The effect of maternal iron deficiency on zinc and copper levels and on genes of zinc and copper metabolism during pregnancy in the rat

Sarah C. Cottin
Guenievre Roussel
Lorraine Gambling
Helen E. Hayes
Valerie J. Currie
Harry J. McArdle

This is an Author Accepted version of the article: Cottin, S., Roussel, G., Gambling, L., Hayes, H., Currie, V., & McArdle, H. (n.d.). The effect of maternal iron deficiency on zinc and copper levels and on genes of zinc and copper metabolism during pregnancy in the rat. *British Journal of Nutrition*, 1-9. Available at: https://doi.org/10.1017/S0007114518003069

Title page

Title: The effect of maternal iron deficiency on zinc and copper levels and on genes of zinc and copper metabolism during pregnancy in the rat.

Author names and affiliations: Sarah C Cottin[3], Guenievre Roussel[2], Lorraine Gambling[1], Helen E Hayes[1], Valerie J Currie[1], Harry J McArdle[1]

[1] Corresponding author: Prof Harry J McArdle, Rowett Institute of Nutrition and Health, University of Aberdeen, Foresterhill House, Ashgrove Rd W, Aberdeen AB25 2ZD, UK: h.mcardle@abdn.ac.uk |tel +44 7711384254

[2] Aix-Marseille Université, Laboratoire de Neurosciences Cognitives, Marseille, France

[1] Rowett Institute of Nutrition and Health, University of Aberdeen, Foresterhill House, Ashgrove Rd W, Aberdeen AB25 2ZD, UK.

[3]Division of Food and Drink, School of Science Engineering and Technology, Abertay University, Dundee UK

Running title: Maternal iron deficiency, zinc and copper

Funded by Scottish Government (Rural and Environmental Scientific and Analytical Services)

Key words (6 words maxi): placenta, liver, iron status, copper, pregnancy.

Conflict of interest: none.

Abstract

Iron deficiency is relatively common in pregnancy and has both short and long term consequences. However, little is known about the effect on the metabolism of other micronutrients. 54 female rats were fed control (50 mg iron/ kg) or iron deficient diets (7.5 mg / kg) prior to and during pregnancy. Maternal liver, placenta and fetal liver were collected at day 21 of pregnancy for copper and zinc analysis, and to measure expression of the major genes of copper and zinc metabolism. Copper levels increased in the maternal liver ($p = 0.002$) and placenta ($p = 0.018$) of iron deficient rats. zinc increased ($p < 0.0001$) and copper decreased ($p = 0.006$) in the fetal liver. Hepatic expression of the copper chaperones ATOX1 ($p = 0.042$) and COX17 ($p = 0.020$) decreased in the iron deficient dams, while the expression of the genes of zinc metabolism was unaltered. In the placenta, iron deficiency reduced the expression of the chaperone for SOD1, CCS ($p = 0.030$), ceruloplasmin ($p = 0.042$), and zinc transport genes, ZIP4 ($p = 0.047$) and ZnT1 ($p = 0.012$). In fetal liver, iron deficiency increased COX17 ($p = 0.020$), ZIP14 ($p = 0.036$) and ZnT1 ($p = 0.0003$), and decreased ZIP4 ($p = 0.004$). The results demonstrate that iron deficiency during pregnancy has opposite effects on copper and zinc levels in the fetal liver. This may, in turn, alter metabolism of these nutrients, with consequences for development in the fetus and neonate.

1 Introduction

Iron deficiency is the most common nutrient deficiency worldwide and is particularly prevalent in children, women of child bearing age and pregnant women. Even in the UK, it is estimated that 23% of women aged 19-64 and nearly half of teenage girls do not reach their lower reference nutrient intake for iron [1]. During development, the fetus relies entirely on maternal supply for iron, zinc and copper, which are critical for development and health. Crosstalks between these three trace elements have been established decades ago [2-4] but the effect of iron deficiency on the metabolism of copper and zinc is still not fully understood, particularly during pregnancy. The ability to carry out work establishing these links is, of course, limited, but micronutrient metabolism is well conserved in mammals, and the rat has been shown to be a very good model for pregnancy and nutrition in humans [3].

Generally, iron deficiency in mammals results in increased copper levels in the blood, gut and liver [5-7]. Circulating ceruloplasmin (CP), produced by the liver, and its gut homolog hephaestin, are copper dependent ferroxidases and appear as an evident link between iron and copper metabolism. Accordingly, iron deficiency in rats induces an increase in serum copper associated with an increase in CP levels [5]. Interestingly, iron deficient rats have higher liver copper levels which correlate with CP expression and its ferroxidase activity, while hepatic CP mRNA remains unchanged, suggesting that copper loading is associated with an increased metallation of the protein [8] . Iron deficiency also increases the expression of the Menkes copper ATPase (ATP7a) in the duodenum [6, 9], suggesting an increased export of copper from the enterocyte to the circulation, while Wilson copper ATPase (ATP7b) expression in the liver is unaltered [6]. In addition, copper and iron may compete for DMT1 for their uptake in the gut. Although copper influx through DMT1 in the enterocyte is minor compared to CTR1 in replete conditions, it is of particular importance during iron deficiency, due to its upregulation and the lack of iron in the lumen, which favours copper uptake [10].

The effect of iron deficiency on zinc status is believed to be less important than on copper status. Iron deficiency appears to reduce zinc requirements in rats, increasing zinc levels in several organs such as liver, spleen, brain or kidney [11, 12], while decreasing apparent zinc absorption [13] and plasma zinc [14]. Noteworthy, marginal iron deficiency (9 and 13 mg iron per kg diet) did not affect zinc status in rat, while it was sufficient to induce copper accumulation in the liver [15]. Because iron and zinc have similar mechanisms of absorption

and transport in the gut, they were originally thought to compete with each other, notably through DMT1. Accordingly, iron treatment reduces the transport of zinc in Caco-2 cells, although to a lesser extent than other divalent cations [16]. However, DMT1's affinity for zinc is much lower than for iron and its contribution to zinc transport and uptake is probably minimal[17].

Two families of zinc transporters have been identified, ZIP and ZnT, generally involved in influx and efflux of the cells, respectively. ZIP stands for Zrt (zinc regulated transporter), Irt (iron regulated transporter)-like protein, and is composed of 14 members in mammals. They are characterised by their ability to transport zinc, but several of them have also been reported to mediate the uptake of iron, as well as manganese [18]. In rats, iron overload increases the expression of several ZIPs in the liver, namely ZIP5 (up), ZIP6, ZIP7 and ZIP10 (down), while ZIP14, the most abundant ZIP in the liver, is upregulated by iron deficiency [19]. On the other hand, the ZnT (zinc transporters) family comprises 10 members so far, and contribute to zinc homeostasis by exporting zinc out of the cell or sequestrating zinc into cellular compartments when zinc levels are low. While there is evidence of direct action of iron on ZIP, which may lead to alterations of zinc metabolism, interactions between iron and ZnT have not been identified.

Metabolic crossroads between iron, copper and zinc during pregnancy are less known. Maternal iron deficiency in rat increases copper levels in the maternal liver, maternal serum, as well as the placenta [20, 21] along with an increase in serum copper and ceruloplasmin [22] . In contrast, we showed that iron deficiency decreased copper levels in the fetal liver, without affecting the fetal expression of the main genes involved in copper metabolism [23, 24]. In humans, iron supplementation has been shown to decrease plasma zinc levels during pregnancy and zinc absorption during lactation in humans [25-27]. Whether iron deficiency, as opposed to overload, affects zinc metabolism in the mother and fetus remains essentially unknown. In this study we investigated the effect of maternal iron deficiency on zinc status in pregnant rats and the offspring, as well as the implication of major genes of zinc metabolism. We also examined the mechanisms through which iron deficiency differentially affects copper metabolism in the mother and fetus and tested the implication of most genes of copper metabolism in the liver of the mother and fetus, as well as in the placenta.

