

Fetal iron levels are regulated by maternal and fetal *Hfe* genotype and dietary iron

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Acknowledgments: we thank Michelle Murphy and Helen Hayes for technical help throughout the study.

Funding: this work was supported by a Biotechnological and Biological Sciences Research Council grant (BB/D521322/1) to SKSS.

Manuscript received on September 7, 2011. Revised version arrived on November 10, 2011. Manuscript accepted on December 5, 2011.

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ABSTRACT

Background

Iron metabolism during pregnancy maintains fetal iron levels at the expense of the mother. The mechanism behind this regulation is still not clear despite recent advances. Here we examine the role of maternal and fetal *Hfe*, its downstream signaling molecule, hepcidin and dietary iron in the regulation of placental iron transfer.

Design and Methods

Hfe wild-type, knockout and heterozygote dams were fed iron deficient (12.5 ppm), adequate (50 ppm) and replete (150 ppm) iron diets and mated with heterozygote males to produce pups of all genotypes. Dams and pups were sacrificed at Day 18 of gestation; serum, placenta, body and liver iron parameters were measured. Protein and mRNA levels of various iron transporter genes were determined in duodenum, liver and placenta by Western blotting and real time PCR.

Results

Maternal liver iron levels were dependent on both dietary iron intake and *Hfe* genotype. Increasing iron levels in the maternal diet resulted in increased total iron in the fetus, primarily in the liver. However, fetuses of *Hfe*-knockout mothers showed further elevation of liver iron levels, concomitant with elevated expression of *Tfr1*, *Dmt1* and *Fpn* in the placenta. *Hfe*-knockout fetuses that express low levels of liver hepcidin accumulated more iron in their liver than wild-type fetuses due to increased ferroportin levels in the placenta.

Conclusions

Maternal and fetal status, as well as dietary iron, is important in regulating iron transfer across placenta. Maternal *Hfe* regulates iron transfer by altering gene expression in the placenta. Fetal *Hfe* is important in regulating placental iron transfer by modulating fetal liver hepcidin expression.

Key words: iron, fetal *Hfe*, dietary, genotype, maternal.

Citation: Balesaria S, Hanif R, Salama MF, Raja K, Bayele HK, McArdle H, and Srai SKS. Fetal iron levels are regulated by maternal and fetal *Hfe* genotype and dietary iron. *Haematologica* 2012;97(5):661-669. doi:10.3324/haematol.2011.055046

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Introduction

Iron homeostasis is important for human health because its deficiency or overload results in anemia or hemochromatosis, respectively. Iron deficiency anemia is the most common nutritional deficiency, affecting nearly 2 billion people worldwide; pregnant women, women of child-bearing age and children are most vulnerable.¹ Iron deficiency in the neonatal and early postnatal period has been associated with impaired brain function and other deleterious neurological effects.² The majority of fetal iron is acquired in the third trimester of gestation; therefore, understanding the mechanism of iron transport across the placenta in this gestational period is important.

Iron transfer from mother to fetus is a regulated process involving iron uptake from the maternal circulation, its transport across the placenta and subsequent transfer into the fetal circulation.³ The mechanisms behind iron transfer from mother to fetus are beginning to be clarified but much is still unknown. As pregnancy progresses, the amount of iron transferred from mother to fetus increases and at term is directed against a concentration gradient. Syncytiotrophoblast cells of the placenta form the barrier between mother and fetus with the apical surface adjacent to the maternal circulation and the basolateral surface bordering the fetal circulation. Proteins localized at the apical membrane of syncytiotrophoblasts include transferrin receptor 1 (TfR1) and HFE.⁴⁻⁸ Diferric transferrin from the maternal circulation binds to TfR1 at the apical surface of the syncytiotrophoblast and this complex is internalized by endocytosis.^{9,10} Acidification of the endosomal compartments causes dissociation of iron from TfR1; iron is subsequently released into the cytoplasm, possibly by DMT1.¹¹ Transfer of iron to the fetal circulation probably occurs via ferroportin (FPN) as this has been shown to localize to the basal membrane of syncytiotrophoblast cells.¹²

HFE is a negative regulator of iron absorption. Mutations in the *HFE* gene result in hereditary hemochromatosis (HH) which is characterized by increased iron absorption and transferrin saturation, leading to hepatic iron overload.¹³ There have been no studies on the relationship between the HH mutation and levels of iron in newborn infants, so the functional consequences of an absence of HFE in placenta are not known. However, it has been shown that in placenta, HFE is associated with TfR1 at the apical membrane of syncytiotrophoblast cells^{7,8} where it is predicted to compete with transferrin for the same binding site on TfR1.¹⁴ HFE is an upstream regulator of liver hepcidin which has been demonstrated to be a negative regulator of intestinal dietary iron absorption and efflux of recycled iron from macrophages. Hepcidin regulates iron levels by binding to and causing the internalization of FPN in hepatocytes, macrophages and enterocytes.^{15,16} The loss of HFE in humans and mice results in low hepcidin expression in the liver.¹⁷⁻²⁰ The precise mechanism by which this occurs is not known; however, it has been proposed that binding of diferric transferrin to the TfR1-HFE complex regulates hepcidin synthesis by releasing HFE to bind to TfR2.^{21,22} As pregnancy progresses, maternal hepcidin levels decrease but return to normal soon after birth.²³ Transgenic mice that over-express hepcidin in the liver show a reduction in placental TfR1 levels; the offspring of these mice are also severely anemic and die shortly after birth.^{24,25} How hepcidin regulates iron

transfer across the placenta in order to cause fetal anemia is still unknown.

In this study, we investigated the role of, on the one hand, maternal and fetal *Hfe* genotype and, on the other hand, dietary iron levels in iron transfer from mother to fetus. Our data demonstrate that fetal *Hfe* status is important in the regulation of placental iron transfer by the modulation of fetal hepcidin expression. Maternal *Hfe* genotype also affects iron transfer to the fetus; this is likely due to increased iron stores at higher dietary iron levels and modulation of gene expression in the placenta.

