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Review

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Materno-fetal iron transfer and the emerging role of ferroptosis pathways

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A running title: **Materno-fetal iron transfer and placental ferroptosis**

Abstract

A successful pregnancy and the birth of a healthy baby depend to a great extent on the controlled supply of essential nutrients via the placenta. Iron is essential for mitochondrial energy supply and oxygen distribution via the blood. However, its high reactivity requires tightly regulated transport processes. Disturbances of maternal-fetal iron transfer during pregnancy can aggravate or lead to severe pathological consequences for the mother and the fetus with lifelong effects. Furthermore, high intracellular iron levels due to disturbed gestational iron homeostasis have recently been associated with the non-apoptotic cell death pathway called ferroptosis. Therefore, the investigation of transplacental iron transport mechanisms, their physiological regulation and potential risks are of high clinical importance. The present review summarizes the current knowledge on principles and regulatory mechanisms underlying materno-fetal iron transport and gives insight into common pregnancy conditions in which iron homeostasis is disturbed. Moreover, the significance of the newly emerging ferroptosis pathway and its impact on the regulation of placental iron homeostasis, oxidative stress and gestational diseases will be discussed.

Abbreviations:

AMP, Adenosine monophosphate; AMPK, AMP-activated protein kinase; BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; BM, basal membrane; 2,2-BP, 2,2-bipyridyl; BPDE, Benzo[a]pyrene-7,8-diol-9,10-epoxide; BSO, buthionine sulfoximine;CP, Ceruloplasmin; CPX, ciclopirox olamine; CTB, cytotrophoblasts; DFO, deferoxamine; DFOM, desferrioxamine mesilatec; DHT, 5α-dihydrotestosterone; DIPSI, Diabetes in Pregnancy Study Group India; DMT1, Divalent metal transporter 1; EC, endothelial cells; ERK, extracellular signal regulated kinases; FC, fetal capillaries; FLVCR1, Feline leuk. virus subg. C receptor 1; FLVCR2, Feline leuk. virus subg. C receptor 2; FPN1, Ferroportin-1; FTH, Ferritin heavy chain; GCL, glutamate-cysteine ligase; GDM, gestational diabetes mellitus; GSH, glutathione; H, Hofbauer cell; HCP1, Heme carrier protein 1; HE, haematoxylin and eosin; HELLP, hemolysis, elevated liver enzymes and low platelet count; HEPC, Hepcidin; HEPH, Hephaestin; HFE, Hereditary hemochromatosis protein; HO1, Heme oxygenase 1; 4-HNE, 4-hydroxynonenal; HO2, Heme oxygenase 2; HRG1, Heme transporter 1; IRE, iron-responsive element; IRP1, Iron regulatory protein 1;IRP2, Iron regulatory protein 2; Irt, Iron-regulated transporter; IUGR, intrauterine growth restriction; IUGR, intrauterine growth restriction; IVS, intervillous space; JNK, c-Jun N-terminal kinases; KO, knock out; LC-MS/MS, Liquid chromatography-tandem mass spectrometer; LOX, lipoxygenases; LRP1, Lipoprotein receptor-related protein 1; MAPK, mitogen-activated protein kinase pathways; MDA, malondialdehyde; MFRN1, β-ME, β-mercaptoethanol; Mitoferrin-1; MVM, microvillous plasma membrane; NADPH, nicotinamide adenine dinucleotide phosphate; NBD, nucleotide-binding domain; NLR, leucine-rich repeat protein; oGTT, oral glucose tolerance test; PCOS, polycystic ovary syndrome; PE, preeclampsia; PMNL, polymorphonuclear leukocytes; PUFA, polyunsaturated fatty acids; PUFA-OOH, phospholipid peroxides;ROS, reactive oxygen species; SCARA5, Scavenger receptor class A member 5; SLA, sex-linked anemia; STB, syncytiotrophoblasts; STEAP3, Metalloreductase STEAP3; STEAP3, six-transmembrane epithelial antigen of the prostate 3; SV, stem villi; tert-BOOH, tert-Butylhydroperoxid; Tf, Serotransferrin; TFR1, Transferrin receptor protein 1; UTR, untranslated region; WHO, world health organization; YAP, yes-associated protein; ZIP14, Zrt- and Irt-like protein 14; ZIP8, Zrt- and Irt-like protein 8; ZP, Zyklopen; Zrt, Zinc-regulated transporter;

