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Hemochromatosis: Evaluation of the dietary iron model and regulation of hepcidin

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ABSTRACT

Our knowledge of iron homeostasis has increased steadily over the last two decades; much of this has been made possible through the study of animal models of iron-related disease. Analysis of transgenic mice with deletions or perturbations in genes known to be involved in systemic or local regulation of iron metabolism has been particularly informative. The effect of these genes on iron accumulation and hepcidin regulation is traditionally compared with wildtype mice fed a high iron diet, most often a 2% carbonyl iron diet. Recent studies have indicated that a very high iron diet could be detrimental to the health of the mice and could potentially affect homeostasis of other metals, for example zinc and copper. We analyzed mice fed a diet containing either 0.25%, 0.5%, 1% or 2% carbonyl iron for two weeks and compared them with mice on a control diet. Our results indicate that a 0.25% carbonyl iron diet is sufficient to induce maximal hepatic hepcidin response. Importantly these results also demonstrate that in a chronic setting of iron administration, the amount of excess hepatic iron may not further influence hepcidin regulation and that expression of hepcidin plateaus at lower hepatic iron levels. These studies provide further insights into the regulation of this important hormone.

1. Introduction

Animal models of disease have helped us understand how mutations in disease-implicated genes contribute to the development of pathology. Most life forms require iron for their proper physiological functioning. Animal models of iron disorders have been instrumental in extending our knowledge of iron regulation and the flux of iron within the body. Transgenic and knockout mice with mutations or deletions in genes that are associated with regulation of iron metabolism are important tools. Studies using these transgenic mice have traditionally compared them to wildtype mice with either diet-induced iron overload [1–4] or anemia [4,5], to better understand the molecular functions of the affected genes. Most studies have used mice fed with a diet containing 2% carbonyl iron for one or two weeks [1–4] as a means of inducing dietary iron overload.

An increase in body iron levels is known to increase levels of the liver-expressed peptide, hepcidin, in mice and humans. Hepcidin, a 25-amino acid peptide, is also known as the master regulator of iron homeostasis, and its synthesis is regulated by many factors (reviewed in [6,7]) besides iron. The 2% carbonyl iron diet increases hepcidin levels not only in the livers of wildtype mice, but also in transgenic mice which have mutations in the genes proposed to be involved in the iron-mediated regulation of hepcidin, namely *Hfe* [3], *Tfr2* [3] and *Hjv* [8]. This suggests that there may be additional pathways that are activated in the presence of excess iron and hence lead to increased hepcidin expression in the livers.

In addition, it has been shown that exposure to a high-iron diet may affect the metabolism of other metals including copper [9,10]. There is also evidence showing that high-iron levels interferes with zinc absorption, where an increase in iron levels may result in a decrease in zinc absorption in both humans and rats [11,12]. A prolonged exposure to a high-iron diet has been shown to be detrimental to the health of rats [13]. Rats fed a high-iron diet had significantly lower body weights and increased liver and heart weights, as compared to controls. In addition, rats fed a high-iron diet also exhibited signs of cardiac hypertrophy and copper deficiency-induced anemia [13]. It was shown that in mice fed a high-iron diet, systemic copper homeostasis is disturbed [10]. Mice fed a high-iron diet had different tissue copper distribution leading to changes in the bioavailability of copper. These changes translate to symptoms of severe copper deficiency [10]. Together these observations suggest that a high-iron diet model may not be the best suited either to the health of the mice or the experimental design.

In order to determine the effect of varying iron levels on the hepcidin response and the optimum concentrations to be used in studies of

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dietary iron overload, we fed mice a range of diets containing between 0.25 and 2% carbonyl iron. These mice were fed the diets for two weeks as previous studies have established that hepatic hepcidin and serum iron levels saturate relatively quickly [4,14]. We examined iron parameters and expression of genes involved in the systemic regulation of iron metabolism. Our results indicate that the 0.25% carbonyl iron diet is sufficient to induce a hepcidin response and the regulatory system appears to be saturated at this iron level.