Design and Methods

Experimental animals

All experimental procedures were approved and conducted in accordance with the UK Animals (Scientific Procedures) Act, 1986. *Hfe* knockout mice (129/Ola-C57BL/6 mixed background strain) were derived by replacing a 2.5 kb BglII fragment of the *Hfe* gene with a 2 kb pgk-neo^r gene as described previously.²⁶ Knockout mice (KOs) were mated with wild-type (WT) mice to produce heterozygotes (Hets). Hets were crossed with each other to produce KO, WT and Het pups. Subsequently, these mice with the same genetic background, of all three genotypes were used for the following experiments.

After weaning, animals were fed regular chow (RM1 diet, SDS, UK). One week after weaning, animals were placed on low (12.5 ppm), or adequate (50 ppm) iron diets. The experimental diets used were based on a dried egg albumin diet²⁷ and conformed to American Institute of Nutrition guidelines for laboratory animals.²⁸ FeSO₄ was added to achieve total levels of Fe. Dietary ingredients were purchased from Mayjex, Ltd. (Chalfont-St Peter, UK), BDH Chemicals (Poole, UK), or Sigma (Poole, UK). Females of all genotypes were mated with Het males three weeks later and remained on their respective diet throughout pregnancy. Pregnancy was confirmed by detection of a vaginal plug and this day was denoted Day 0. On Day 18 of gestation, dams from each group were anesthetized with pentobarbitone. The fetuses were delivered by caesarean section, decapitated and weighed before dissection. Fetal sex was not determined at any point in this study. Maternal and fetal livers, maternal duodenum and placenta were removed, weighed and rapidly frozen in liquid nitrogen before being stored at -80°C.

Iron, non-heme iron and serum iron measurements

Whole fetuses (minus liver) were dried at 50°C for 72 h and subsequently weighed. The dried carcasses were digested in 1% (v/v) nitric acid (VWR) for 24 h at room temperature before heating to 80°C for a further 24 h to ensure complete digestion. The digests were centrifuged at 3000g and supernatants were analyzed for Fe using ICP-MS. Briefly, supernatants were diluted 1:50 using 1% nitric acid and 0.1% TX100 containing ~5 ppb of the internal standards scandium and rhodium. The Fe concentrations were measured from calibration curves (0-2500 ppb) of the standards.

Livers were dried at 50°C for 72 h and subsequently weighed. Quantitative measurement of non-heme iron was performed according to the method of Torrance and Bothwell.²⁹ Results were reported as micrograms iron/gram of tissue dry weight. Serum iron was measured using an iron binding assay kit (Pierce). Results were reported as micrograms iron/deciliter.

RNA extraction and RT-PCR

Duodenal, hepatic and placental RNA was extracted with TRIzol (Invitrogen) according to the manufacturer's instructions.

Total RNA (1 µg) was reverse transcribed using the Verso cDNA reverse transcription kit (ThermoFisher Scientific) according to the manufacturer's instructions. Real time PCR reactions were run using a Lightcycler (Roche) with β-actin as an internal standard. Each reaction was performed in duplicate and contained 10 pmoles of specific primers, 1 x SYBR Green Mastermix (Qiagen) and 1 µL of cDNA in a 20 µL reaction. Samples without cDNA were included as negative controls. Mouse PCR primer-pairs were as follows: *β-actin*: 5'-GACGGCCAAGTCATCACTATT-3' (forward); 5'-CCACAGGATTCCATACCCCAAGA-3' (reverse); *Tfr1*: 5'-CAGAAAAGTTCCTCAGCTCAACCA-3' (forward); 5'-GTTCAATTCAACGCATGGGTAAG-3' (reverse); Total (IRE plus non-IRE form) *Dmt1*: 5'-GGCTTTCTTATGAGCATTGCC-TA-3' (forward); 5'-GGAGCACCCAGAGCAGCTTA-3' (reverse); *ferroportin*: 5'-CCAGCATCAGAACAACACAG-3' (forward); 5'-ACTGCAAAGTGCCACATCC-3' (reverse); *Hamp-1*: 5'-CCTATCTCCATCAACAGATG-3' (forward); 5'-AACAGATAC-CACACTGGGAA-3' (reverse); *ceruloplasmin*: 5'-TGCAAACC-TATTCCCTCATAAAAGT-3' (forward); 5'-GGCACTCAACAT-CAAAAGTCCC-3' (reverse). Cycle threshold (Ct) values were obtained for each gene of interest and the β-actin internal standard. Gene expression was normalized to β-actin and represented as ΔCt values. For each sample the mean of the ΔCt values was calculated. Relative gene expression was normalized to controls with an arbitrary expression level of 1.0.

Western blot analysis

Placental protein samples (40 µg) were heated in 1 x sample loading buffer (0.125M Tris-HCl, pH 6.8, 4% SDS, 20% (v/v) glycerol, 0.6M β-mercaptoethanol and 0.02% bromophenol blue) at 95°C for 5 min, resolved on a 10% SDS polyacrylamide gel and transferred to polyvinylidene difluoride membranes (BioRad, Hercules, USA). Membranes were blocked for 1 h at room temperature with 5% non-fat dry milk in PBS-T (PBS/0.1% Tween 20) and incubated at 4°C overnight with 1:500 dilution of ferroportin antibody (Alphadiagnostics, San Antonio, USA) and 1:10,000 dilution of β-actin antibody (Abcam, Cambridge, UK). Cross reactivity was detected using an HRP-linked secondary antibody (1:4000; GE Healthcare, Buckingham, UK) and ECL reagent (Pierce, Rockford, USA). Band densities were quantified using Quantity One 1-D Analysis software (BioRad).