2 Material and methods

2.1 Animal procedures

Experiments were performed using female Rowett Hooded rats from 3 different studies of identical design. The protocol and animal procedures have recently been described in detail [28] and were approved by the Rowett Institute Ethics Committees and conducted in accordance with the UK Animals (Scientific Procedures) Act 1986. Briefly, 54 weanling females were fed a control diet (AIN93, containing 860 mg/kg Zn and 170 mg/kg Cu) for 2 weeks and were then randomised into two groups. 30 remained on the control diet (Fe = 50mg/kg) for 4 weeks until mating and 24 switched to an iron deficient (Fe = 7.5 mg/kg) diet for the same duration. They were mated with males of the same strain and remained on the same diet until they were killed at day 21 of gestation. Dams were killed by stunning and cervical dislocation and the fetuses by decapitation. Animals were housed under 12;12 light dark cycle with a constant temperature (22 ^{o}C). No adverse effects were recorded as a result of this dietary intervention.

Livers from the dams and fetuses, as well as the placentas, were collected and immediately frozen in liquid nitrogen. They were kept in metal free vials and stored at -80°C until analysis. Whenever possible, placentas and fetal livers from the 3 male median weights in each litter were pooled together and ground in liquid nitrogen. Ground tissues from maternal liver, pooled placentas and pooled fetal livers were used for iron, copper and zinc content (control n = 24, iron deficient n = 30) and gene analysis (control n = 21, iron deficient n = 24). It should be noted that control and iron deficient samples from each of the three animal studies were analysed together, and results from each experiment were then pooled together for data analysis.

2.2 Assessment of copper and zinc status

Total copper and zinc were measured in maternal liver, placenta and fetal liver by inductively coupled plasma mass spectrometry (ICP-MS) as previously described (28). Samples were digested in 2 % HNO_3, 0.5% HCl, and metal concentrations measured using an Agilent 7700X spectrophotometer (Agilent Technologies) equipped with a MicroMist nebulizer, nickel sampler and skimmer cones. Intra-assay CV% were 10.8% (Fe), 11.1% (Cu) and 5.2% (Zn).

2.3 RNA extraction and real-time RT-qPCR

20-30 mg of frozen placenta, maternal and fetal liver ground tissue was homogenized after grinding, using a T25 Ultra-Turrax ®. RNA was isolated with the RNeasy ® Mini Kit (Qiagen) and 200 ng were reverse transcribed on a PTC-100® Thermal Cycler (Bio-Rad Laboratories,

Hemel Hempstead, UK) using the Applied Biosystems® TaqMan® RT reagent kit (Life technologies, Paisley, UK). cDNA fragments were amplified by real-time qPCR (7700 Sequence detection system, Life technologies) using the primers (TaqMan®, ThermoFisher Scientific) described in **Table 1**. The most relevant genes of copper [24] and zinc metabolism were selected. Specifically, ZIP-1 is ubiquitously expressed and was shown to bind iron with high affinity [29]. ZIP-4 is crucial during development and for the absorption of maternal zinc (30). ZIP8 and 14 are closely related and have both been shown to mediate iron transport in addition to zinc [31, 32]. ZnT-1 (ubiquitous), ZnT-4 and ZnT-5 are sensitive to maternal zinc status and may play an important role in fetal zinc supply from the maternal diet [33, 34] . The gene expression was normalized to 18s rRNA (HS99999901_s1) in all samples. This was chosen even though the levels of expression were higher than the test genes because it was which was the only gene that was consistently unaffected by the nutritional treatments (Roussel, G, McArdle, HJ, unpublished data).

The experiment was performed and data interpreted according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines [35]: 18sRNA expression did not significantly vary between the iron deficient and control groups in all tissues (Mann-Whitney test, data not shown). Linear amplification was observed for 18s and all genes of interest across their range of expression.

2.4 **Statistical analysis**

Iron, copper and zinc content are expressed in μg/g dry tissue. The material used in this paper were obtained from 3 separate experiments. To minimize variations between the three animal studies, as well as between each PCR run, the expression of copper and zinc genes was expressed as percentage of the average gene expression of the control group for each of the 3 experiments. The normality of the distribution and equality of variances were analysed with GraphPad Prism (version 5.04). Data were analysed with IBM SPSS statistics 21.0 software using unpaired t-tests when data were normally distributed and otherwise using non-parametrical tests (Mann-Whitney U).

3 Results

3.1 Copper and Zinc levels

The effect of maternal iron deficiency on growth, placental:fetal ratio, placenta and liver weights, hematocrit and iron levels in this study has been recently reported (28). Specifically, iron levels were decreased by 152.6 µg/g (~ -30%), 604.0 µg/g (~ -60%) and 369.4 µg/g dry tissue (~ -75%), in the placenta, fetal and maternal liver, respectively [(28)]. Maternal liver and placenta weight were not affected by iron deficiency while fetal weight and fetal liver weight were significantly decreased by ~13% and ~25%, respectively [(28)]. Maternal iron deficiency increased copper levels by 1.44 µg/g dry tissue (95% CI [+0.34 , +2.54] ; p = 0.002) and 3.45 µg/g (95% CI [-0.42 , +7.32] ; p = 0.018) in maternal liver and placenta, respectively. In contrast, iron deficiency decreased copper levels in the fetal liver by 9.64 µg/g (95% CI [-15.85 , -3.42], p = 0.006) (**Figure 1A**, Mann-Whitney), while zinc levels were increased by +81.6 µg/g (95% CI [50.0 , 113.1], p < 0.0001). Zinc levels in maternal liver and placenta were not significantly altered by maternal iron deficiency (**Figure 1B**, T-test). Changes in absolute levels of iron, zinc and copper followed a similar pattern (Table 2), apart from the absolute zinc levels that were reduced by 4.08 µg (95% CI [-1.64 , -6.52] ; p = 0.003) in the fetal liver.

3.2 Zinc and copper genes expression

Maternal iron deficiency decreased the hepatic expression of the copper chaperones ATOX1 by -10.5% (95% CI [-20.7 , -0.4] ; p = 0.042) and COX17 by -13.8% (95% CI [-25.3 , -2.3] ; p = 0.020) in the dams. In contrast, the hepatic expression of COX17 was increased by +15.0% (95% CI [+2.5 , +27.5] ; p = 0.020) in the fetus. In the placenta, iron deficiency led to a decrease in CCS, the chaperone that delivers Cu to SOD1, by -9.5% (95% CI [-18.0 , -1.0] ; p = 0.030) and CP by -15.0% (95% CI [-29.4 , -0.5] ; p = 0.042) expression (**Figure 2**).

The maternal hepatic expression of the genes related to zinc metabolism were not affected by iron deficiency. In the placenta, ZIP4 was reduced by -23.7% (95%CI [-47.0 , -0.4] ; p = 0.047) and ZnT1 by -12.3% (95%CI [21.8 , -2.8] ; p = 0.012) in the iron deficient group compared to control (**Figure 3**). In fetal liver, iron deficiency also decreased ZIP4 by – 17.0% (95%CI [-28.1 , -5.8] ; p = 0.004), while ZIP14 and ZnT1 were increased by +10.0% (95%CI [+0.7 , +19.3] ; p = 0.036) and by +18.7% (95%CI [+9.0 , +28.4] ; p = 0.0003), respectively (**Figure 3**).

The effect of maternal iron deficiency on copper and zinc metabolism observed in the current study is summarized in **Table 3**.

4 Discussion

In this paper, we have examined the interactions between iron, copper and zinc during pregnancy in the rat. The study has some limitations, in that the data can only be extrapolated to humans to a limited extent. However, given how well conserved micronutrient metabolism is between species, it is likely that the same results would be observed in humans with iron deficiency. We restricted our measurements to liver and placenta, as these are the major organs involved in micronutrient metabolism[3]. Iron deficiency altered copper, but not zinc, concentrations in the maternal liver and placenta, confirming that the metabolism of copper is more sensitive to iron deficiency than that of zinc [15]. However, maternal iron deficiency had an important impact on both copper and zinc levels in the fetal liver, which were reduced by ~15% and augmented by ~25%, respectively.

