Short Communication

Hepatic Iron Deposition Does Not Predict Extrahepatic Iron Loading in Mouse Models of Hereditary Hemochromatosis

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Hereditary hemochromatosis is characterized by tissue iron loading and associated organ damage. However, the phenotype can be highly variable. The relationship between iron loading of different organs and the temporal nature of its deposition is still not well understood. We examined the progression of tissue iron loading in three mouse models to advance our understanding of the natural history of iron deposition in hereditary hemochromatosis. Wild-type, *Hfe*^{-/-}, *Tfr2*^{-/-}, and *Hfe*^{-/-}/*Tfr2*^{-/-} mice were analyzed at 3, 5, 10, 26, and 52 weeks, respectively. Hepatic, splenic, cardiac, and pancreatic iron concentrations were determined. Expression of both iron-regulatory and fibrosis genes was determined by quantitative real-time PCR in livers and hearts of 52-week-old mice. In all models, hepatic iron increased rapidly, plateauing before 10 weeks at different levels, depending on the genotype. Iron deposition in the pancreas and heart occurred after maximal iron loading of the liver was reached and was most marked in the *Hfe*^{-/-}/*Tfr2*^{-/-} mice. Although a significant positive correlation was identified between pancreatic and cardiac iron in all models at 52 weeks, there was no correlation between hepatic and either pancreatic or cardiac iron. There is variability in the timing and extent of tissue iron loading within a genotype, suggesting that hepatic iron levels in hereditary hemochromatosis may not accurately predict the iron content of other organs. (Am J Pathol 2012, 181:1173–1179; <http://dx.doi.org/10.1016/j.ajpath.2012.06.025>)

The iron overload disorder, hereditary hemochromatosis (HH), covers the spectrum of disease that results from mutations in several genes, including *HFE*, *TFR2*, *HJV*, or *HAMP*.¹ Iron overload resulting from mutations in these genes occurs because of dysregulation of the iron-regulatory hormone, hepcidin.² Hepcidin, encoded by the *HAMP* gene, is largely responsible for the systemic regulation of iron homeostasis; it negatively regulates iron absorption and recycling by reducing surface levels of the iron export protein, ferroportin, on duodenal enterocytes and macrophages.³

There is a wide phenotypic variation among patients with HH of the same genotype. The reasons for these variations are not fully understood, although several factors are known to contribute. Sex is a major factor affecting phenotype, with iron overload-related disease being significantly more prevalent among males.⁴ Environmental influences, such as alcohol consumption and diet, can also influence phenotype.^{5–7} Genetic modifiers have also been implicated in the phenotypic variation of *HFE*-HH; however, the individual modifiers described only account for a small proportion of the observed variation.⁸

Few studies in humans have attempted to measure the progression of iron overload in untreated individuals with *HFE*-HH. Andersen et al⁹ concluded that serum transferrin saturation and ferritin only increased moderately during a 25-year period. Other studies have likewise con-

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cluded that serum measures of iron loading do not alter significantly over time in *HFE*-HH.^{10,11} The differential iron loading of extrahepatic tissues in HH has not been well studied. It is well-known that the liver accumulates iron in HH, and other organs, such as the pancreas and heart, also load with iron, but the temporal nature of the iron deposition and relationships between different tissues is not well understood.

Iron overload in patients with *HFE*-HH or *TFR2*-HH results from reduced expression of hepcidin relative to body iron stores.^{12,13} A form of juvenile HH can be caused by a combination of mutations in both *HFE* and *TFR2*.¹⁴ We have previously shown that mice lacking both *Hfe* and *Tfr2* develop severe hepatic iron overload because of a marked deficiency in hepcidin.¹⁵ We also demonstrated a gradient of increasing hepatic iron loading from *Hfe*^{-/-} to *Tfr2*^{-/-} to double *Hfe*^{-/-}/*Tfr2*^{-/-} mice at the age of 5 weeks. We showed that the degree of iron loading in these mouse models of HH was inversely related to the expression of hepcidin in relation to iron stores.¹⁵