Statistical analysis

All of the results are presented as mean ±SEM. Statistical analysis was calculated by two-tailed unpaired Student's t-tests. $P \leq 0.05$ was considered significant.

Results

Duodenal and hepatic iron transporter gene expression increases during pregnancy

Serum iron levels were measured in both non-pregnant and pregnant WT and KO dams to observe the effects pregnancy has on circulating iron levels and to see whether there are differences between WT and KO dams. Serum iron levels decreased markedly during pregnancy, and both WT and KO dams had significantly lower levels compared with their non-pregnant counterparts on both iron deficient and adequate diets (Figure 1A). No difference was observed between pregnant WT and KO dams at either 12.5 or 50 ppm dietary iron (iron deficient and iron adequate diets, respectively). In the non-pregnant state, serum iron levels at 50 ppm were significantly high-

er in KO than WT dams. This difference was not observed at 12.5 ppm dietary iron.

In order to assess whether the differences in serum iron levels during pregnancy were attributable to differences in iron transporter gene expression, we measured duodenal *Dcytb*, *Dmt1* and *Fpn* mRNA levels. During pregnancy, regardless of maternal genotype, all three genes were significantly up-regulated compared to their non-pregnant counterparts (Figure 1B); however, this was only true at 50 ppm dietary iron. No difference was observed at 12.5 ppm between pregnant and non-pregnant dams (*data not shown*). The levels of *Dcytb*, *Dmt1* and *Fpn* expression were the same in both WT and KO pregnant dams at 50 ppm dietary iron suggesting that during pregnancy the increased demand for iron overrides the genotypic difference. In the non-pregnant state, only *Dmt1* appeared to be significantly up-regulated in the duodenum of KO dams compared with WT.

We measured non-heme liver iron levels in non-pregnant and pregnant WT and KO dams ingesting 12.5 and 50 ppm iron diets to determine whether iron storage during pregnancy is genotype and diet specific. During pregnancy, liver iron levels decreased markedly in both WT and KO dams ingesting an iron adequate diet. Although we found decreased liver iron levels at 50 ppm during pregnancy, KO dams still retained more iron compared to their WT pregnant counterparts (Figure 1C). Interestingly, at 12.5 ppm dietary iron, no difference was observed between non-pregnant and pregnant WT dams, indicating that once a certain minimum limit is obtained, no further iron is mobilized from maternal liver. This suggests there is a lower limit threshold for liver iron loss during pregnancy. Liver iron levels in non-pregnant KO dams were much higher than in WT dams at both diets tested (Figure 1C).

The expression levels of *Tfr1*, *Fpn*, ceruloplasmin (Cp) and *Hamp-1* were assessed in the livers of non-pregnant and pregnant dams to see whether their mRNA levels were altered during pregnancy. The expression of *Tfr1* and Cp increased during pregnancy for both WT and KO dams at 50 ppm iron; conversely *Hamp-1* levels were barely detectable during pregnancy in both WT and KO dams (Figure 1D). No change was observed in any of the genes tested at 12.5 ppm (*data not shown*). Liver *Fpn* expression was up-regulated in KO dams during pregnancy at 50 ppm dietary iron, suggesting that maternal liver *Fpn* expression was up-regulated to increase iron supply to the fetus; no change was observed in WT dams.

Maternal Hfe genotype and dietary iron intake determine fetal development

Measurement of fetal body weight showed that growth was dependent on maternal diet, increasing with increasing dietary iron intake. Body weight was also influenced by maternal *Hfe* status. Overall, KO dams appeared to produce pups with higher body weight than WT. It would, therefore, appear that the *Hfe* status of the mothers and the iron content of their diet are essential for maintaining high fetal body weight during pregnancy (Table 1). Dietary iron and *Hfe* genotype did not alter the number of fetuses born per litter (*data not shown*).

Maternal Hfe status affects iron transport across the placenta and fetal liver iron stores