4.1 Effect of maternal iron deficiency on copper metabolism

Maternal iron deficiency leads to the hepatic accumulation of copper in the dams, while copper levels are decreased in the fetal liver, as previously described by our group, as well as others [20, 21, 23]. Interestingly, iron deficiency decreased the expression of the chaperones ATOX1 and COX17 in the maternal liver, contrasting with our previous findings, where no change in ATOX1 mRNA levels was observed [23]. It is important to note that in our previous experiment, COX17 was not measured and ATOX1 was only measured in 6 samples. Further, the previous study used Northern blotting to detect expression levels, which is less sensitive than using RT-PCR. The present study included 45 samples, which allows us to detect smaller changes with greater sensitivity.

ATOX1 does not seem to be involved in copper uptake but is a copper chaperone which interacts with ATP7A and ATP7B and delivers copper to these pumps and hence to trans-Golgi network [36] or possibly the efflux pathway of the placenta [37]. Moreover, ATOX1 appears essential to perinatal copper homeostasis [38] for synthesis of ceruloplasmin or export from the cell into the portal circulation (ATP7A in gut) or into the bile for excretion (ATP7B). Understandably, the inhibition of ATOX1 expression reduces the trafficking of ATPases [39], thus reducing copper export and causing its accumulation in the cell [40]. Therefore, the small but significant reduction in ATOX1 expression in the iron deficient liver could be responsible – at least partially – for the increase in copper levels.

In addition, the hepatic expression of COX17, a chaperone delivering Cu to the cytochrome oxidase complex in the respiratory chain, was also decreased. The impairment of mitochondrial function and the reduction of cytochrome c activity (which requires both iron and copper to function) is well established in iron deficient tissues, including in the liver [41, 42], though whether this is cause or consequence is not certain at this time. There are little data on the direct effects of copper on either ATOX1 or COX17 expression, so the possibility that these changes are a response to changes in copper levels, rather than copper levels being changed by the alterations in gene expression also cannot be excluded in these experiments

As previously observed [23], maternal iron deficiency led to the increase of copper levels in the placenta (~19%), to a greater extent than the maternal liver (~10%). This was accompanied by the reduction of CCS expression, another chaperone channeling Cu for incorporation into the Cu-Zn superoxide dismutase (SOD) complex, an essential component of the anti-oxidant cell system. Copper has been shown to modulate the degradation of CCS [43] and copper supplementation in humans reduces CCS mRNA levels in peripheral mononuclear cells [44]. It is likely that the downregulation of CCS expression observed here is due to the presence of elevated copper levels in the placenta, rather than a direct consequence of reduced iron levels. Our findings suggest that maternal iron deficiency leads to the impairment of SOD activity (closely related to CCS expression) and the anti-oxidant defense of the placenta, as observed in liver and brain cells upon copper accumulation [45, 46].

Ceruloplasmin (CP) is involved in iron export in the liver, oxidizing Fe(II) to Fe(III) for incorporation into transferrin, while its homolog zyklopen performs the same function in placenta from mice and humans [47] . Whether the ferroxidase mRNA measured in the placenta is zyklopen, Cp or a combination of both remains uncertain, since the sequences are very similar and because the primers are from a commercial supplier. However it is likely that we measured the mRNA coding for a multicopper oxidase responsible for iron export [48] . The effect that the reduced placental expression might have on iron transport is not clear, and contrasts with previous findings [48] . However, it is important to note that Cp protein and enzyme levels do not correlate with mRNA levels [49] and that changes in Cp mRNA expression observed in the present study may therefore have little physiological impact. Maternal iron

deficiency is partly alleviated in the fetus, due to increased expression of transferrin receptors [50, 51], which would imply that ferroxidase expression or activity is not rate limiting.

Copper transport across the placenta is less well understood. Copper appears to be taken up from the maternal circulation through CTR1 and excreted through ATPases [52, 53]. It has been suggested that ATP7A is responsible for the efflux of copper from the basolateral membrane into the fetal circulation, while ATP7B is responsible for excreting copper back into the maternal circulation at the apical side [54]. Whether ceruloplasmin and/or zyklopen are involved in copper efflux and/or delivery to the fetal liver, as we have previously [54] suggested for transport of copper from the mother to the placenta [55], and whether this could explain the differences observed in copper levels between placenta and fetal liver remains to be clarified.

4.2 **Effects of maternal iron deficiency on zinc metabolism**

The effect of iron deficiency on zinc metabolism in the pregnant rat is less marked than for copper but is nonetheless significant. Zinc levels in the maternal liver and placenta were not changed but were markedly increased in the fetal liver.

In the maternal liver, the expression of the genes of zinc metabolism tested were unchanged, which is consistent with the lack of effect on zinc levels but contrasts with previous findings in male rats, where iron deficiency led to an increase in hepatic ZIP14 expression [19]. It should be noted, however, that the authors also observed a small decrease in zinc levels which was not seen in the present study, nor in their other study of a similar design [19] . Taken together, these results imply that iron deficiency during pregnancy is unlikely to lead to changes in zinc metabolism that are physiologically important in the maternal liver.

While zinc levels were unchanged in the placenta, ZIP4 and ZnT4, genes of zinc cellular uptake and exit, respectively, were both decreased in the placenta. The results do not fully match those seen in mice given a Zn deficient diet, where ZnT4, but also ZIP1, ZnT1, and 5 were reduced in placenta [33]. It is possible that there was a mild reduction in zinc supply to and in efflux from the placenta, with a neutral net effect on zinc levels in the placenta. Zinc metabolism and transport in the placenta is still not entirely understood, and the role and localization of ZIP4 in the placenta is not known. ZIP4 is crucial for zinc cellular uptake in the small intestine and is upregulated upon zinc deficiency in mice [56]. It is also abundantly expressed in other tissues involved in nutrient absorption and reabsorption (stomach, colon, kidney) [57]. While ZIP4 expression could not be compared with intestinal or kidney expression in the present study, it

is important to note that it was higher in the placenta compared to maternal liver (~2 fold) and fetal liver (~15 fold, data not shown). This suggests that the changes observed in placental expression could be biologically relevant, and further research is warranted to clarify the role of ZIP4 in placenta. If it were involved in the return of Zn from fetal to maternal circulation, then decreased expression could result in increased levels of Zn in the fetal liver. Interestingly, neither ZIP8 nor ZIP14, both of which have previously been implicated in iron metabolism as well as that of Zn [32], are altered in the placenta or maternal liver, which would argue against the changes in fetal liver Zn levels being a direct consequence of competition with iron for these transporters.

Iron deficiency leads to an increase in zinc levels in the fetal liver, but also a lesser decrease in iron levels compared to the decrease observed in the maternal liver [58]. Both these changes may be explained, at least partially, by an upregulation of ZIP14, which mediates the uptake of zinc, as well as non-transferrin-bound iron [31], and thus could help protect the fetus against iron deficiency. It is important to note that this does not rule out other mechanisms, such as an increased expression of transferrin receptors, as we have previously observed in the placenta of rats and women [50, 51]. On the other hand, the reduced ZIP4 expression in the fetal liver is more likely to be a consequence of the increase in zinc levels, as has been seen in Zn overload in observed in the intestine and visceral yolk sac of mice [56]. In addition, as described earlier in this discussion, ZIP4 expression in the fetal liver was fairly low compared to the other tissues, indicating that its changes may have little biological relevance.

Contrasting with our results, ZnT1 hepatic upregulation has been reported in zinc deficient rats in association with zinc depletion in the liver [59], although this was only observed at the protein level. Whether the increase in ZnT1 mRNA expression in the fetal liver would lead to an increase in protein levels is uncertain, and whether this could lead to more zinc efflux back into the fetal circulation is unlikely in the present study. The reasons why ZnT1 upregulation is associated with a marked increase in zinc levels are not known and the role of ZnT1 in fetal liver zinc metabolism needs to be clarified.