1 Introduction

The placenta is a vital tissue during pregnancy as it is the connecting organ between the fetus and the *ex utero* world. Furthermore, it is a fetal organ and temporarily enables the mutual exchange between the embryo and mother. Besides its main function of physiological materno-fetal exchange of nutrients and waste products, the placenta is also responsible for the prevention of maternal immune rejection as well as for maintaining pregnancy through the secretion of gestational hormones. The placenta maintains the balance between nutrition and fetal growth control through a selective and regulated supply of macronutrients such as carbohydrates, proteins, lipids and critical micronutrients like minerals, vitamins and iron as the most abundant trace element in the human body [1]. Placental nutrient transport does not only play a role in fetal development, but also affects the newborn's life by metabolic programming until adulthood [2].

Due to the progressive increase of iron requirements during pregnancy and the high prevalence of pre-existing anemia in women of reproductive age, pregnant women are particularly vulnerable to iron deficiency [3]. Gestational iron deficiency has been found to sustainedly impact the developing brain by metabolic programming depending on the window of vulnerability during fetal development [4–6]. Hence, iron supplementation has become a routine recommendation for all women throughout pregnancy. Therefore, the WHO recommends routine iron supplementation of 30 – 60 mg per day throughout pregnancy [7]. However, the risks and benefits of prophylactic iron supplementation in each pregnant woman, especially in those who are not iron deficient, remains a subject of controversy. Maternal iron stores are generally improved by iron supplementation, but often no changes are observed in cord ferritin and maternal ferritin levels. A small observational study found only a weak

relationship with neonatal ferritin levels [8], while another investigation including pregnant women (n=381) in the second and third trimester showed a clear benefit of iron supplementation [9]. In the latter study 65% of women were taking iron supplements and had significantly higher serum ferritin concentrations compared to those lacking the iron supplementation [9]. Of note, the success of folate supplementation was clearly higher compared to iron supplementation. Furthermore, a more recent study in China including 3702 mother-newborn pairs, detected a strong correlation between maternal and neonatal ferritin levels in women whose plasma ferritin levels fell below a threshold of depletion (<13.6 µg/L serum ferritin). Below this threshold every unit of decrease in maternal serum ferritin corresponded to a 2.4-unit drop in cord ferritin, suggesting a biphasic relationship between maternal and fetal serum ferritin levels [10]. Hence, the reduction of placental iron transfer towards the fetus in iron deficiency supports the theory of a materno-fetal iron homeostasis that is prioritizing placental iron status against fetal supply under iron deficient conditions [11]. According to the view of Paracelsus "The dose makes the poison", Dewey and Oaks suggested an U-shaped curve describing an increased risk of adverse birth outcomes in early and late pregnancy if maternal hemoglobin concentrations are high [12]. Several randomized controlled and observational studies are questioning the value of iron supplementation in non-anemic and iron-replete women. Herein a high iron status in women was associated with the delivery of small-for-gestational-age infants after shorter duration of gestation and the development of gestational hypertension [13,14]. However, most studies are based on healthy pregnancies, neglecting that conditions with underlying placental abnormalities, such as intrauterine growth restriction (IUGR) and gestational diabetes mellitus (GDM), may disrupt the normal regulatory mechanism of the placenta and could negatively affect fetal iron transfer [15]. To date

there is only indirect but rather comprehensible evidence for a causative relationship between increased iron status and hypertensive disorders such as preeclampsia (PE) [16,17] and GDM (see section 2.4). PE was previously suspected to be associated with high iron status and ferroptosis [18], a distinct form of programmed cell death triggered by iron-dependent accumulation of phospholipid peroxides [19]. Ferroptosis was defined by the Stockwell Lab in 2012 [19], but was characterized as a cysteine caused cell death long before in the 50 and 60s [20–23]. While cell death is essential for tissue homeostasis, ferroptosis differs from other forms of cell death by the morphological and molecular mechanisms and is most closely related to autophagy [19,24–26]. A detailed description of ferroptotic pathways in the placenta is provided in section 3.