What effects do maternal genotype and diet have on

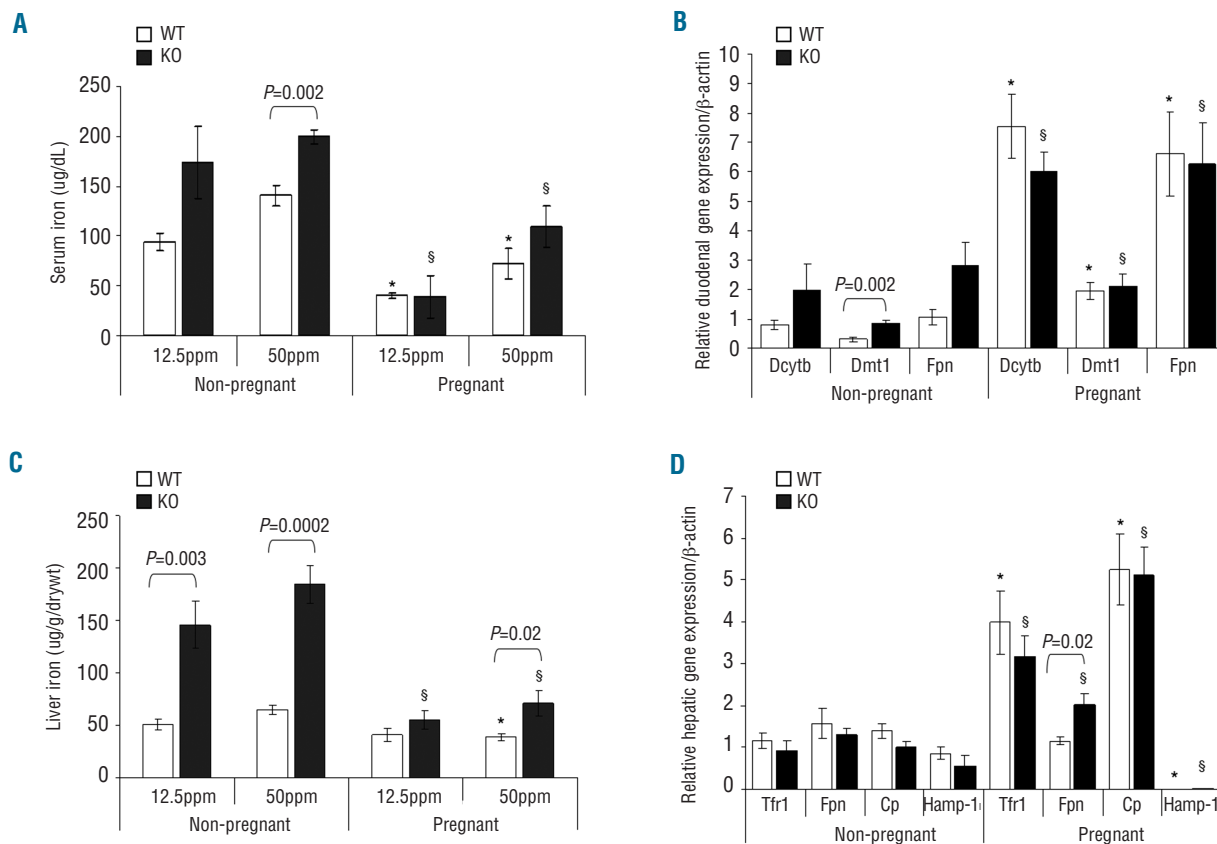


Figure 1. (A) Serum iron levels in non-pregnant and pregnant WT and KO dams ingesting 12.5ppm and 50ppm dietary iron. Mean \pm SEM presented: * $P < 0.05$ for WT non-pregnant vs. pregnant at 12.5 and 50ppm dietary iron ($n \geq 4$); $^{\S}P < 0.05$ for KO non-pregnant vs. pregnant at 12.5 and 50 ppm dietary iron ($n \geq 4$). (B) Duodenal transporter mRNA levels in non-pregnant and pregnant WT and KO dams ingesting 50ppm dietary iron. Mean \pm SEM presented: * $P < 0.05$; WT non-pregnant vs. pregnant for *Dcytb*, *Dmt1* and *Fpn* ($n \geq 4$); $^{\S}P < 0.05$; KO non-pregnant vs. pregnant for *Dcytb*, *Dmt1* and *Fpn* ($n \geq 4$). (C) Non-heme liver iron levels in non-pregnant and pregnant WT and KO dams ingesting 12.5ppm and 50ppm dietary iron. Mean \pm SEM presented: * $P < 0.001$ for WT non-pregnant vs. pregnant at 50ppm dietary iron ($n \geq 4$); $^{\S}P < 0.001$ for KO non-pregnant vs. pregnant at 12.5 ppm and 50 ppm dietary iron ($n \geq 4$). (D) Hepatic transporter mRNA levels in non-pregnant and pregnant WT and KO dams ingesting 50ppm dietary iron. Mean \pm SEM presented: * $P < 0.05$; WT non-pregnant vs. pregnant for *Tfr1* and *Cp* ($n \geq 4$); $^{\S}P < 0.05$; KO non-pregnant vs. pregnant for *Tfr1*, *Fpn* and *Cp* ($n \geq 4$).

iron transfer to the fetus? We wanted to see whether loss of maternal *Hfe* could promote increased iron transport across the placenta. To address this, we mated WT and KO dams with Het males and measured body iron in addition to non-heme liver iron in Het fetuses. Our results show that maternal genotype affects the amount of iron being transferred to the fetus (Figure 2). At 12.5 ppm iron, the amount of iron in Het pups born from KO dams was significantly higher than those born from WT dams (Figure 2A). This, however, was not a factor when dietary iron levels were adequate at 50 ppm. Iron levels in fetuses were higher in pups derived from dams fed an adequate iron diet than those fed an iron deficient diet.

Non-heme iron was measured in fetal liver in order to assess fetal iron storage during the latter stages of gestation (Figure 2B). It appeared that little iron was stored in the liver of fetuses when the dams were fed a low-iron diet, independent of maternal genotype. However, when dietary iron was adequate, liver iron levels were significantly increased in fetuses from both WT and KO dams compared with those on an iron deficient diet. In addition, fetal liver iron levels in pups derived from KO dams were significantly higher than those derived from WT dams on the adequate diet.

Table 1. Total body weight (in milligrams) of fetuses born from dams of varying genotypes (WT, Het, KO) fed varying iron diets (12.5 and 50 ppm) during pregnancy.

Maternal genotype Dietary Fe (ppm)	Fetal body weight (mg)		
	WT	Het	KO
12.5	973.9 ^a \pm 134.9 n=33	971.1 ^a \pm 121.0 n=51	1058.8 ^b \pm 91.6 n=16
50	1018.6 ^c \pm 111.9 n=58	1117.0 ^d \pm 94.4 n=31	1090.1 ^d \pm 115.8 n=68

Mean \pm SD presented; a statistically significant difference ($P < 0.001$) between pups derived from each maternal genotype on each diet is indicated by a different superscript letter.

In order to assess whether the effects of iron transport to the fetus were due to maternal serum and liver iron levels or *Hfe* genotype, WT dams were iron loaded to the same degree as KO dams. WT dams were placed on 150 ppm iron whereas KO dams were fed a diet consisting of 50 ppm iron. Serum iron levels of both WT and KO dams were equivalent at 104.4 \pm 18.0 and 108.8 \pm 20.6 mg/dL, respectively (mean \pm SEM; $P = 0.8$). Liver iron levels of both WT and KO dams were also equivalent at 66.3 \pm 5.3 and

70.8±12.2 µg/g/drywt, respectively (mean ±SEM; $P=0.9$). Liver iron levels of Het pups were then assessed (Figure 2C). Fetal liver iron levels were significantly higher in Het pups derived from KO dams on 50 ppm iron than Het pups derived from WT dams on 150 ppm iron, suggesting an effect of loss of *Hfe* rather than maternal serum and liver iron levels on iron transfer to the fetus.