In summary, this study has shown that iron deficiency has opposite effects on copper and zinc levels in the fetal liver, and this is associated with changes in the expression of genes of copper and zinc metabolism in the placenta, as well as in the fetus. It is important to note that some of these effects may not be directly caused by iron deficiency, but rather by changes in the metabolism of the other two nutrients. The results further demonstrate that micronutrient metabolism, especially during pregnancy, is tightly interlinked and underscores the importance

of considering all of the micronutrients when trying to alleviate deficiencies in one of them. It would be important to consider that the symptoms caused by deficiencies in one micronutrient (here iron) may not be caused directly by that deficiency, but by other deficiencies or overloads occurring as a consequence of the primary defect.

5 Acknowledgements

HJM, LG and SCC designed the research; LG, HEH, VJC and SCC conducted the research; SCC analyzed the data and wrote the manuscript; GR and HJM reviewed the manuscript. HJM, GR and SCC take responsibility of data interpretation and presentation. All authors read and approved the final manuscript. We are grateful for the technical assistance of Gill Campbell (ICPMS analysis). This work was supported by Scottish Government (Rural and Environment Science and Analytical Services).

Tables

Table 1: List of genes analysed by PCR using TaqMan® Gene Expression Assays (ThermoFisher Scientific)

Gene	Protein	Assay ID
ATOX1	Antioxidant 1 copper chaperone	Rn00584459_m1
COX17	Cytochrome c oxidase copper chaperone	Rn00585530_m1
CCS	Copper chaperone for superoxide dismutase	Rn00584772_m1
CP	Ceruloplasmin	Rn00561049_m1
ATP7A	ATPase copper transporting alpha (Menkes)	Rn00583815_m1
ATP7B	ATPase copper transporting beta (Wilson)	Rn00560862_m1
SLC31A1 / CTR1	Copper transporter 1	Rn00683634_m1
SLC39A1 / ZIP1	ZRT/IRT-like protein 1	Rn01458936_g1
SLC39A4 / ZIP4	ZRT/IRT-like protein 4	Rn01505595_g1
SLC39A8 / ZIP8	ZRT/IRT-like protein 8	Rn01748352_m1
SLC39A14 / ZIP14	ZRT/IRT-like protein 14	Rn01468336_m1
SLC30A1 / ZnT1	Zinc Transporter 1	Rn00575737_m1
SLC30A4 / ZnT4	Zinc Transporter 4	Rn00597094_m1
SLC30A5 / ZnT5	Zinc Transporter 5	Rn01493867_m1

Table 2: Organ weight, water content and absolute iron, copper and zinc content in the maternal liver, placenta and fetal liver of control and iron deficient rats at d21 of gestation

	Control	**Iron deficient**
Maternal Liver		
Organ weight (g)[1]	10.94 ± 0.39	11.60 ± 0.35
Water content (%)[2]	70.04 ± 0.26	71.22 ± 0.24**
Absolute iron content (mg)[1]	1.96 ± 0.29	0.70 ± 0.12††
Absolute copper content (µg)[1]	44.77 ± 1.20	51.10 ± 1.48*
Absolute zinc content (µg)[1]	305.9 ± 8.2	326.8 ± 8.5
Placenta		
Organ weight (g) [3]	0.512 ± 0.017	0.551 ± 0.009
Water content (%)[2]	87.83 ± 0.24	88.10 ± 0.27
Absolute iron content (mg)[3]	39.07 ± 1.88	28.47 ± 1.66***
Absolute copper content (µg)[3]	1.17 ± 0.10	1.52 ± 0.09
Absolute zinc content (µg)[3]	5.52 ± 0.20	5.60 ± 0.19
Fetal Liver		
Organ weight (g)[1]	0.313 ± 0.009	0.234 ± 0.009***
Water content (%)[2]	78.23 ± 0.21	79.26 ± 0.18*
Absolute iron content (mg)[1]	80.37 ± 2.38	20.08 ± 1.14†††
Absolute copper content (µg)[1]	3.62 ± 0.27	1.90 ± 0.15***
Absolute zinc content (µg)[1]	23.37 ± 0.87	19.27 ± 0.85**

[1] n = 16 (8 control, 8 Fe deficient), [2] n = 54 (24 control, 30 Fe deficient), [3] n = 38 ; Data are mean ± SEM; *, ** and *** indicate significant differences compared to control at $p < 0.05$, $p < 0.01$ and $p < 0.05$ (independent t-test), †† and ††† indicate significant differences compared to control at $p < 0.01$ and $p < 0.01$ (Mann-Whitney U test).

Table 3: Summary of the effect of maternal iron deficiency on copper and zinc metabolism in maternal liver, placenta and fetal liver at d21 of pregnancy

Gene	Maternal Liver	Placenta	Fetal Liver
Cu[1]	↑↑	↑	↓
ATOX1[2]	↓	=	=
COX17[2]	↓	=	↑
CCS [2]	=	↓	=
CP[2]	=	↓	=
ATP7A[2]	=	=	=
ATP7B[2]	=	=	=
CTR1[2]	=	=	=
Zn[1]	=	=	↑↑
ZIP1[2]	=	=	=
ZIP4[2]	=	↓	↓
ZIP8[2]	=	=	=
ZIP14[2]	=	=	↑
ZnT1[2]	=	=	↑
ZnT4[2]	=	↓	=
ZnT5[2]	=	=	=

The concentrations of copper, zinc, and the expression of genes of copper and zinc metabolism were either greatly increased (↑↑), increased (↑), decreased (↓) or unchanged (=) by maternal iron deficiency. [1] n = 54 (control n = 24, iron deficient n = 30); [2] n = 45 (control n = 21, iron deficient n = 24).

Figure Legends:

Figure 1: The effect of maternal iron deficiency on copper (A) and zinc (B) levels in maternal liver, placenta and fetal liver 21d after mating (n = 54)

Data are expressed as mean percentage of control and error bars are SEM. Results are significantly different between the control (Ctrl, n = 24) and iron deficient (Fe Def, n= 30) groups at $P < 0.0001$ (****) (independent t-test), < 0.01 (††) and < 0.05 (†) (Mann-Whitney test).

Figure 2: The effect of maternal iron deficiency on the expression of genes related to copper metabolism (n=45) 21d after mating

Data are expressed as mean percentage of control and error bars are SEM. * Results are significantly different between the control (Ctrl, n = 21) and iron deficient (Fe Def, n = 24) groups at $P < 0.05$ (Mann-Whitney test).

Abbreviations: Antioxidant 1 copper chaperone (ATOX1), cytochrome c oxidase chaperone (COX17), ATPase copper transporting alpha (Menkes) (ATP7A), ATPase copper transporting beta (Wilson) (ATP7B); copper chaperone for superoxide dismutase (CCS), ceruloplasmin (CP), copper transporter 1 (CTR1).

Figure 3: The effect of maternal iron deficiency on the expression of genes related to copper metabolism (n=45) 21d after mating

Data are expressed as mean percentage of control and error bars are SEM. Results are significantly different between the control (Ctrl, n = 21) and iron deficient (Fe Def, n = 24) groups at $P < 0.01$ (**) and < 0.05 (*) (Mann-Whitney test).

Abbreviations: ZRT/IRT-like protein 1 (ZIP1), ZRT/IRT-like protein 4 (ZIP4), ZRT/IRT-like protein (ZIP8), ZRT/IRT-like protein 14 (ZIP14), Zinc Transporter 1 (ZnT1), Zinc Transporter 4 (ZnT4), Zinc Transporter 5 (ZnT5).

References

References

1. Miller R, Spiro A, Stanner S (2016) Micronutrient status and intake in the UK – where might we be in 10 years' time? *Nutr Bull* **41**(1), 14-41.