We have used three mouse models of HH with mild, intermediate, and severe iron loading phenotypes, *Hfe*^{-/-}, *Tfr2*^{-/-}, and *Hfe*^{-/-}/*Tfr2*^{-/-} mice, respectively, to investigate iron loading over time and in different tissues to advance our knowledge of the natural history of tissue iron deposition and disease in human HH. We show that significant iron loading of the pancreas and heart occurs in mice lacking both *Hfe* and *Tfr2* and that hepatic iron content in 1-year-old mice does not predict iron loading of other organs, in particular the pancreas and heart.

Materials and Methods

Animals

Animal experiments were approved by the Queensland Institute of Medical Research Animal Ethics Committee. All animals had free access to water and food under standard conditions and received humane care. The *Hfe*^{-/-}, *Tfr2*^{-/-}, and *Hfe*^{-/-}/*Tfr2*^{-/-} mice backcrossed to the C57BL/6J background for 10 generations have been previously described.¹⁵ Male mice were weaned at 21 days and maintained on standard laboratory chow ad libitum (Norco/Goldmix rat and mouse nuts; iron content, 120.1 mg/kg; Norco Stockfeeds, South Lismore, Australia). Wild-type (WT), *Hfe*^{-/-}, *Tfr2*^{-/-}, and *Hfe*^{-/-}/*Tfr2*^{-/-} mice were fasted overnight before being sacrificed at the ages of 3 (*n* = 6 per group), 10 (*n* = 4 to 5 per group), 26 (*n* = 5 to 6 per group), and 52 (*n* = 9 to 12 per group) weeks, and tissues were collected for analysis. Mice used in the 5-week time point (*n* = 5 to 6 per group) formed part of a previous study, and the collection of tissues was performed in the same way.¹⁵ The regions of tissue collected for iron content and other measurements were consistent between mice.

Serum and Tissue Iron Measurements

Serum transferrin saturation was measured using an iron/total iron-binding capacity reagent set (Pointe Scientific, Canton, MI), according to the manufacturer's instruc-

tions. Tissue iron concentrations were measured in the liver, spleen, heart, and pancreas, according to the method of Torrance and Bothwell.¹⁶

Real-Time PCR Analysis of mRNA Transcripts

Primer pairs for detecting *Hamp* and *Hprt* mRNA transcripts have been previously described.^{17,18} Primer sequences for detecting collagen type I α 1 (*Col1a1*) and α -smooth muscle actin (*Acta2*) mRNA transcripts were as follows: murine (m)*Col1a1*, 5'-AGAGGCGAAGGCAACAGTCG-3' (forward) and 5'-AAGCAGGCAGGGCCAATGTC-3' (reverse); and m*Acta2*, 5'-ACCCAGGCATTGCTGACAGG-3' (forward) and 5'-GGGGGACTTAGAAGCATTTGCGG-3' (reverse). The RNA isolation, cDNA synthesis, and real-time PCR were performed as previously described,¹⁹ except the quantitation of mRNA transcripts in liver was determined by normalization to respective *Hprt* levels.

Histological Detection of Fibrosis and Iron

The presence of fibrosis was assessed in liver sections by histochemical staining for collagen using picrosirius red. Sections were scanned using the Aperio ScanScope XT (Aperio, Brisbane, Australia) at $\times 40$ magnification, and picrosirius red staining was quantified using ImageScope Version 10.0.36.1805 software and the ColorDeconvolution_version 9 algorithm calibrated to the sections, as previously described.¹⁹ The presence of iron was detected by staining with Perls' Prussian Blue, as previously described.¹⁹

Statistical Analysis

Variables were compared between groups using either two- or one-way analysis of variance. Post-hoc analysis to compare differences between individual groups was performed using the Fisher's least significant difference method. Statistical analyses were performed using SPSS Statistics version 19 (IBM, Armonk, NY), and graphs were prepared using GraphPad Prism 5 (GraphPad Software, San Diego, CA). Because of the assumption of equal variance between groups not being satisfied for all of the outcome variables, a variance-stabilizing transformation was performed for variables composing the natural log of (1 + *x*), where *x* is the variable.