2. Methods

2.1. Animals and diets

Wildtype male C57BL6/J mice were purchased from the Animal Resource Centre (Murdoch, Western Australia). Animal experimentation was performed as per the guidelines and approval of the QIMR Berghofer (QIMRB) Animal Ethics Committee. The mice were housed under a 12 h light/dark cycle, were provided food and water ad libitum. Mice (n = 5) at the age of 4 weeks were fed either a control diet (AIN93G, Specialty Feeds), or a high-iron diet containing either 0.25%, 0.5%, 1% or 2% carbonyl iron for two weeks. The diets (Specialty Feeds, Glen Forest, Western Australia) were prepared by mixing 2% carbonyl iron diet (SF07-082, Specialty Feeds) with iron-deficient diet (SF01-017, Specialty Feeds). The 1% carbonyl iron diet was composed of 1:1 ratio of 2% carbonyl iron diet (SF07-082, Specialty Feeds) and iron-deficient diet (SF01-017, Specialty Feeds). This was then used to make 0.5% and 0.25% through serial dilution. At the end of two weeks the animals were sacrificed and tissues collected for further analysis.

2.2. Measurement of iron indices

Iron parameters (serum iron concentrations, hepatic iron concentration (HIC), splenic iron concentration (SIC), cardiac iron concentration (CIC), duodenal iron concentration (DIC) and pancreatic iron concentrations (PIC)) were measured as described previously [15,16].

2.3. Real-time quantitative PCR

Total RNA isolated from the liver, spleen and duodenum was used to prepare cDNA using a SensiFAST™ kit, (Bioline, Sydney, NSW, Australia). Real-time quantitative-PCR (qPCR) was performed using SensiFAST™ SYBR No-Rox (Bioline) as described previously [5].

2.4. Perls' staining

Tissues were fixed in 10% formalin and processed and sectioned by the Histology Facility at QIMRB. Perls' Prussian blue staining for iron was performed on the tissues as described by McDonald et al. [17]. The slides were scanned using the Aperio AT Turbo (Aperio, Vista, CA) at 40× magnification and scans were analyzed using Imagescope software (Aperio).

2.5. Western blotting

Liver tissue (100 mg) was homogenized in a lysis buffer containing phosphatase inhibitors [18] using the Precellys Evolution tissue homogenizer (Bertin Instruments, Montigny-le-Bretonneux, France). Protein lysate (20 μg) was electrophoresed on 10% SDS-polyacrylamide gels and transferred onto a nitrocellulose membrane using the Trans-Blot Turbo apparatus (Bio-Rad Laboratories, Gladesville, NSW, Australia). After blocking with 10% non-fat milk for 2 h at room temperature, the membranes were incubated with primary antibodies (anti-Tfr2 [19] 1:10,000); anti-pSmad (Cell Signalling Technology, Danvers, MA) 1:1000, anti Smad1 (Cell Signalling Technology) 1:1000 and anti-β actin (Sigma-Aldrich 1:20,000) overnight at 4 °C. The membrane was then washed and incubated with anti-rabbit IgG-horseradish peroxidase (1:10000, Invitrogen, Life Technologies) diluted in 10% non-fat milk for 1 h at room temperature. The excess secondary antibody was washed off and the blots were incubated with chemiluminescent substrate (Lumina Forte; Merck Millipore, Kilsyth, Victoria, Australia) for 5 min at room temperature. The blots were then exposed to X-ray film (Fujifilm, Brookvale, NSW, Australia). Films were developed using the Minolta film processor (Konica Minolta Medical and Graphic, Tokyo, Japan).

2.6. Immunohistochemistry

Formalin-fixed paraffin-embedded liver sections were deparaffinised in xylene and rehydrated in graded ethanol. Following heat-induced epitope retrieval (10 mM Tris/1 Mm EDTA pH 9.0 solution), sections were blocked with 20% heat-inactivated fetal bovine serum for 1 h. Sections were incubated with anti-4 hydroxynonenal (4HNE) (1:250; Abcam, Cambridge, MA) overnight at 4 °C and staining detected using the Dako EnVision+ Dual Link System-HRP (DAB+) kit (Agilent Technologies, Santa Clara, CA). Images were acquired at 10× magnification using the Nikon Eclipse TS2 microscope (Nikon Australia, Rhodes, Australia).