2. Collins JF, Prohaska JR, Knutson MD (2010) Metabolic crossroads of iron and copper. *Nutr Rev* **68**(3), 133-147.

3. Gambling L, Kennedy C, McArdle HJ (2011) Iron and copper in fetal development. **22**(6), 637-644.

4. Whittaker P (1998) Iron and zinc interactions in humans. **68**(2), 446S.

5. Owen CA,Jr (1973) Effects of iron on copper metabolism and copper on iron metabolism in rats. *Am J Physiol* **224**(3), 514-518.

6. Ravia JJ, Stephen RM, Ghishan FK *et al.* (2005) Menkes copper ATPase (ATP7A) is a novel metal-responsive gene in rat duodenum and immunoreactive protein is preent on brush border and basolateral membrane domains. *J Biol Chem* **280**(43), 36221-36227.

7. Sourkes TL, Lloyd K, Birnbaum H (1968) Inverse relationship of heptic copper and iron concentrations in rats fed deficient diets. *Can J Biochem* **46**(3), 267-271.

8. Ranganathan PN, Lu Y, Jiang L *et al.* (2011) Serum ceruloplasmin protein expression and activity increases in iron-deficient rats and is further enhanced by higher dietary copper intake. *Blood* **118**(11), 3146-3153.

9. Collins JF, Franck CA, Kowdley KV *et al.* (2005) Identification of differentially expressed genes in response to dietary iron deprivation in rat duodenum.*Am J Physiol* **288**(5), G971.

10. Jiang L, Garrick MD, Garrick LM *et al.* (2013) Divalent metal transporter 1 (Dmt1) mediates copper transport in the duodenum of iron-deficient rats and when overexpressed in iron-deprived HEK-293 cells. *J Nutr* **143**(12), 1927-1933.

11. Shukla A, Agarwal KN, Shukla GS (1990) Effect of latent iron deficiency on the levels of iron, calcium, zinc, copper, manganese, cadmium and lead in liver, kidney and spleen of growing rats. *Experientia* **46**(7), 751-752.

12. Shukla A, Agarwal KN, Shukla GS (1989) Effect of latent iron deficiency on metal levels of rat brain regions. *Biol Trace Elem Res* **22**(2), 141-152.

13. Kaganda J, Matsuo T, Suzuki H (2003) Development of iron deficiency decreases zinc requirement of rats. *J Nutr Sci Vitaminol (Tokyo)* **49**(4), 234-240.

14. Yokoi K, Kimura M, Itokawa Y (1991) Effect of dietary iron deficiency on mineral levels in tissues of rats. *Biol Trace Elem Res* **29**(3), 257-265.

15. Stangl GI & Kirchgessner M (1998) Effect of different degrees of moderate iron deficiency on the activities of tricarboxylic acid cycle enzymes, and the cytochrome oxidase, and the iron, copper, and zinc concentrations in rat tissues. *Z Ernahrungswiss* **37**(3), 260-268.

16. Tallkvist J, Bowlus CL, Lonnerdal B (2000) Functional and molecular responses of human intestinal Caco-2 cells to iron treatment. *Am J Clin Nutr* **72**(3), 770-775.

17. Illing AC, Shawki A, Cunningham CL *et al.* (2012) Substrate profile and metal-ion selectivity of human divalent metal-ion transporter-1. *J Biol Chem* **287**(36), 30485-30496.

18. Guerinot ML (2000) The ZIP family of metal transporters. *Biochim Biophys Acta* **1465**(1-2), 190-198.

19. Nam H & Knutson MD (2012) Effect of dietary iron deficiency and overload on the expression of ZIP metal-ion transporters in rat liver. *Biometals* **25**(1), 115-124.

20. Sherman AR & Moran PE (1984) Copper metabolism in iron-deficient maternal and neonatal rats. *J Nutr* **114**(2), 298-306.

21. Sherman AR & Tissue NT (1981) Tissue iron, copper and zinc levels in offspring of iron-sufficient and iron-deficient rats. *J Nutr* **111**(2), 266-275.

22. Gambling L, Dunford S, McArdle HJ (2004) Iron deficiency in the pregnant rat has differential effects on maternal and fetal copper levels. *J Nutr Biochem* **15**(6), 366-372.

23. Gambling L, Andersen HS, Czopek A *et al.* (2004) Effect of timing of iron supplementation on maternal and neonatal growth and iron status of iron-deficient pregnant rats. *J. Physiol.* **561**, 195-203.

24. Lenartowicz M, Kennedy C, Hayes H *et al.* (2015) Transcriptional regulation of copper metabolism genes in the liver of fetal and neonatal control and iron-deficient rats. **28**, 51-59.

25. Fung EB, Ritchie LD, Woodhouse LR *et al.* (1997) Zinc absorption in women during pregnancy and lactation: a longitudinal study. *Am J Clin Nutr* **66**(1), 80-88.

26. Hambidge KM, Krebs NF, Jacobs MA *et al.* (1983) Zinc nutritional status during pregnancy: a longitudinal study. *Am J Clin Nutr* **37**(3), 429-442.

27. Fischer-Walker CF, Kordas K, Stoltzfus RJ *et al.* (2005) Interactive effects of iron and zinc on biochemical and functional outcomes in supplementation trials. *Am J Clin Nutr* **82**(1), 5-12.

28. Cottin SC, Gambling L, Hayes HE *et al.* (2016) Pregnancy and maternal iron deficiency stimulate hepatic CRBPII expression in rats. *J Nutr Biochem* **32**, 55-63.

29. Segawa S, Shibamoto M, Ogawa M *et al.* (2014) The effect of divalent metal cations on zinc uptake by mouse Zrt/Irt-like protein 1 (ZIP1). *Life Sci* **113**(1-2), 40-44.

30. Andrews GK (2008) Regulation and function of Zip4, the acrodermatitis enteropathica gene. *Biochem Soc Trans* **36**(Pt 6), 1242-1246.

31. Liuzzi JP, Aydemir F, Nam H *et al.* (2006) Zip14 (Slc39a14) mediates non-transferrin-bound iron uptake into cells. *Proc Natl Acad Sci U S A* **103**(37), 13612-13617.

32. Wang CY, Jenkitkasemwong S, Duarte S *et al.* (2012) ZIP8 is an iron and zinc transporter whose cell-surface expression is up-regulated by cellular iron loading. *J Biol Chem* **287**(41), 34032-34043.

33. Helston RM, Phillips SR, McKay JA *et al.* (2007) Zinc transporters in the mouse placenta show a coordinated regulatory response to changes in dietary zinc intake. *Placenta* **28**(5-6), 437-444.

34. Liuzzi JP, Bobo JA, Cui L *et al.* (2003) Zinc transporters 1, 2 and 4 are differentially expressed and localized in rats during pregnancy and lactation. *J Nutr* **133**(2), 342-351.

35. Bustin SA, Benes V, Garson JA *et al.* (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem* **55**(4), 611-622.

36. Kambe T, Weaver BP, Andrews GK (2008) The genetics of essential metal homeostasis during development. *Genesis* **46**(4), 214-228.

37. Hardman B, Michalczyk A, Greenough M *et al.* (2007) Distinct functional roles for the Menkes and Wilson copper translocating P-type ATPases in human placental cells. *Cell Physiol Biochem* **20**(6), 1073-1084.

38. Hamza I, Faisst A, Prohaska J *et al.* (2001) The metallochaperone Atox1 plays a critical role in perinatal copper homeostasis. *Proc Natl Acad Sci U S A* **98**(12), 6848-6852.

39. Hamza I, Prohaska J, Gitlin JD (2003) Essential role for Atox1 in the copper-mediated intracellular trafficking of the Menkes ATPase. *Proc Natl Acad Sci* **100**(3), 1215-1220.

40. Safaei R, Maktabi MH, Blair BG *et al.* (2009) Effects of the loss of Atox1 on the cellular pharmacology of cisplatin. *J Inorg Biochem* **103**(3), 333-341.