Results

Mouse Models of HH Have Different Maximal Hepatic Iron Levels

The WT, *Hfe*^{-/-}, *Tfr2*^{-/-}, and *Hfe*^{-/-}/*Tfr2*^{-/-} male mice on the C57BL6 background were sacrificed at 3, 5, 10, 26, and 52 weeks, respectively, and tissues were obtained for analysis. Serum transferrin saturation was measured in all mice and was similar between all groups at 3 weeks. At all other time points, the mouse models of HH had elevated transferrin saturation compared with WT

Figure 1. Longitudinal analysis of tissue iron deposition in mouse models of HH. HIC (A), CIC (B), PIC (C), and SIC (D) values are determined in 3- ($n = 6$ per group), 5- ($n = 5$ to 6 per group), 10- ($n = 4$ to 5 per group), 26- ($n = 5$ to 6 per group), and 52- ($n = 9$ to 12 per group) week-old WT, *Hfe*^{-/-}, *Tfr2*^{-/-}, and *Hfe*^{-/-}/*Tfr2*^{-/-} mice, respectively. Pancreatic tissue is not available for the 5- and 10-week time points. Error bars indicate SEM. At the 52-week time point, HIC (E), CIC (F), PIC (G), and SIC (H) values are shown for WT ($n = 10$), *Hfe*^{-/-} ($n = 12$), *Tfr2*^{-/-} ($n = 9$), and *Hfe*^{-/-}/*Tfr2*^{-/-} ($n = 12$) mice, respectively. Data are shown as box-and-whisker plots, showing median values, quartiles, and range, and plotted on a log scale. Variables are log-transformed before statistical analyses. Statistically significant differences (one-way analysis of variance post-hoc comparisons: $P < 0.05$) are denoted as a compared with WT, b compared with *Hfe*^{-/-}, and c compared with *Tfr2*^{-/-}. Conc., concentration.

(see Supplemental Figure S1 at <http://ajp.amjpathol.org>). Tissue iron concentrations were determined in the liver, spleen, and heart for all mice and also in the pancreas for the 3-, 26-, and 52-week old mice, respectively. Pancreatic tissue was not available from the 5- and 10-week time points. Because of the unequal variance of the data for tissue iron concentrations, the data were log-transformed for statistical analysis. The raw values for tissue iron concentrations over time are presented for each tissue in Figure 1, A–D. Two-way analysis of variance of the log-transformed data revealed that there was a strongly significant interaction between age and genotype for the hepatic iron concentration (HIC; $P < 0.01$), splenic iron concentration (SIC; $P < 0.01$), and cardiac iron concentration (CIC; $P = 0.05$) and a weakly significant interac-

