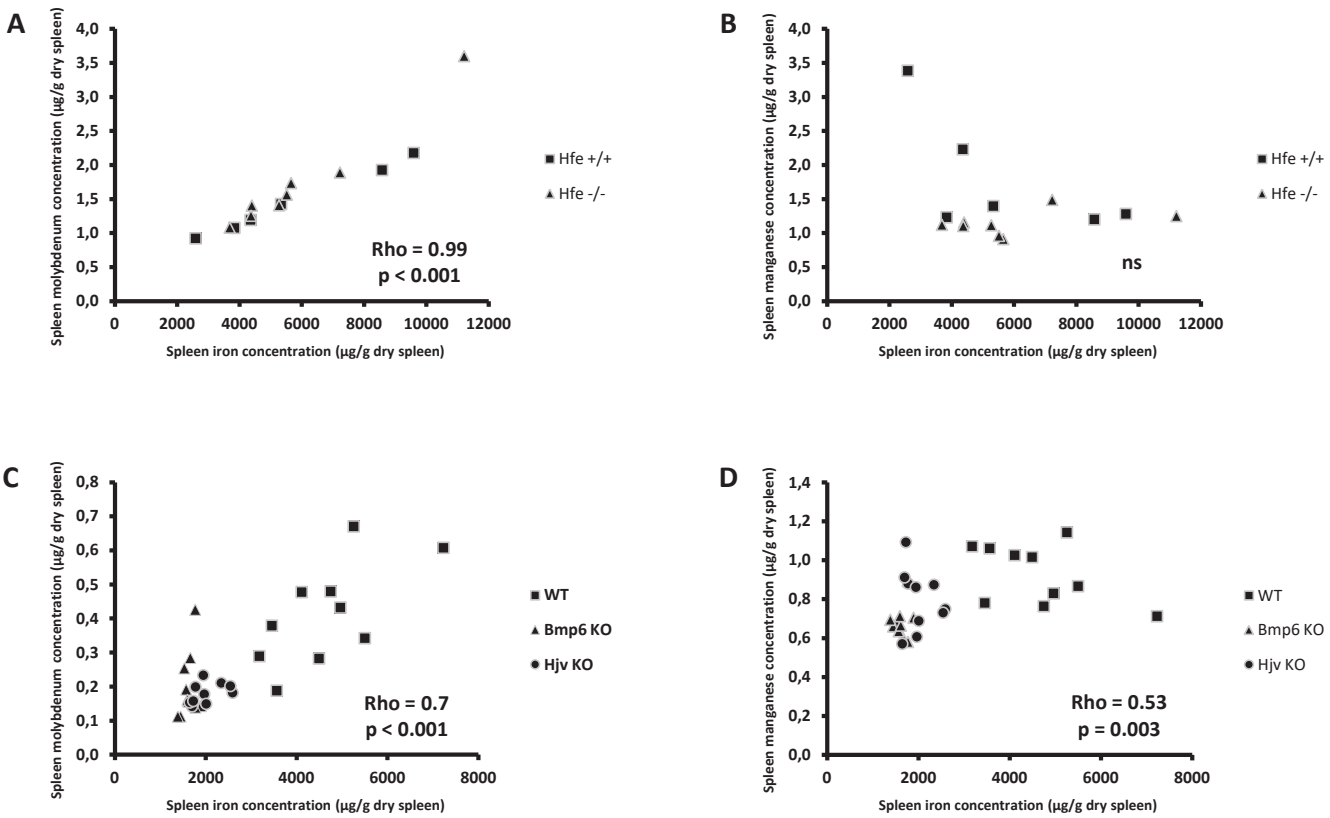

Figure 6.