41. Masini A, Salvioli G, Cremonesi P *et al.* (1994) *Dietary Iron Deficiency in the Rat. I. Abnormalities in Energy Metabolism of the Hepatic Tissue.* pp. 46-52. http://www.sciencedirect.com/science/article/pii/0005272894900205.

42. Walter PB, Knutson MD, Paler-Martinez A *et al.* (2002) Iron deficiency and iron excess damage mitochondria and mitochondrial DNA in rats. *Proc Natl Acad Sci* **99**(4), 2264-2269.

43. Bertinato J & L'Abbé MR (2003) Copper Modulates the Degradation of Copper Chaperone for Cu,Zn Superoxide Dismutase by the 26 S Proteosome. *J Biol Chem* **278**(37), 35071-35078.

44. Araya M, Andrews M, Pizarro F *et al.* (2012) Chaperones CCS, ATOX and COXIV responses to copper supplementation in healthy adults. *Biometals* **25**(2), 383-391.

45. Ozcelik D & Uzun H (2009) Copper Intoxication; Antioxidant Defenses and Oxidative Damage in Rat Brain. *Biol Trace Elem Res* **127**(1), 45-52.

46. Zhang SS, Noordin MM, Rahman SO *et al.* (2000) Effects of copper overload on hepatic lipid peroxidation and antioxidant defense in rats. *Vet Hum Toxicol* **42**(5), 261-264.

47. Chen H, Attieh ZK, Syed BA *et al.* (2010) Identification of Zyklopen, a New Member of the Vertebrate Multicopper Ferroxidase Family, and Characterization in Rodents and Human Cells. *J Nut* **140**(10), 1728-1735.

48. Danzeisen R, Fosset C, Chariana Z *et al.* (2002) Placental ceruloplasmin homolog is regulated by iron and copper and is implicated in iron metabolism. *Am J Physiol* **282**(3), C478-C483.

49. McArdle HJ, Mercer JF, Sargeson AM *et al.* (1990) Effects of cellular copper content on copper uptake and metallothionein and ceruloplasmin mRNA levels in mouse hepatocytes. *J Nutr* **120**(11), 1370-1375.

50. Cornock R, Gambling L, Langley-Evans S *et al.* (2013) The effect of feeding a low iron diet prior to and during gestation on fetal and maternal iron homeostasis in two strains of rat. *Reprod Biol Endocrinol* **11**(1), 32.

51. Garcia-Valdes L, Campoy C, Hayes H *et al.* (2015) The impact of maternal obesity on iron status, placental transferrin receptor expression and hepcidin expression in human pregnancy. *Int J Obes (Lond)* **39**(4), 571-578.

52. Kuo Y, Zhou B, Cosco D *et al.* (2001) The copper transporter CTR1 provides an essential function in mammalian embryonic development. *Proc Natl Acad Sci* **98**(12), 6836-6841.

53. McArdle HJ, Andersen HS, Jones H *et al.* (2008) Copper and Iron Transport Across the Placenta: Regulation and Interactions. *J Neuroendocrinol* **20**(4), 427-431.

54. Hardman B, Manuelpillai U, Wallace EM *et al.* (2004) *E*xpression and Localization of Menkes and Wilson Copper Transporting ATPases in Human Placenta. *Placenta* **25**. 512-517.

55. Hilton M, Spenser DC, Ross P *et al.* (1995) Characterisation of the copper uptake mechanism and isolation of the ceruloplasmin receptor/copper transporter in human placental vesicles. *Biochim Biophys Acta* **1245**(2), 153-160.

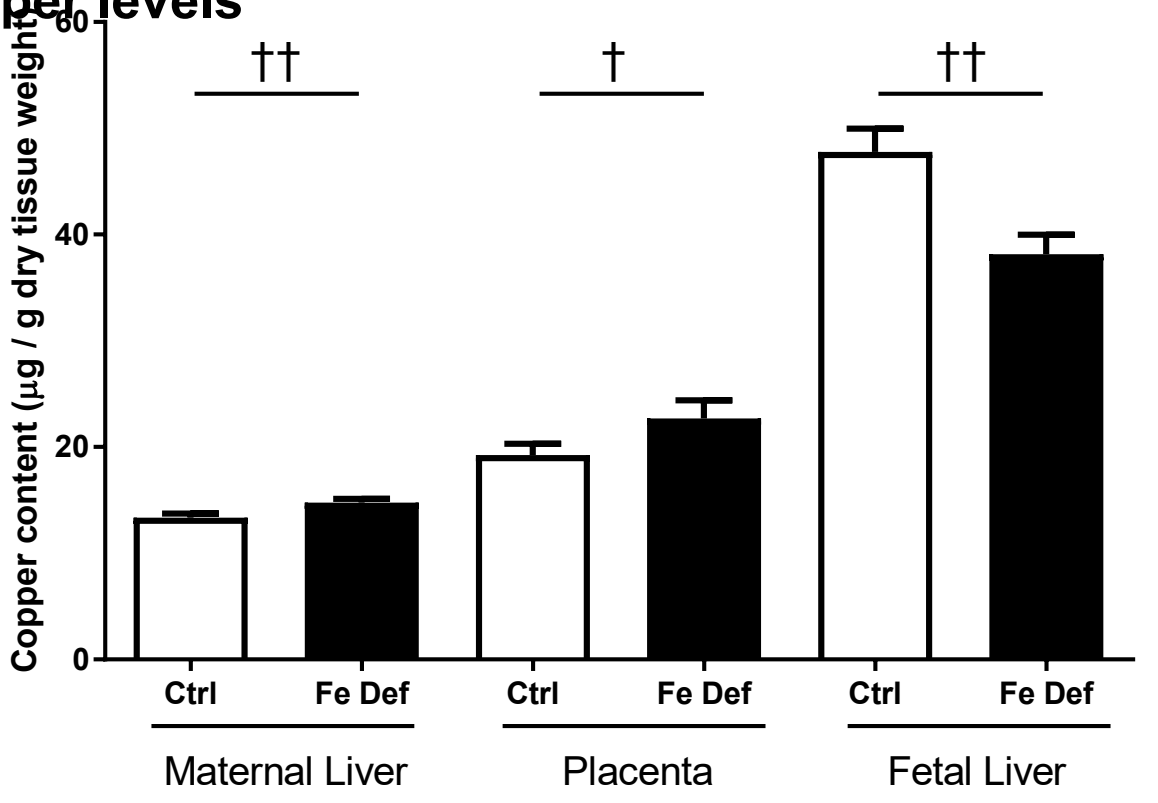
56. Dufner-Beattie J, Wang F, Kuo YM *et al.* (2003) The acrodermatitis enteropathica gene ZIP4 encodes a tissue-specific, zinc-regulated zinc transporter in mice. *J Biol Chem* **278**(35), 33474-33481.

57. Wang K, Zhou B, Kuo YM *et al.* (2002) A novel member of a zinc transporter family is defective in acrodermatitis enteropathica. *Am J Hum Genet* **71**(1), 66-73.

58. Gambling L, Charania Z, Hannah L *et al.* (2002) Effect of Iron Deficiency on Placental Cytokine Expression and Fetal Growth in the Pregnant Rat. *Biol Reprod* **66**(2), 516-523.

59. Jou MY, Hall AG, Philipps AF *et al.* (2009) Tissue-specific alterations in zinc transporter expression in intestine and liver reflect a threshold for homeostatic compensation during dietary zinc deficiency in weanling rats. *J Nutr* **139**(5), 835-841.