3. Results

3.1. Differences in body weights and oxidative stress in mice fed varying concentrations of iron

The first observation that we made was that the mice fed the diet with high concentrations of iron i.e. 1% and 2%, had a lower body weight than mice fed lower iron concentrations (Fig. 1A). No significant differences in the liver and spleen weights of the mice were observed (data not shown). The decrease in the body weights of the mice fed iron diets with higher iron concentrations could be due to either the mice eating less or the increased iron in the diet being directly detrimental to health. An increase in iron levels is also known to result in an increase in reactive oxygen species leading to oxidative stress and associated DNA, lipid and protein damage [20]. Thus, we examined the mRNA levels of two markers of oxidative stress, superoxide dismutase 1 and 2 (*Sod1* and *Sod2*) in the livers of these mice (Fig. 1B and C). Interestingly, the mRNA levels of both *Sod1* and *Sod2* were significantly higher in the livers of mice fed a diet containing 1% or 2% carbonyl iron. We next measured the levels of oxidative damage in the livers of these mice by staining with 4-hydroxy-2-nonenal (4-HNE). 4-HNE is a known marker of oxidative damage, and lipid peroxidation. We did not observe any differences in the 4-HNE staining in the livers of these mice (Supplementary Fig. 1). In the absence of any detectable oxidative damage, it is more likely the weight loss is due to the mice eating less of the high-iron diet.

3.2. Iron parameters in the mice fed an increasingly iron-rich diet

We measured the iron parameters including tissue iron concentrations for liver (HIC), spleen (SIC), heart (CIC), duodenum (DIC) and pancreas (PIC). We also measured the total serum iron and transferrin saturation in the mice fed diets with different concentrations of iron. We saw a gradual and significant increase in the HICs (Fig. 2A) with increasing percentage of carbonyl iron in the diets. Interestingly, the SIC (Fig. 2B), total serum iron (Fig. 2F) and transferrin saturation (Fig. 2G) reached maximal levels at 0.25% of carbonyl iron in the diet. The level of iron in the heart (Fig. 2C), duodenum (Fig. 2D) and pancreas (Fig. 2E) increased significantly in the mice fed a diet containing 1.0% or more iron as compared to the controls. This gradual increase in the amount of iron being accumulated in the liver was also reflected in the Perls' staining for iron (Fig. 3). We examined the localization of iron in the duodenum, heart, liver, pancreas and the spleen of these mice (Fig. 3). There were no differences in the localization of iron in the

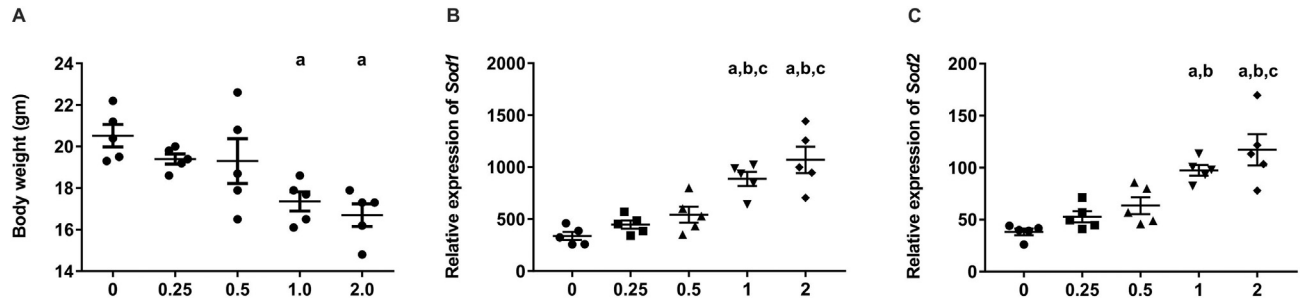

Fig. 1. The effect of increasing dietary iron on the body weights of mice. (A) Body weights of 6-week-old male mice (n = 5) were measured. Mice fed a diet containing 1% or more iron have significantly lower body weights as compared to mice fed either a control, 0.25% or 0.5% iron diet. Relative mRNA expression of Sod1 (B), and Sod2 (C) was measured in the livers of 6-week-old male mice fed a control diet (0) or a diet containing 0.25%, 0.5%, 1.0% or 2.0% carbonyl iron. There was a significant increase in the expression of Sod1 and Sod2 in the livers of the mice fed a diet containing > 1% carbonyl iron.