tion for the pancreatic iron concentration (PIC; $P = 0.08$). To distinguish the effect of genotype, each time point was considered separately. The tissue iron concentrations at the age of 52 weeks are presented graphically (Figure 1, E–H), and the multiple comparisons of the log-transformed data at all ages are also presented (see Supplemental Tables S1–S5 at <http://ajp.amjpathol.org>). The HIC increased rapidly in *Hfe*^{-/-}/*Tfr2*^{-/-} mice, reaching maximal levels of 3000 to 4000 μg/g by the age of 10 weeks (Figure 1A). At 26 weeks, HIC was at similar levels in *Hfe*^{-/-}/*Tfr2*^{-/-} mice, suggesting that hepatic iron levels had plateaued by the age of 10 weeks (Figure 1A). By 52 weeks, the HIC had decreased slightly compared with the levels observed at 26 weeks, to just <3000 μg/g, but remained high compared with the other genotypes. In *Tfr2*^{-/-} mice, the HIC also plateaued by the age of 10 weeks, but at a lower concentration (approximately 2000 μg/g) compared with *Hfe*^{-/-}/*Tfr2*^{-/-} mice, and remained at a similar level until 52 weeks (Figure 1A). In *Hfe*^{-/-} mice, the HIC had reached maximal levels by the age of 5 weeks at approximately 1000 μg/g, between that of WT and *Tfr2*^{-/-} mice (Figure 1A). These results suggested that hepatic iron loading in mouse models of HH on the C57BL/6 background was rapid and that maximal loading was attained by 5 to 10 weeks. However, these peak HICs varied between different genotypes, with low, intermediate, and high iron concentrations in *Hfe*^{-/-}, *Tfr2*^{-/-}, and *Hfe*^{-/-}/*Tfr2*^{-/-} mice, respectively (Figure 1, A and E).

Iron Deposition in Other Organs Occurs after the Liver and Is Marked in *Hfe*^{-/-}/*Tfr2*^{-/-} Mice

Cardiac iron accumulation was slower than hepatic iron loading in mouse models of HH. Although, at the age of 52 weeks, the CICs of *Hfe*^{-/-} and *Tfr2*^{-/-} mice were modestly elevated compared with WT, they did not reach statistical significance (Figure 1, B and F). The *Hfe*^{-/-}/*Tfr2*^{-/-} mice had a significant difference in CIC at the ages of 26 and 52 weeks, compared with all other genotypes (Figure 1, B and F). These results were consistent with the phenotype of *Hfe*^{-/-}/*Tfr2*^{-/-} mice, resembling juvenile HH, that was characterized by greatly increased cardiac iron and risk of cardiomyopathy, as observed in the published cases of combined HFE- and TFR2-related HH.¹⁴ The PIC was minimal in all groups at the age of 3 weeks. By 26 weeks, all of the mouse models of HH had significant elevations in PIC compared with WT that was also seen at 52 weeks. The *Hfe*^{-/-}/*Tfr2*^{-/-} mice had the most dramatic elevation in PIC, with a highly significant 7.8-fold elevation at 26 weeks and a 30-fold elevation at 52 weeks compared with WT (Figure 1, C and G). At the age of 52 weeks, there was striking variability in the PIC between mice of the same genotype. For example, the 52-week-old *Hfe*^{-/-}/*Tfr2*^{-/-} mice had a PIC ranging from 752 to 8343 μg/g. This variability was also discernable in the color of the pancreas, ranging from a slight orange discoloration at one end of the spectrum to a chocolate brown coloration at the other end of the spectrum. The SIC increased with age in all genotype groups (Figure 1D). The increase in SIC was slowest in the *Hfe*^{-/-}/*Tfr2*^{-/-} mice,

Figure 2. Hepatic hepcidin expression. Real-time qPCR is used to quantitate expression of *Hamp1* mRNA in 52-week-old WT ($n = 10$), $Hfe^{-/-}$ ($n = 12$), $Tfr2^{-/-}$ ($n = 9$), and $Hfe^{-/-}/Tfr2^{-/-}$ ($n = 12$) mice, respectively. **A:** Absolute levels of *Hamp1* are shown relative to *Hprt*. **B:** *Hamp1* mRNA is shown in relation to iron stores as a ratio of *Hamp1*/HIC and presented on a log scale. Data are shown as box-and-whisker plots, showing median values, quartiles, and range. *Hamp1*/HIC values are log-transformed before statistical analyses. Statistically significant differences (one-way analysis of variance post-hoc comparisons: $*P < 0.05$).

being significantly lower than in all other groups at the ages of 26 and 52 weeks (Figure 1, D and H). These results were consistent with the relative iron sparing of macrophages that was observed in humans and mice with HH.^{20,21}