Iron transporter expression in KO placentas is elevated at adequate dietary iron levels

The expression of various key iron regulatory genes was measured in the placenta of Het pups derived from both WT and KO dams on low and adequate iron diets in order to clarify the molecular mechanisms of iron acquisition from the mother (Figure 3). The levels of *Tfr1*, *Fpn* and *Dmt1* expression were unchanged at 12.5 ppm iron; maternal genotype had no effect (Figure 3A). This, however, was not the case at 50 ppm iron where *Tfr1*, *Fpn* and *Dmt1* expression in the placenta were elevated approximately 3-fold in pups derived from KO dams compared with WT (Figure 3B). Placental *FPN* protein levels were also measured by Western blotting (Figure 3C). It appears that there are higher *FPN* protein levels in placenta of Het pups derived from KO dams at 50 ppm than WT. Placental non-heme iron levels were also measured (Figure 3D) and higher iron levels were observed in Het pups from KO dams when fed an adequate diet.

Fetal genotype controls iron absorption from mothers fed a high iron diet

If maternal genotype is a factor affecting iron transfer across placenta, could fetal genotype also play a role? To address this, Het dams were mated with Het males and total body iron as well as non-heme liver iron was measured in fetuses with a WT and KO genotype (Figure 4). We found that there was less iron in fetuses from dams on a low iron diet than those from dams on an adequate diet (Figure 4A). In addition, fetal genotype appeared to have no effect on liver iron storage at either low or adequate iron diets (Figure 4B). Here again, there was less liver iron in those fetuses derived from dams on a low iron diet than those on the adequate diet. At these dietary iron concentrations, we also found no difference in the levels of placental *Tfr1*, *Fpn* and *Dmt1* mRNA expression in both KO and WT pups (*data not shown*). At the protein level, also no difference in *FPN* expression was detected (*data not shown*).

When Het dams were fed a high iron diet consisting of 150 ppm iron, fetal iron measurements showed no difference between KO or WT pups (*data not shown*). However, when fetal non-heme liver iron was measured, KO pups had higher levels than WT (Figure 5A) suggesting increased iron acquisition and storage in these fetuses. This suggests strongly that fetal iron regulatory genes might regulate iron absorption from the mother. Consequently, hepcidin mRNA was measured in the livers of WT and KO pups (Figure 5B). Liver hepcidin expression was barely detectable in KO pups compared with WT. In addition, *Fpn* mRNA levels were measured in both WT and KO pup placenta. *Fpn* mRNA levels were found to be significantly lower in WT than in KO pups (Figure 5C). Placental *FPN* protein expression mirrored its mRNA expression, being lower in WT than in KO pups (Figure 5D); this was confirmed by quantification of band intensity (Figure 5E).

Discussion

Most of the iron transferred to the developing fetus takes place during the last trimester of pregnancy. It is clear that iron transfer is strictly regulated. In a previous study using iron supplementation and deficiency during pregnancy, we were able to infer by correlation data that fetal liver plays a central role in regulating the fetal iron supply.³⁰ In this study, using a mating strategy between *Hfe*^{-/-}, *Hfe*^{+/-} and *Hfe*^{+/+} mice, we have been able to confirm that fetal iron levels are indeed involved. This regulation by fetal liver iron levels involves expression of hepcidin and its negative effect on placental ferroportin protein levels, thereby modulating iron efflux. In the case of *Hfe* KO fetuses, placental ferroportin levels are increased due to low expression of hepcidin, thereby resulting in increased iron levels when dams are fed an iron replete diet. In addition to this, we have also shown that maternal *Hfe* status

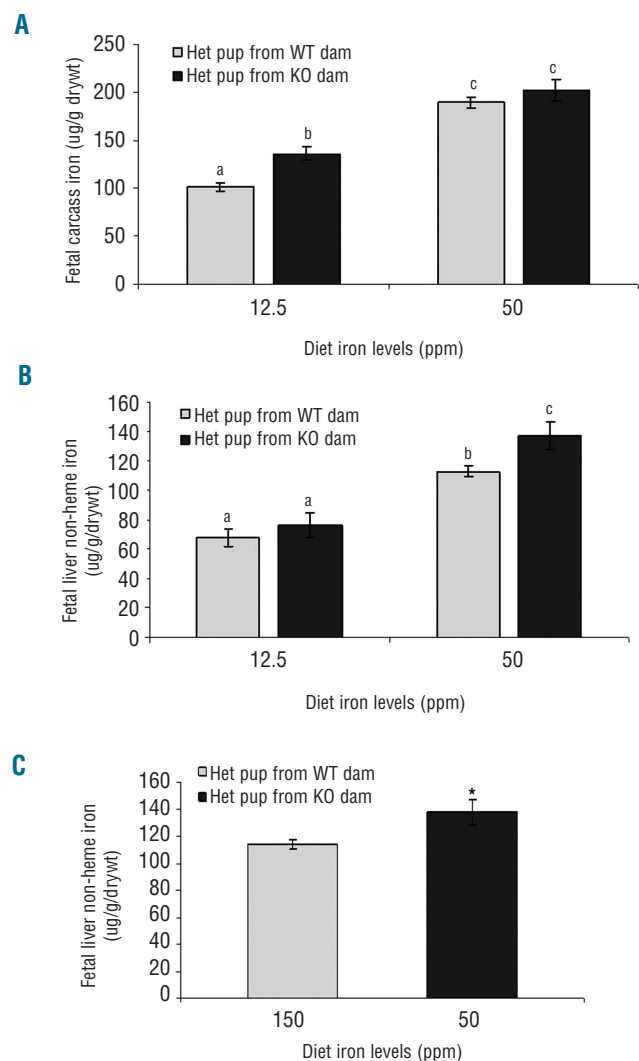


Figure 2. (A) Fetal carcass iron (total body iron minus liver) and (B) non-heme liver iron levels of Het pups derived from either WT or KO dams fed 12.5 or 50 ppm iron diets. Mean ±SEM presented; bars with different letter superscripts are significantly different ($P<0.001$, $n\geq 7$). (C) Non-heme liver iron levels of Het pups derived from WT dams on 150 ppm diet and Het pups derived from KO dams on 50 ppm diet. Mean ±SEM presented; * $P=0.02$; $n\geq 27$.