Figure 1: A - Effect of maternal iron deficiency on copper levels



B - Effect of maternal iron deficiency on zinc levels

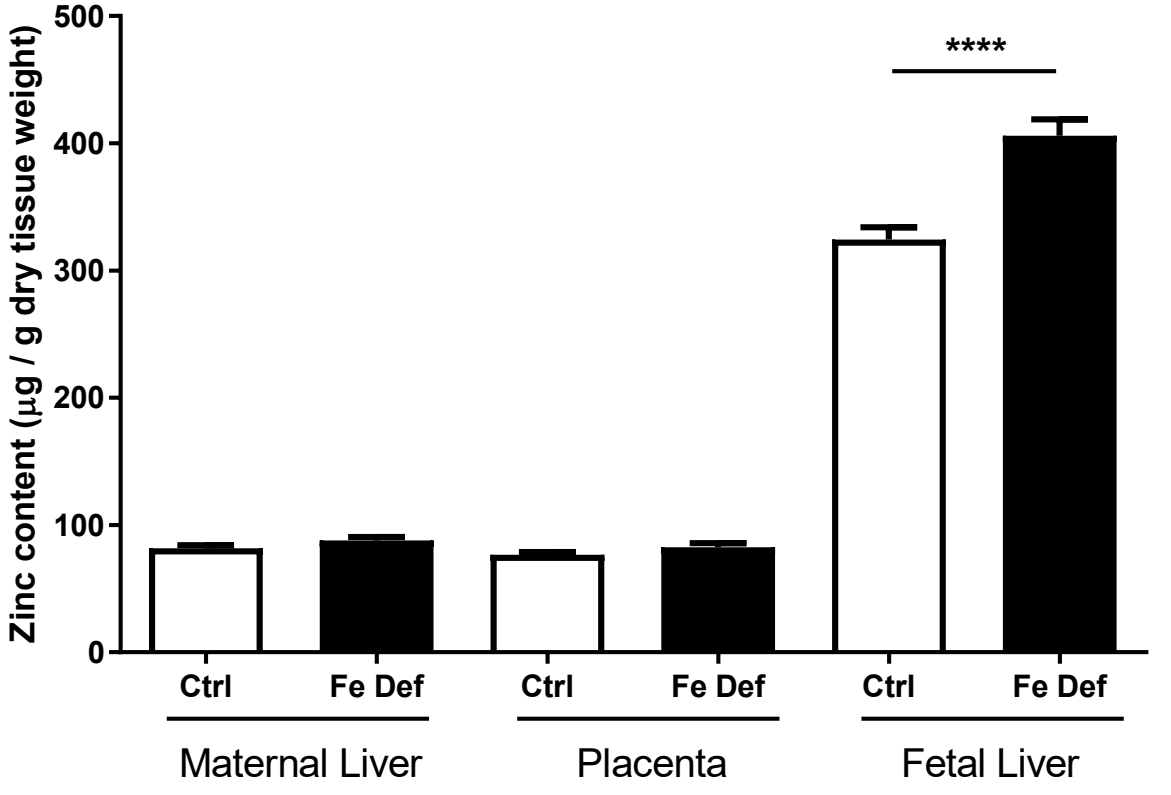
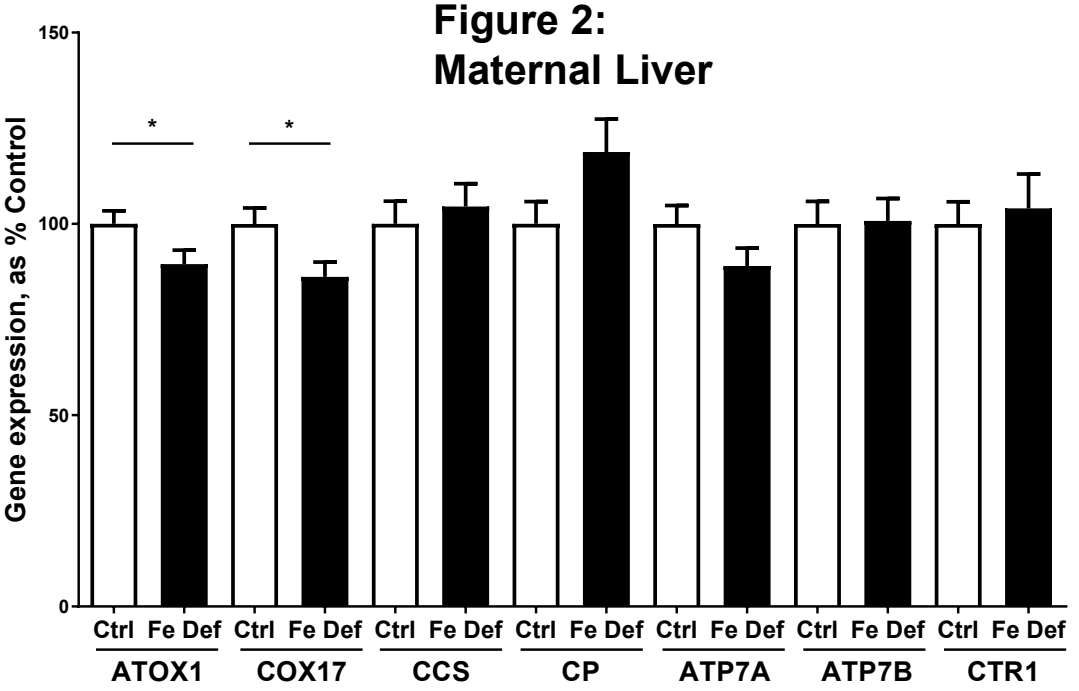
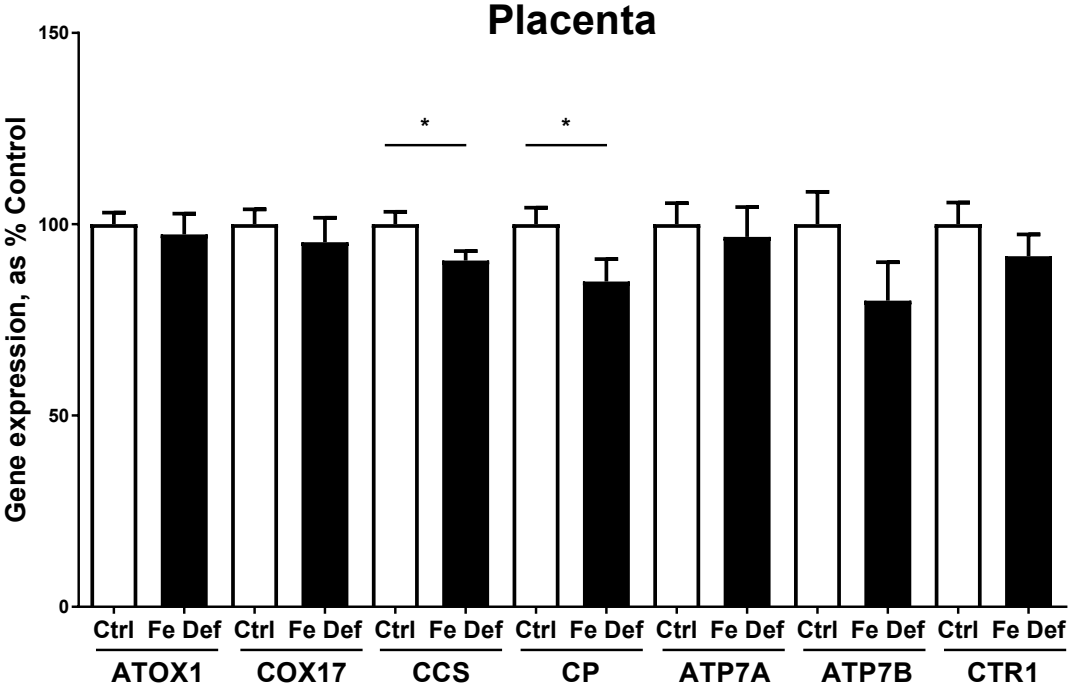


Figure 2:


Maternal Liver
Gene expression, as % Control
150
100
50
0
*
*
Ctrl Fe Def Ctrl Fe Def Ctrl Fe Def Ctrl Fe Def Ctrl Fe Def Ctrl Fe Def Ctrl Fe Def
ATOX1 COX17 CCS CP ATP7A ATP7B CTR1