Extent of Iron Overload Is Inversely Related to Hepatic Hepcidin Expression

In human HH, advanced disease and organ damage occurred after many years of elevated iron levels. Therefore, to address the effects of long-term iron storage, we studied the 52-week-old group in more detail. At the age of 52 weeks, all of the mouse models of HH had a significantly higher iron content of the liver and pancreas compared with WT (Figure 1, E and G). The differences between each genotype were also significantly different, with a progressive increase in HIC and PIC from $Hfe^{-/-}$ to $Tfr2^{-/-}$ to $Hfe^{-/-}/Tfr2^{-/-}$ mice (Figure 1, E and G). The $Hfe^{-/-}/Tfr2^{-/-}$ mice also had a significant elevation in CIC and a significantly lower SIC compared with all other genotype groups (Figure 1, F and H). The basis for the increased tissue iron levels in these mouse models of HH was investigated by measuring the expression of *Hamp1* mRNA in the liver by real-time quantitative PCR (qPCR). The absolute levels of *Hamp1* were similar in all four genotype groups (Figure 2A). Levels of *Hamp1* were also analyzed relative to hepatic iron stores by calculating the *Hamp1*/HIC ratio. When analyzed relative to hepatic iron stores, *Hamp1* mRNA levels were significantly lower in all three mouse models of HH when compared with WT (Figure 2B). The log (*Hamp1*/HIC) ratio was also significantly lower in the $Hfe^{-/-}/Tfr2^{-/-}$ mice compared with the $Hfe^{-/-}$ and $Tfr2^{-/-}$ mice.

Cardiac and Pancreatic Iron Contents Correlate with Each Other in Mouse Models of HH

The pattern of tissue iron deposition was investigated in the mouse models of HH by analyzing the correlations between the log-transformed tissue iron concentrations. Linear regressions between hepatic, splenic, cardiac,

and pancreatic iron concentrations were performed in all genotype groups at the ages of 26 and 52 weeks, respectively. At 26 weeks, there were no significant correlations observed between the iron contents of tissues, with the exception of CIC and PIC in the $Hfe^{-/-}/Tfr2^{-/-}$ mice, in which a significant positive correlation was observed ($r = 0.92$, $P = 0.01$; see Supplemental Table S6 at <http://ajp.amjpathol.org>). At 52 weeks, there were significant positive correlations between CIC and PIC in all three mouse models of HH (Figure 3, A–D: $Hfe^{-/-}$ $r = 0.78$, $P < 0.01$; $Tfr2^{-/-}$ $r = 0.96$, $P < 0.01$; and $Hfe^{-/-}/Tfr2^{-/-}$ $r = 0.91$, $P < 0.01$). The SIC also correlated with the CIC and PIC in the $Hfe^{-/-}/Tfr2^{-/-}$ mice at 52 weeks (see Supplemental Table S7 at <http://ajp.amjpathol.org>). There were no significant correlations between the HIC and the iron content of any other tissue in all genotypes at both 26 and 52 weeks (see Supplemental Tables S6 and S7 at <http://ajp.amjpathol.org>). These results suggested that, in these mouse models of HH, the hepatic iron content was not a good predictor of the iron content of

Figure 3. Tissue iron correlations. Correlations between PIC and CIC values are performed for 52-week-old WT (**A**), $Hfe^{-/-}$ (**B**), $Tfr2^{-/-}$ (**C**), and $Hfe^{-/-}/Tfr2^{-/-}$ (**D**) mice. The PIC and CIC values are plotted on log scales. Values are log-transformed before statistical analyses. The correlation coefficient squared (r^2) and P value for each correlation are shown. There are strong correlations between PIC and CIC for all three mouse models of HH. Perl's staining of liver (**E** and **H**), pancreas (**F** and **I**), and heart (**G** and **J**) sections from two 52-week-old $Hfe^{-/-}/Tfr2^{-/-}$ mice, showing strikingly different patterns of tissue iron deposition. **E–G:** Mouse 1. **H–J:** Mouse 2. Slides are scanned at $\times 40$ magnification.