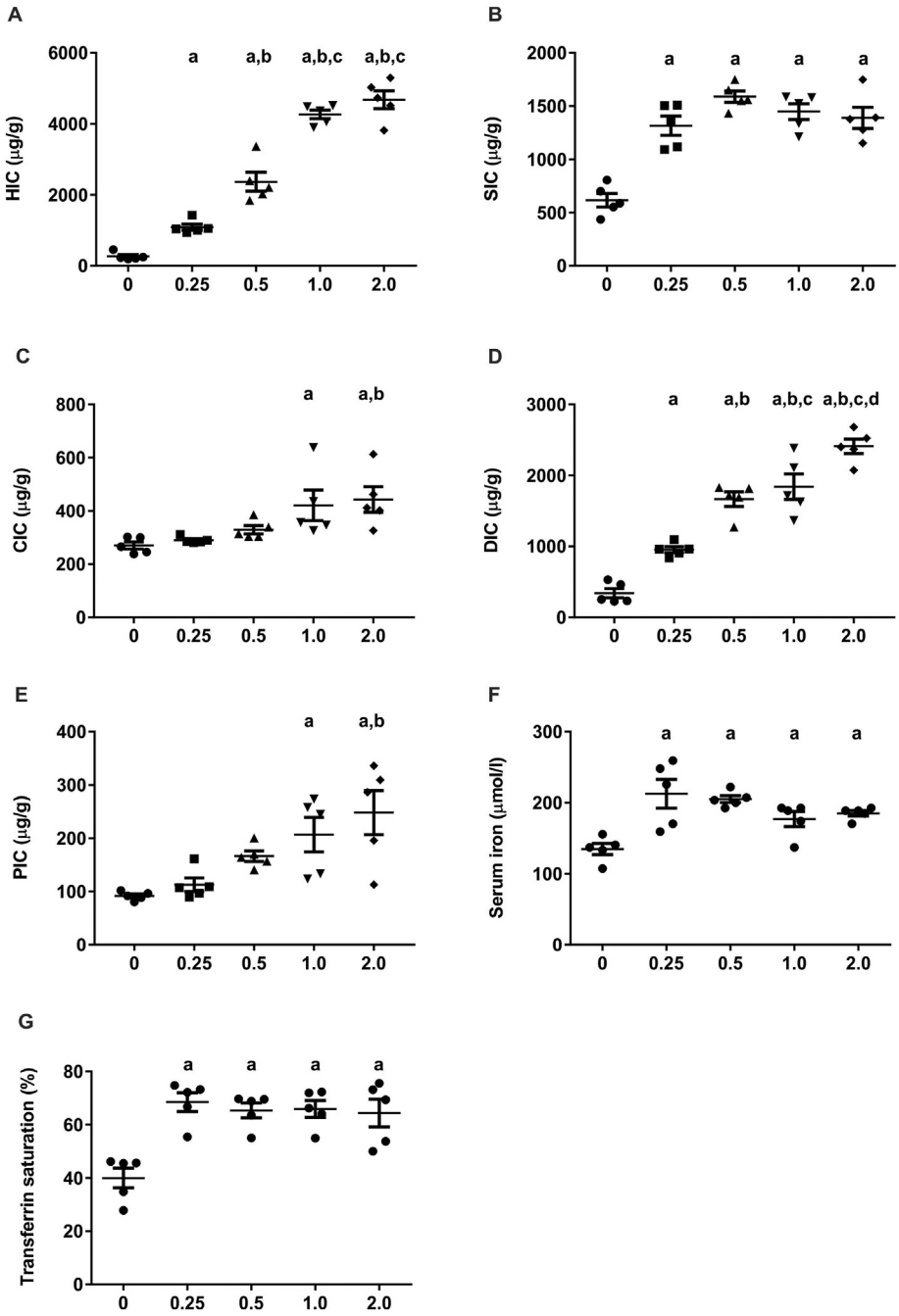

Fig. 2. Iron parameters in mice fed a high-iron diet. The hepatic iron concentrations (A), splenic iron concentration (B), cardiac iron concentration (C), duodenal iron concentration (D), pancreatic iron concentration (E), total serum iron (F) and transferrin saturation (G) were measured in 6-week-old male mice (n = 5) fed control and high-iron diets. The splenic iron, transferrin saturation and serum transferrin levels plateau at 0.25% carbonyl iron containing diet. Data are shown as dot plots, showing the mean and the standard error of the mean (SEM). Statistically significant differences (one-way ANOVA using Tukey's multiple comparison test; $p < 0.05$) are denoted as (a) compared to the control diet, (b) compared to diet containing 0.25% iron diet and (c) compared to diet containing 0.5% iron diet.