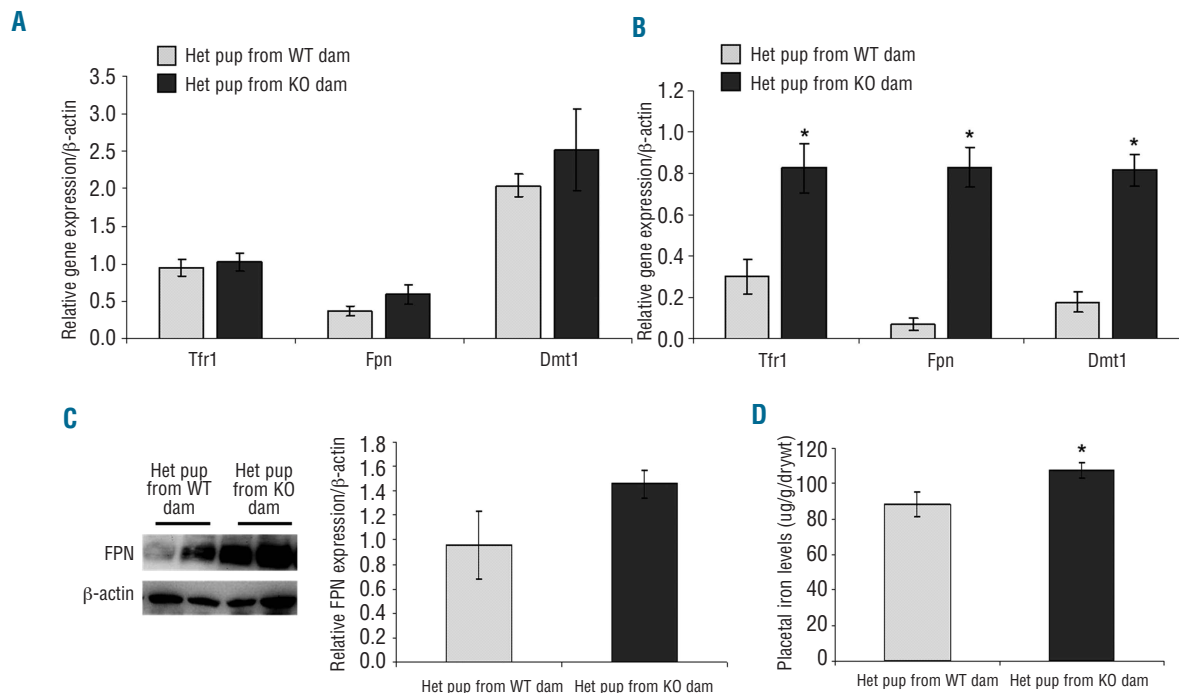


Figure 3. Placental gene expression in Het pups derived from WT and KO dams fed (A) 12.5 ppm and (B) 50 ppm dietary iron. Mean \pm SEM presented; * $P < 0.05$, $n \geq 5$. (C) Western blot analysis of placental ferroportin expression (upper panel) of Het pups derived from WT and KO dams fed 50 ppm iron diet. Lanes 1 and 2: Het pups from WT dams. Lanes 3 and 4: Het pups from KO dams. Band intensity was quantified by densitometry, corrected for loading using β -actin as a control and represented graphically, $n = 3$. (D) Non-heme placental iron levels of Het pups derived from WT and KO dams on 50 ppm iron diet. Mean \pm SEM presented: * $P = 0.04$; $n = 8$.

plays a role in the transfer of iron across placenta, but this is independent of maternal hepcidin expression during pregnancy.

As in previous studies, we have also shown that deletion of *Hfe* in non-pregnant mice results in increased serum iron and hyperaccumulation of iron in the liver.^{26,31} However, during pregnancy, increases in serum iron and liver iron are observed only when dietary iron is adequate. When dietary iron levels are low (12.5 ppm), maternal liver iron levels drop to low levels. What is interesting is that maternal iron levels in WT dams are the same when they are fed both the iron deficient and adequate diets, a 4-fold difference in dietary iron levels, whereas fetal iron levels are significantly higher in iron adequate fed dams. Therefore, we can conclude that during iron deficiency, the mother maintains its liver iron at the expense of the fetus, as has been suggested previously.^{32,33} Previous studies have shown that iron supplementation and adequate iron ingestion during the first two trimesters of pregnancy are associated with better pregnancy outcome and higher fetal weights;³⁴⁻³⁷ this present study confirms this. Our results demonstrate that the combination of maternal iron ingestion and *Hfe* status affects fetal body weight with dams ingesting an iron deficient diet producing fetuses with the lowest body weight. Pups derived from KO dams appeared to have higher body weights than those derived from WT dams when fed both deficient and adequate diets. This has also been demonstrated recently in a human study; mothers with *HFE* variants were more likely to give birth to babies of a higher weight than controls; although this was true only for babies with *HFE* mutations.³⁸ In agreement with this, we found that when high