Figure 4. Analysis of fibrosis in liver and heart. Markers of fibrosis are measured in the livers (A–C) and hearts (D–F) of 52-week-old WT ($n = 10$), $Hfe^{-/-}$ ($n = 12$), $Tfr2^{-/-}$ ($n = 9$), and $Hfe^{-/-}/Tfr2^{-/-}$ ($n = 12$) mice, respectively. The proportion of liver tissue stained for picrosirius red is quantitated (A and D). Real-time qPCR is used to quantitate the expression of *Col1a1* (B and E) and *Acta2* (C and F) mRNA and shown relative to *Hprt*. Data are shown as box-and-whisker plots, showing median values, quartiles, and range. No significant differences between the groups are observed using one-way analysis of variance.

other tissues, in particular the heart and pancreas. In contrast, the levels of iron in the heart and pancreas correlated well with each other. To investigate the lack of correlation between HIC and the iron contents of the heart and pancreas in individual $Hfe^{-/-}/Tfr2^{-/-}$ mice, we performed Perls' Prussian Blue staining of tissue sections to visualize the distribution of iron. There were significant positive correlations between the measured iron concentration and the percentage of tissue stained with Perls' Prussian Blue for all tissues (data not shown). We observed a striking difference in iron distribution between tissues in the $Hfe^{-/-}/Tfr2^{-/-}$ mice. Some mice had prominent iron deposition in the heart and pancreas, with relative sparing of the liver (Figure 3, E–G), whereas other mice had prominent iron deposition in the liver, with relative sparing in the heart and pancreas (Figure 3, H–J). These two divergent phenotypes were difficult to explain and were seen in mice from the same litters, with the same genetic background that would have effectively had the same environmental influences.

Iron Overload Is Not Associated with Tissue Damage in Mouse Models of HH

In human HH, long-term deposition of iron in the liver and heart can lead to organ damage, including hepatic fibrosis and cardiomyopathy. In contrast, such pathological consequences of long-term iron loading have not been observed in mouse models of HFE-HH and HJV-associated HH.^{22–24} We evaluated the long-term effects of hepatic and cardiac iron loading in our mouse models by measuring markers of fibrosis in 52-week-old mice. Liver and heart sections from WT, $Hfe^{-/-}$, $Tfr2^{-/-}$, and $Hfe^{-/-}/Tfr2^{-/-}$ mice were stained with picrosirius red for colla-

gen, and positive staining was quantitated. In addition, real-time qPCR was used to quantitate the expression of *Col1a1* and *Acta2* mRNA. In the liver, picrosirius red staining was similar between the different genotype groups (Figure 4A). The expression levels of *Col1a1* and *Acta2* were also similar between the different groups (Figure 4, B and C). These results suggested that, although iron loading was increased in these mouse models of HH and was present for a relatively long time, it did not result in significant liver damage. A similar result was seen in the heart, where picrosirius red staining and the expression levels of *Col1a1* and *Acta2* were not statistically different between the genotype groups (Figure 4, D–F).

Discussion

The natural history and progression of tissue iron loading in human HH are not well understood, although it is accepted that loading of the liver occurs first. The mechanisms controlling the temporal loading of iron in other organs are less clear. The pancreas and heart are known to load iron in HH. The extent and location of tissue iron loading in HH are highly variable; some individuals accumulate large amounts of iron in multiple organs and develop clinical disease, whereas other individuals accumulate little or no iron and are clinically unaffected. The genetic cause of iron overload is one factor that influences the severity of iron overload. Juvenile HH, caused by mutations in either *HJV* or *HAMP* or a combination of *HFE* and *TFR2* mutations, is distinguishable from the more common form of HFE-HH by the more rapid accumulation of iron, earlier onset, and increased incidence of cardiac iron loading with complications, including cardiomyopathy and hypogonadism.^{14,25} The incidence of re-