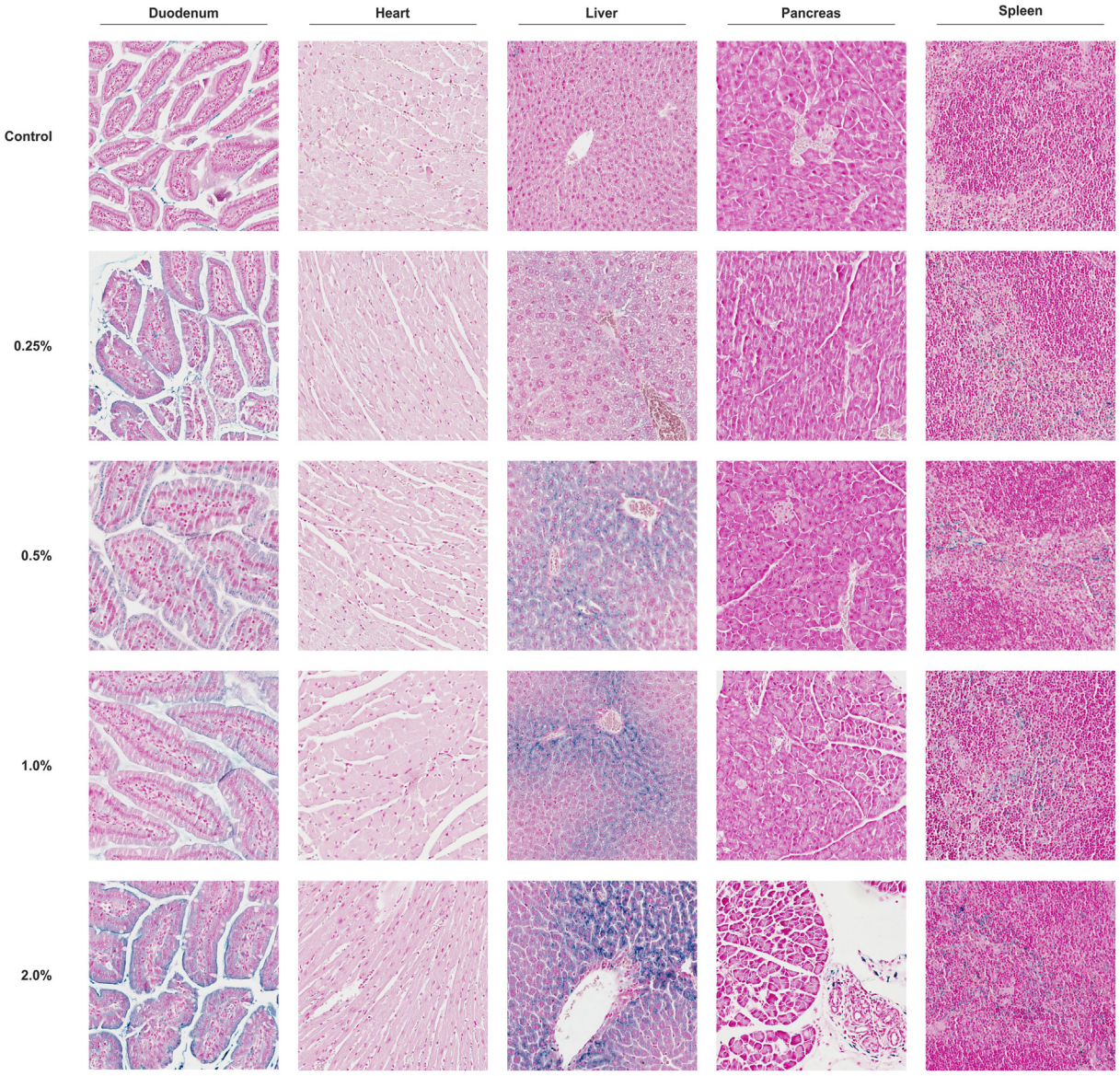

Fig. 3. Localisation of iron in mouse tissues. Perls' Prussian blue staining for iron was performed on sections of duodenum, heart, liver, pancreas and spleens from 6-week-old male mice (n = 5 per group) fed a control diet (0) or a diet containing 0.25%, 0.5%, 1.0% or 2.0% carbonyl iron. All images were taken at a magnification of 40×.

livers of mice fed diets with different concentrations of iron. Interestingly, as observed in dry tissue iron concentration measurements, iron deposition in other tissues (heart, pancreas and duodenum) increased as the concentration of iron in the diets increased (Fig. 3).