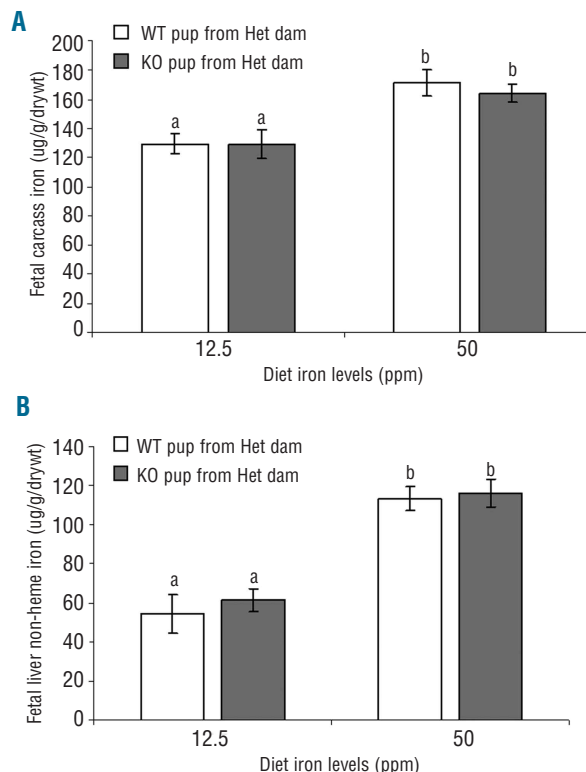


Figure 4. (A) Fetal carcass iron (total body iron minus liver) and (B) non-heme liver iron levels of WT and KO pups born from Het dams fed 12.5 or 50 ppm iron diets. Mean \pm SEM presented; bars with different letter superscripts are significantly different ($P < 0.05$, $n \geq 7$).

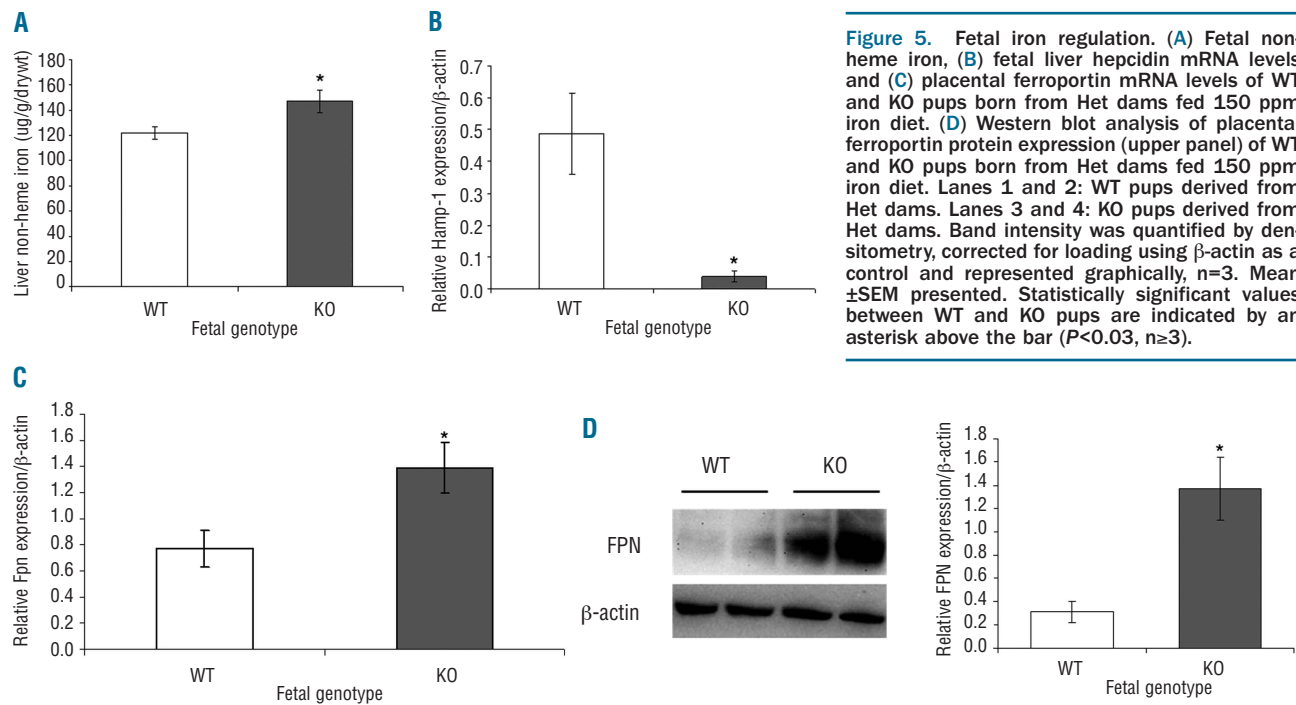


Figure 5. Fetal iron regulation. (A) Fetal non-heme iron, (B) fetal liver hepcidin mRNA levels and (C) placental ferroportin mRNA levels of WT and KO pups born from Het dams fed 150 ppm iron diet. (D) Western blot analysis of placental ferroportin protein expression (upper panel) of WT and KO pups born from Het dams fed 150 ppm iron diet. Lanes 1 and 2: WT pups derived from Het dams. Lanes 3 and 4: KO pups derived from Het dams. Band intensity was quantified by densitometry, corrected for loading using β -actin as a control and represented graphically, $n=3$. Mean \pm SEM presented. Statistically significant values between WT and KO pups are indicated by an asterisk above the bar ($P<0.03$, $n\geq 3$).

maternal iron was ingested, KO pups had higher weights than either WT or Het pups regardless of maternal genotype (*data not shown*). Hfe KO dams, therefore, appear to be better able to provide iron to their pups than their WT counterparts.