duced glucose tolerance is also significantly higher in patients with juvenile HH compared with *HFE*-HH,²⁵ suggesting that greater iron loading of the pancreas is causing or contributing to pancreatic dysfunction. The literature on subjects with *TFR2*-HH indicates an intermediate phenotype between that of juvenile HH and *HFE*-HH.^{26,27} The differences in the severity of iron loading between *HFE*, *TFR2*, and juvenile forms of HH have been supported by our previous study on mouse models of these diseases; at 5 weeks, we showed a gradient of increased hepatic iron from *Hfe*^{-/-} to *Tfr2*^{-/-} to *Hfe*^{-/-}/*Tfr2*^{-/-} mice.¹⁵ The severity of hepatic iron loading was explained by reduced expression of hepatic hepcidin in relation to iron stores in these mouse models.¹⁵ We have extended these studies to analyze the natural history of iron loading over time and in various organs. We found that liver iron loading was rapid and, by the age of 5 to 10 weeks, iron levels in all three models of HH had plateaued. The level at which maximal iron loading had occurred was, however, different between the three mouse models, with *Hfe*^{-/-} mice plateauing at relatively low iron concentrations compared with the intermediate and high levels attained in the *Tfr2*^{-/-} and *Hfe*^{-/-}/*Tfr2*^{-/-} mice, respectively. The mechanism regulating this maximal iron loading concentration in the liver is unclear; however, it is likely related to differences in the expression of hepcidin. Indeed, we found that hepcidin was reduced relative to hepatic iron content in all of the mouse models of HH, with the lowest levels observed in the *Hfe*^{-/-}/*Tfr2*^{-/-} mice. One explanation for the plateauing of iron levels in the liver could be that as the liver loads with iron, hepcidin synthesis increases, even in the mouse models of HH, until hepcidin synthesis increases sufficiently to limit further duodenal iron absorption. The increase in hepcidin per unit of iron, however, varies between the three models of HH, resulting in differing maximal iron concentrations. This explanation, however, does not explain the increased iron absorption required for the deposition of iron into other organs, as observed in the *Hfe*^{-/-}/*Tfr2*^{-/-} mice. Regardless, it appears that the capacity for the liver to load iron in *Hfe*^{-/-}/*Tfr2*^{-/-} mice is greatly increased. Our data suggest that once the liver has reached its maximal capacity in *Hfe*^{-/-}/*Tfr2*^{-/-} mice, iron accumulates in other organs. At the age of 10 weeks, the iron content of the heart in the *Hfe*^{-/-}/*Tfr2*^{-/-} mice, although marginally elevated, was not statistically significant when compared with the other groups. By 6 months, the pancreas and the heart of the *Hfe*^{-/-}/*Tfr2*^{-/-} mice had accumulated significant amounts of iron, suggesting a more gradual accumulation of iron in these organs once the liver had reached its capacity. The *Hfe*^{-/-} and *Tfr2*^{-/-} mice also start to load iron in other organs, but at a reduced rate compared with the *Hfe*^{-/-}/*Tfr2*^{-/-} mice, and have significant deposition of iron in the pancreas by the age of 6 months.