3.3. Expression of genes involved in iron homeostasis in the livers of mice fed an increasing iron-rich diet

Next, we examined the changes in the hepatic expression of iron metabolism-related genes in these mice. The mRNA levels of *bone morphogenetic protein 6* (*Bmp6*) (Fig. 4A) and hepcidin (Fig. 4B) increased significantly in mice fed with increased iron diets as compared to the mice fed a control diet. However, the increase in *Bmp6* and *Hamp* mRNA expression plateaued with the 0.25% carbonyl iron diet and did not increase any further in mice fed 0.5%, 1% or 2% iron-rich diet. This suggests that 0.25% carbonyl iron saturates iron-mediated hepcidin regulation. Interestingly at a 2% carbonyl iron diet, the mRNA expression of *DNA-binding protein inhibitor 1* (*Id1*), another molecule regulated by the bone morphogenetic protein-small mothers against decapentaplegic (BMP-SMAD) pathway, was significantly higher as compared to all other groups (Fig. 4C). This increase with 2% carbonyl iron in the diet suggests that excess iron may activate alternative pathways, which may affect the Bmp-Smad pathway as well. We also examined the mRNA expression of genes involved in iron transport in the livers of these mice. We observed an increase in the expression of the iron exporter ferroportin (*Fpn1*) (Fig. 4D) and a decrease in the expression of transferrin receptor 1 (*Tfr1*) (Fig. 4E) which correlates with cellular iron loading. The *Hamp*/HIC ratio also decreased significantly with increasing iron in the diet (Fig. 4F), suggesting that hepatic iron levels may not be contributing to the regulation of hepcidin in a chronic setting.

3.4. Effect of increasing iron levels on SMAD-signalling

We analyzed the protein expression levels of TFR2, Smad1 and phospho-Smad1/5 in total liver homogenates. TFR2 protein levels increase with an increase in the amount of iron due to increased stability of the protein [21]. As expected the amount of TFR2 protein increases