In order to understand the mechanism of regulation of iron homeostasis during pregnancy, we studied gene expression of the main iron transporter genes in the liver and intestine. In the liver, during pregnancy, iron transporter gene expression altered to compensate for a loss in iron stores by increasing *Tfr1*, *Fpn* and *Cp* mRNA levels. We also tested the role of genotype and found that there was differential expression of only *Fpn* in WT and KO dam liver. The increase in *Fpn* expression in KO dams may serve to release hepatic iron for delivery to the fetus. Increased *Tfr1* expression in maternal liver during pregnancy was similar in both WT and KO dams. An increase in maternal liver *Tfr1* expression has previously been shown to be inversely correlated with maternal iron status during pregnancy in the rat.^{23,34} Increased CP oxidase activity in serum has also been previously reported during iron deficiency in pregnancy.^{39,40} In support of this, we found increased *Cp* mRNA levels in both WT and KO pregnant dams compared with their non-pregnant counterparts. This may serve to change the valency of iron as it shuttles between the mother and fetus. During the third trimester of pregnancy, hepcidin levels are barely detectable²³ and no difference in hepcidin mRNA expression was observed between pregnant WT and KO dams in this study.

Depletion of liver iron stores during pregnancy has been shown to induce rapid iron intake by the maternal gut.^{30,41} In support of this, we found increased expression of *Dmt1*, *Dcytb* and *Fpn* in the duodenum of pregnant WT and KO mice compared to their non-pregnant counterparts. Previous studies in rats have shown increased *Dcytb* and *Dmt1* mRNA levels in the last trimester of pregnancy;

however, no changes in *Fpn* expression were found.²³ We also found that non-pregnant KO dams showed increased *Dmt1* expression; this may account for the increased iron absorption seen in the KO mouse, as has been described previously.⁴² We did not find any differences in duodenal gene expression for *Dmt1*, *Dcytb* and *Fpn* during pregnancy between WT and KO dams, suggesting that pregnancy and maintenance of maternal iron levels override all other factors.

Maternal *Hfe* status may be critical for regulating iron transport across the placenta to the fetus; the heterozygote offspring of KO dams had higher iron levels than heterozygote pups from WT dams at both dietary levels. In addition, when WT dams were loaded to the same degree as KO dams, higher liver iron levels were observed in pups derived from KO dams, indicating that iron transfer to the fetus is not due solely to maternal iron levels but is likely attributable to loss of *Hfe* in the dam. In an attempt to explain this observation, we measured the expression levels of major iron transport genes *Tfr1*, *Dmt1* and *Fpn* in the placenta. When maternal dietary iron intake was limited, expression of these genes was maximal regardless of maternal genotype. When maternal dietary iron was adequate, *Tfr1*, *Dmt1* and *Fpn* expression was down-regulated in the placentas of pups derived from WT dams. *Tfr1* has been shown to be up-regulated in the placentas of iron deficient rats⁴³ and this was true for those pups derived from WT dams on 12.5 ppm *versus* 50 ppm (cf *Tfr1* Figure 3A and B; *Tfr1* arbitrary value of 1 at 12.5 ppm vs. 0.3 at 50 ppm). This regulation, however, was lost in those pups derived from KO dams that had equivalent levels of *Tfr1* and *Fpn* mRNA at both dietary iron levels. FPN protein levels were also increased in placenta of pups derived from KO dams on an adequate diet compared with those derived from WT, indicative of increased iron transfer to the fetus; this accounts for the increased liver iron stores

seen in those pups derived from KO dams ingesting 50 ppm iron. Interestingly, placental non-heme iron levels were lower in pups derived from WT than those derived from KO on 50 ppm dietary iron. It would appear that KO dams are unable to regulate the amount of iron transported across the placenta; this is manifested in the inability to down-regulate both *Tfr1* and *Fpn* in the placenta when dietary iron levels are adequate and when placental iron and fetal iron stores are high. The driving force behind this increase in placental gene expression in pups derived from KO dams is still not clear; however, it is unlikely to be due to maternal hepcidin levels as the hepatic expression of hepcidin was negligible in both maternal genotypes. We propose that some other as yet unidentified factor under the regulation of the *Hfe* gene is involved in these changes in placental gene expression.

Interestingly, fetal genotype also appears to play a role in iron acquisition. Our results show that KO pups accumulate more iron in the liver than their WT counterparts when maternal dietary intake is high. WT pups produce hepcidin in the liver whereas KO pups fail to do so. It has been suggested previously that liver iron levels in the fetus regulate iron uptake across placenta;³⁰ our data support this. Our results suggest that normal regulation of iron absorption in KO pups is lost, thus enabling increased iron transport even though liver iron stores are near full capacity. Fetal hepcidin has been shown to be decreased in the livers of iron deficient rats compared with rats on an iron-supplemented diet;^{30,44} this indicates that, in WT animals, fetal hepcidin could contribute to the regulation of iron

transport. Our data shows that this effect of fetal hepcidin at higher dietary iron levels may be directed primarily at placental ferroportin. Martin *et al.*²⁴ showed that hepcidin transgenic embryos were anemic because they expressed reduced *Tfr1* levels in the placenta but with no change in ferroportin levels. It would be interesting to look at fetal iron metabolism in *Hamp* KO pups and assess gene changes in placentas of these fetuses.

Taken together, our results show that adequate iron intake during pregnancy is crucial for maintaining maternal iron stores and healthy fetal development. Maternal *Hfe* appears to play a major role in iron acquisition by the fetus. *Hfe* KO fetuses lose the ability to control their iron absorption when maternal dietary intake is high. This may be partly due to dynamic changes in hepcidin-ferroportin interaction in the placenta. Taken together, our data suggests that there may be an evolutionary advantage to the *Hfe* mutation during pregnancy: high maternal iron produces healthier offspring with the caveat that if this mutation is inherited, it may not be so advantageous later in life.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

Financial and other disclosures provided by the authors using the ICMJE (www.icmje.org) Uniform Format for Disclosure of Competing Interests are also available at www.haematologica.org.

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