Despite the accumulation of significant amounts of iron in the livers of *Hfe*^{-/-}/*Tfr2*^{-/-} mice during a 1-year period, we failed to detect any evidence for an increase in fibrosis, as measured by picrosirius red staining for collagen and gene expression for *Col1a1* and *Acta2*. This supports and expands on studies of other mouse models

of adult and juvenile HH that have also reported a lack of any pathological consequences resulting from long-term exposure to elevated tissue iron stores.^{22–24} One recent study, however, has reported iron-induced liver injury in another mouse model with disruption of *Hfe* and *Tfr2*.²⁸ Similar to our study, the authors reported increased liver iron and reduced hepcidin expression in mice, with disruption of both *Hfe* and *Tfr2*. In contrast to our study, markers of liver fibrosis and liver injury were increased in the *Hfe*/*Tfr2*-disrupted mice at the age of 11 weeks. The presence of liver injury at this age is unexpected and may be the result of several factors. Namely, the background strain of mouse used, AKR, versus the C57BL/6J used in our study, may have contributed to the differences observed in liver injury. Strain background has had a profound effect on the iron status of mice,²⁹ and the AKR strain stores significantly more iron than the C57BL/6J strain. A previous study showed that *Hfe*^{-/-} mice on the AKR background load 3.6 times more iron than *Hfe*^{-/-} mice on the C57BL6 background.²⁹ The increased basal iron levels in the AKR strain may predispose the mice to increased liver injury, and the excess iron deposition caused by disruption of *Hfe* and *Tfr2* may be enough to induce fibrosis.

The mechanisms that regulate hepatic iron loading and the subsequent switch to iron deposition in the pancreas and heart are unclear. However, our study shows that the distribution of iron between these three tissues is highly variable within each of the three HH models. We found that, although there was a strong correlation between the iron content of the pancreas and heart in 1-year-old mice of all three HH genotypes, the iron contents of the pancreas and heart did not correlate with those of the liver. Furthermore, the levels of iron in the pancreas and heart were increasing, whereas hepatic iron content remained stable. These results suggest that the iron content of the liver is not a good predictor of iron loading in the heart and pancreas. They also suggest a common mechanism for iron loading of the pancreas and heart that differs from the liver. A possible mechanism for the loading of iron into the pancreas and heart may involve the uptake of non-transferrin-bound iron. During the development of iron overload, when transferrin becomes fully saturated, non-transferrin-bound iron could be absorbed by the heart and pancreas. This could start at various ages and possibly intermittently at first, during periods of food intake or diurnal hepcidin lows. The variability in levels of non-transferrin-bound iron in mice over time may partly account for the variability observed in the distribution of tissue iron. Further experiments will be required to determine whether this is the case. Whether the observed variability in tissue iron loading in mouse models of HH translates to the human condition remains to be determined. In the clinical situation, hepatic iron stores are often accounted for in the diagnosis and management of patients with HH, but pancreatic and cardiac iron loading are rarely, if ever, measured. Based on our findings, it is possible that some patients with HH who have relatively low levels of hepatic iron could have relatively high levels of undetected pancreatic and cardiac iron that may confer a higher risk of developing diabetes

and cardiomyopathy. To our knowledge, the differences in the distribution of iron between the liver, pancreas, and heart have not been previously reported in patients with HH or in mouse models of these disorders. However, a similar relationship between pancreatic and cardiac iron in patients with thalassemia major has been observed.³⁰ Similar to our study, Noetzli et al³⁰ observed a significant correlation between pancreatic and cardiac iron, with loading of the pancreas preceding the heart and a lack of correlation with hepatic iron stores in patients with thalassemia major.³⁰ The variability in tissue iron distribution in the *Hfe*^{-/-}/*Tfr2*^{-/-} mice is difficult to explain, because mice of the same genotype with divergent phenotypes are essentially genetically identical. In addition, some of the mice with divergent phenotypes were littermates who had been housed together and had effectively the same environmental influences. Taking these points together, our data suggest a nongenetic, nonenvironmental basis for the differences in tissue iron distribution between *Hfe*^{-/-}/*Tfr2*^{-/-} mice. A more thorough analysis of the mechanisms underlying these differences is warranted.

In conclusion, we have shown that there is wide variation in the distribution of tissue iron stores in mouse models of HH and that hepatic iron stores do not correlate with pancreatic or cardiac iron stores. This could have implications in the clinical management of patients with HH.

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