Placenta
Gene expression, as % Control
150
100
50
0
*
*
Ctrl Fe Def Ctrl Fe Def Ctrl Fe Def Ctrl Fe Def Ctrl Fe Def Ctrl Fe Def Ctrl Fe Def
ATOX1 COX17 CCS CP ATP7A ATP7B CTR1

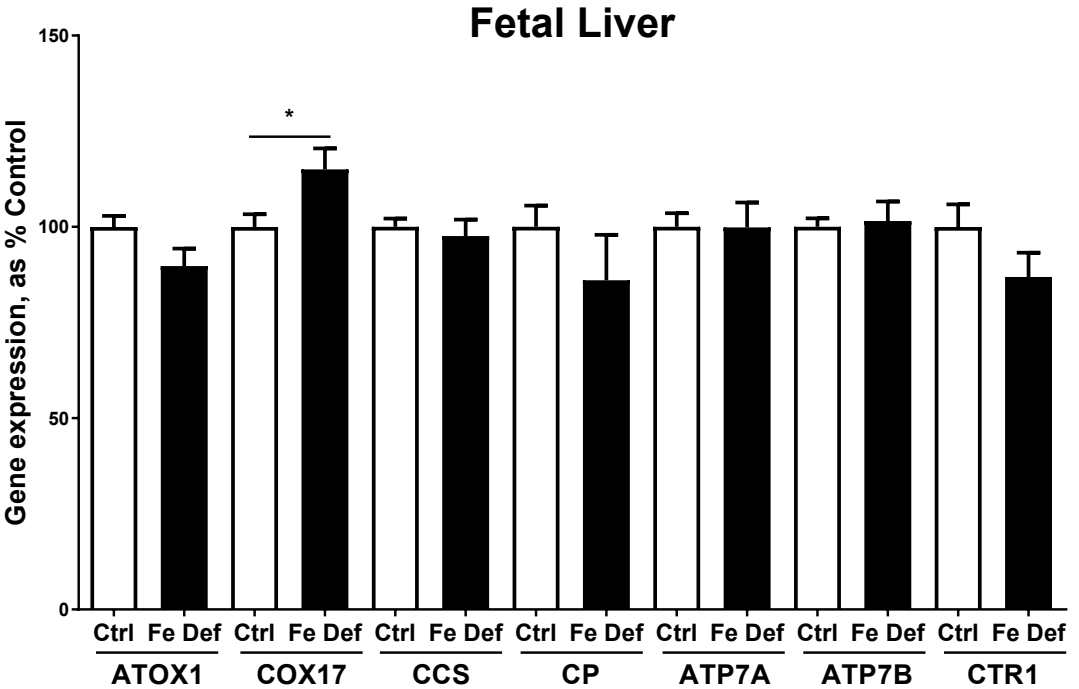
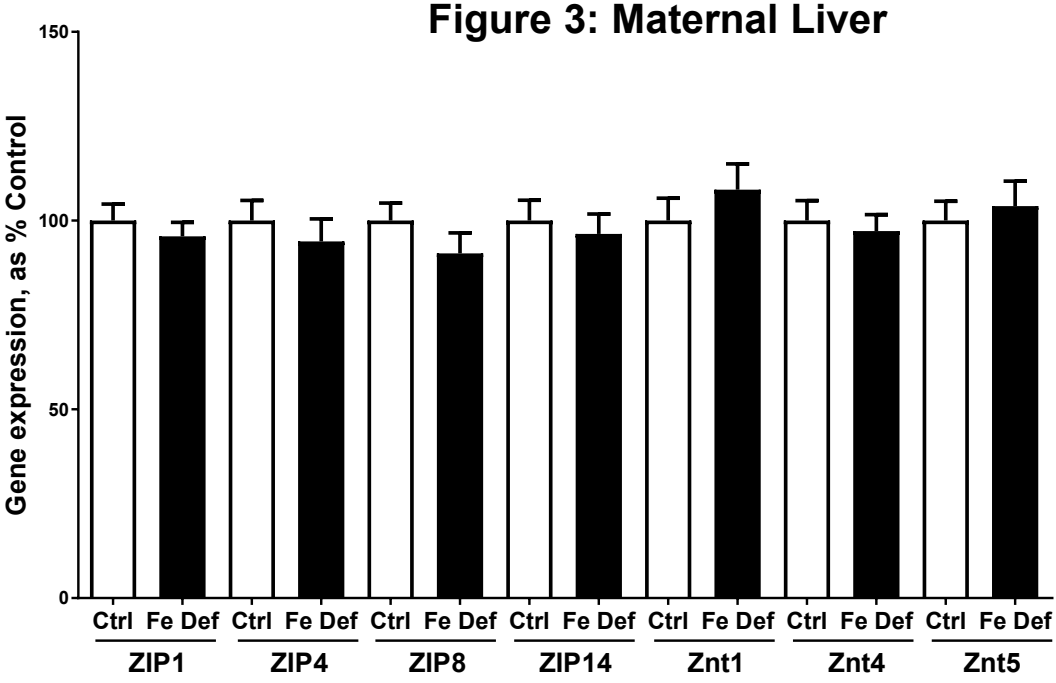
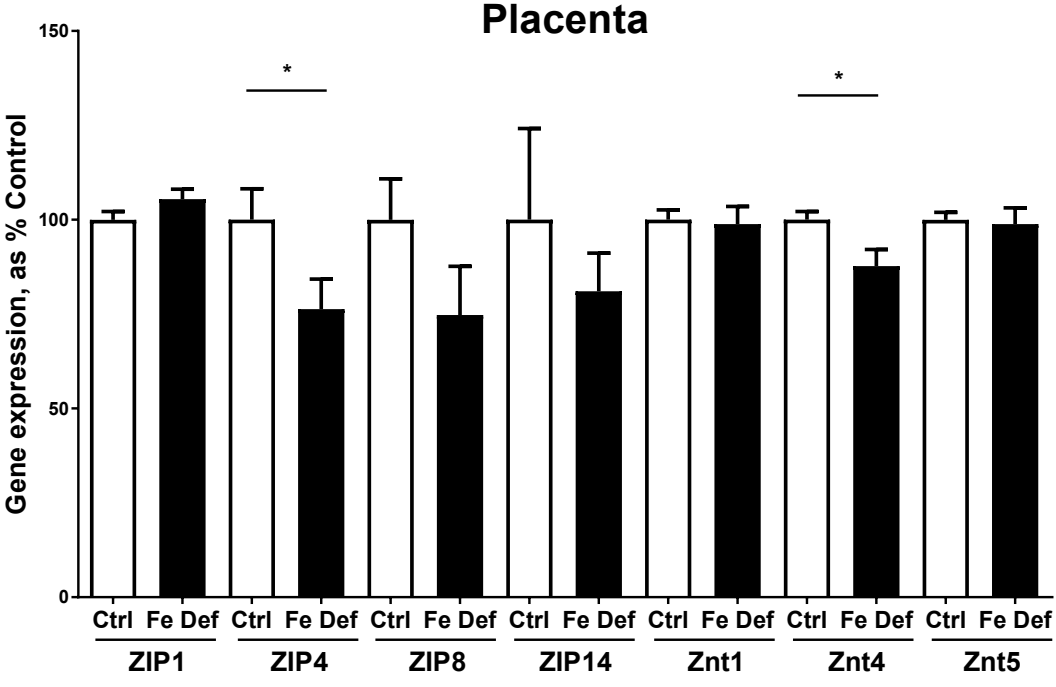

Fetal Liver
Gene expression, as % Control
150
100
50
0
*
Ctrl Fe Def Ctrl Fe Def Ctrl Fe Def Ctrl Fe Def Ctrl Fe Def Ctrl Fe Def Ctrl Fe Def
ATOX1 COX17 CCS CP ATP7A ATP7B CTR1

Figure 3: Maternal Liver



Placenta



Fetal Liver