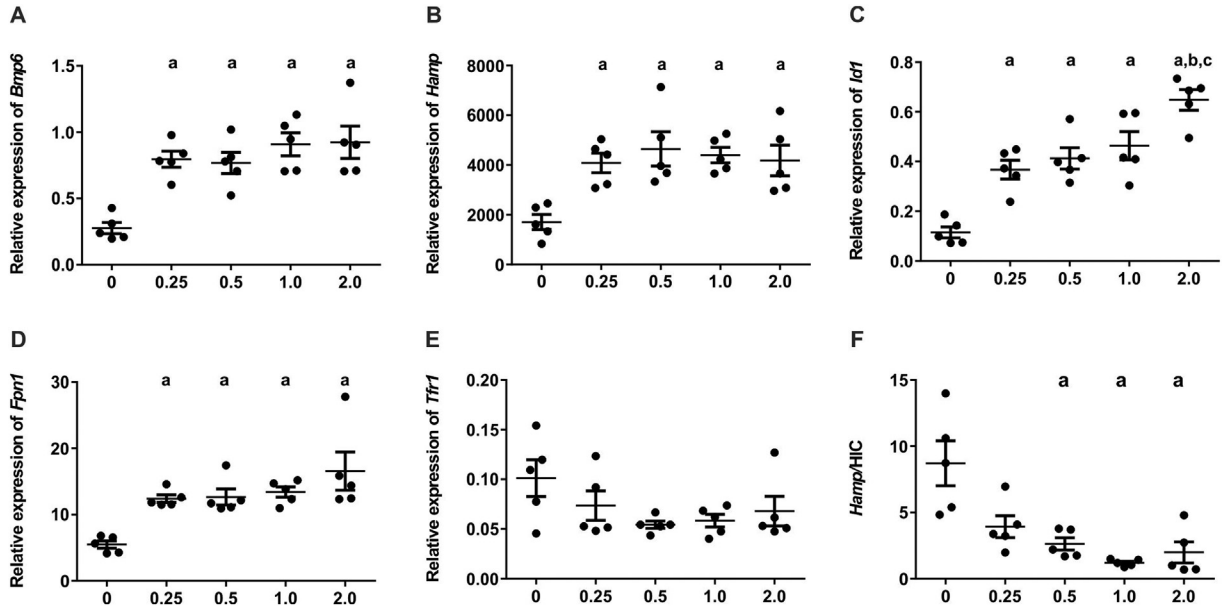

Fig. 4. Expression of iron homeostasis genes in the livers. Relative mRNA expression of *Bmp6* (A), *Hamp* (B), *Id1* (C), *Fpn1* (D) and *Tfr1* (E) was measured in the livers of 6-week-old male mice fed a control diet (0) or a diet containing 0.25%, 0.5%, 1.0% or 2.0% carbonyl iron. The *Hamp*/HIC (F) ratio was also measure in the livers of these mice. The relative expression of both *Bmp6* (A) and *Hamp* (B) plateau at a diet containing 0.25% carbonyl iron indicating that 0.25% iron-rich diet is sufficient to induce an increase in hepcidin levels. Data are shown as dot plots, showing the mean and the standard error of the mean (SEM). Statistically significant differences (one-way ANOVA using Tukey's multiple comparison test; $p < 0.05$) are denoted as (a) compared to the control diet, (b) compared to diet containing 0.25% iron diet and (c) compared to diet containing 0.5% iron diet.

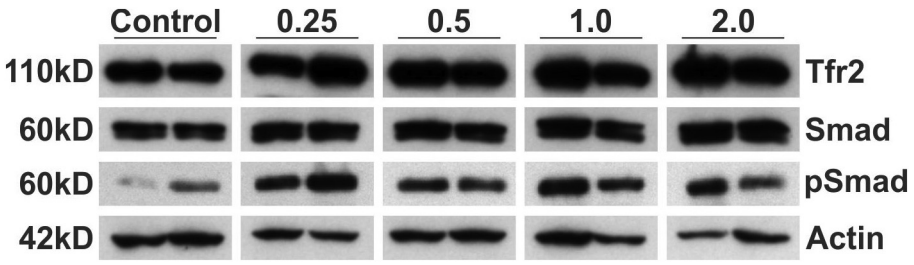

Fig. 5. TFR2 expression and Smad signalling in the livers of mice fed a high-iron diet. (A) Immunoblotting was performed using 10 µg homogenates from the measured from the livers of 6-week-old male mice fed a control diet (0) or a diet containing 0.25%, 0.5%, 1.0% or 2.0% carbonyl iron containing diet. The blot was probed with antibodies against TFR2, total SMAD, pSMAD and β-actin (loading control). This is a representative image of a blot performed at least three times and on all five mice from each group.

with increasing iron in the diet (Fig. 5). We did not observe any changes in the amount of total Smad protein expression in the livers. An increase in the levels of pSmad1/5 in mice fed a high-iron diet as compared to the mice fed a control diet was observed. This suggested an increase in Bmp-Smad signalling as expected. Surprisingly the increase in pSMAD1/5 plateaued with the 0.25% carbonyl iron diet and did not increase any further in mice fed 0.5%, 1% or 2% iron-rich diet.

4. Discussion

Iron homeostasis is regulated by the liver-expressed hormone hepcidin. This 25-amino acid peptide is regulated by several stimuli that may increase or decrease the amount of hepcidin being produced by the liver [6]. Iron-mediated modulation of hepcidin is primarily regulated by liver-expressed proteins including HFE, TFR2, HJV and TMPRSS6 [7]. Mutations in these genes lead to disruption in iron homeostasis leading to either iron overload or anemia. Using transgenic mouse models with disruptions in these genes, we and other researchers have been able to identify dysregulation of hepcidin as the underlying cause of the disrupted iron homeostasis [19,22–25]. The most common model used to study the effect of the HH genes on iron homeostasis is by comparing the transgenic mice with WT mice fed a high-iron diet. In the high iron diet model, mice are usually fed a 2% carbonyl iron diet for one to two weeks. Studies have shown that an increase in iron levels in the diet also affects copper and zinc homeostasis [10–13]. We compared mice fed a diet with varying composition of carbonyl iron (from 0.25% to 2%) for two weeks and compared its effect to mice fed a control diet.

Mice which were fed with a diet containing > 1% carbonyl iron had significantly lower body weights, higher hepatic iron levels and increased amount of mRNA levels of enzymes (*Sod1* and *Sod2*) involved in decreasing reactive oxygen species (ROS). These results indicate that a higher amount of iron in the diet may increase ROS in the livers. We also examined the livers of these mice for evidence of oxidative damage using 4-HNE. There were no differences in the staining as seen in Supplemental Fig. 1, suggesting that the *Sod1* and *Sod2* increase were able to decrease the ROS and protect the livers from oxidative damage.

As expected with increasing iron concentrations in the diets, we observed a rise in the amount of iron deposited in the liver and other organs in mice (Figs. 2 and 3). Surprisingly this extra iron loading did not correlate with a further increase in the expression of *Bmp6* or hepcidin in the livers (Fig. 4). Previous studies have established that an increase in iron levels increases *Bmp6* mRNA levels and this eventually leads to an increase in hepcidin levels [4,26]. Although it has been suggested that both tissue and circulating iron may be involved in the regulation of hepcidin [4], it is unclear whether this increase in hepcidin is due to an increase in the serum iron levels or the hepatic iron levels. Even though the HIC increased over time, the transferrin saturation did not change significantly and the hepcidin levels remained same [4]. It has been proposed that the *Bmp6* levels correlate with the

hepatic iron concentrations [4]. As we can see in Figs. 2 and 4, although the HIC increases linearly, *Bmp6* and hepcidin levels stay the same, suggesting that in cases of prolonged iron loading (as in the case of hemochromatosis patients), HIC may not be involved in the regulation of iron-mediated regulation of hepcidin. These results indicate that our understanding of how the body regulates iron homeostasis in response to body iron levels is incomplete. According to the current hypothesis/model, as body iron levels increase, the signalling through the iron sensing molecules: Hfe, Tfr2, and Hjv, leads to an increase in hepcidin expression; however it is now apparent that although HIC keeps on increasing, hepcidin levels have plateaued. The iron-mediated BMP-SMAD pathway signalling appears to be saturated with the 0.25% carbonyl iron diet, as the SMAD phosphorylation levels do not increase any further (Fig. 5).

The liver *Hamp*/HIC ratio is used to define the "inappropriate" hepcidin levels in hemochromatosis patients and mice models and to determine the appropriate signalling in the iron-mediated hepcidin regulation. We observed that the *Hamp*/HIC ratio significantly decreased as the concentration of iron in the diet increased in the WT mice (Fig. 4F), this suggests that contrary to the current notion hepatic iron levels may not be contributing to the regulation of hepcidin in a chronic setting. Furthermore, our study suggests that the *Hamp*/HIC ratio should not be used to define the hepcidin response, as in a chronic setting, HIC does not affect liver hepcidin expression.

These results also support previous observations made in similar studies. Pigeon et al. [27] fed mice a diet containing either 0.5%, 1.0% or 3.0% iron rich diet and observed that an increase in iron levels in the diet led to an increase in the hepcidin mRNA levels as measured by northern blot. No differences were observed in the levels of hepcidin in mice fed different concentrations of iron, suggesting a saturation level. Similarly, Corradini et al. [4] observed that when mice were fed an iron rich diet, the Tf saturation and *Hamp* levels plateau within 24 h of feeding the mice, but the HIC increases all along the feeding period, up to 3-weeks. Daba et al. [14] also observed that in mice fed an iron rich diet, *Bmp6*, *Hamp*, Tf saturation and serum transferrin all plateaued within one week of feeding an iron-rich diet, whereas the HIC levels kept increasing. These results suggest that the current hypothesis about the role of hepatic iron levels in modulation of systemic *Hamp* levels needs to be revisited. These observations support the view that the hepatic iron levels affect *Hamp* production in an acute setting and in a more chronic setting (as observed in hemochromatosis patients) there may be other factors contributing to *Hamp* production.

The results from this study indicate that a 0.25% iron-rich diet is sufficient to induce a hepcidin response and any further increases in the amount of iron in the diet does not contribute to iron-mediated hepcidin signalling. In addition to the liver, other tissues including heart, pancreas and duodenum keep accumulating iron whereas the splenic iron seems to plateau at 0.5% iron-containing diet. We also show that an increase of iron in the diet beyond a concentration of 0.5% leads to a significant increase in the mRNA expression of enzymes involved in oxidative stress without leading to any obvious oxidative damage. This is indicative of an increase in oxidative stress and may explain the increase in hepcidin levels observed in *Tfr2*, *Hfe*, or *Hjv* mice when fed a high-iron diet.

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Transparency document

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Disclosure

The authors disclose no conflicts of interest.

Authorship contributions

GR and VNS designed the study; GR and ES performed the experiments, GR, ES and VNS analyzed the data; GR and VNS wrote the manuscript, GR, ES and VNS critically reviewed the manuscript.

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