The exact mechanism of transplacental iron transport, its potential association with ferroptosis and with highly prevalent gestational disorders such as PE, GDM and fetal loss has been largely ignored so far. The present review provides an overview on the concepts of materno-fetal nutrient transfer, placental iron regulation and on the emerging role of ferroptosis pathways in pregnancy diseases. The current knowledge on ferroptosis is discussed in the context of highly prevalent gestational disorders according to previously defined hallmarks, such as 1) the availability of redox-active iron in maternal and fetal circulations, 2) the oxidation of polyunsaturated fatty acids (PUFA)-containing lipids that are highly abundant in the placenta and 3) the loss of lipid peroxide repair capacity by GPX4.

2 Placental iron homeostasis

2.1 The human placental barrier and associated transport mechanisms

The placenta is the first organ to be formed in humans and other mammals [27]. In the late first trimester maternal blood enters the intervillous space by spiral arteries (Figure

1A purple) and submerges the finger-like structure called chorionic villi (Figure 1A bright pink). As soon as a feto-placental circulation is established within the placental villi, fetal and maternal blood come very close (Figure 1B, maternal blood marked with green arrows and fetal blood with arrowheads). However, the two bloodstreams are always separated by the so-called placental barrier [28]. The microvillous structure of the chorion increases the effective surface area and represents the functional unit of the placenta, where most transport, metabolic and endocrine activities are localized. Virtually all materno-fetal and feto-maternal exchange processes take place across the placental barrier of the chorionic villi. The chorionic villi are surrounded by syncytiotrophoblasts (STB), a multinuclear epithelial surface layer that is in direct contact with the maternal blood (Figure 1A blue monolayer; Figure 1B STB labeled multinuclear hematoxylin positive circles). Between the STB and the basal lamina (trophoblastic basement membrane) are the villous cytotrophoblasts (CTB), that can fuse and hence act as precursors for STB. The stroma is composed of connective tissue cells, such as mesenchymal cells that differentiate to fibroblasts in the last trimester, connective tissue fibers, tissue macrophages (Hofbauer cells, H; Figure 1B) and fetal vessels [28]. Interestingly, it is speculated that Hofbauer cells play a role in iron storage and homeostasis [29]. However, there are two important cell layers in the chorionic villi to coordinate the nutrient transfer, namely the fetal capillary endothelial cells (EC, orange cells; Figure 1A) and the STB. The EC form the fetal vessels (FC, fetal capillaries Figure 1B), which allow the unrestricted diffusion of molecules like glucose and amino acids (<204 Da) through the paracellular route [30,31]. In contrast, STB represent the limiting barrier for charged macro- and microelements due to the tight syncytium composition of two polarized plasma membranes, the microvillous plasma membrane (MVM; Figure 1A labeled in upper panel) facing the maternal side

and the basal membrane (BM; Figure 1A upper panel) directed towards the fetal capillary. Small hydrophobic molecules cross cell membranes easily, so their transplacental flux depends principally on the concentration and electrochemical gradient. The main factor maintaining such a gradient is the rate of blood circulation on both sides of the membrane [2]. Hence, exchange of molecules such as the respiratory gas O_2 and lipophilic drugs are flow-limited, thus changes in maternal or fetal blood flow have a strong impact on their net flux [32]. Since the STB represent a true syncytium, no intercellular spaces exist in the transporting epithelium of the human placenta. Recently, stereological analysis of transmission electron microscope images showed a coverage of the basal lamina surface area of 69% by STB and 31% by CTB. Furthermore, the visualization of the three-dimensional structure revealed that basal membrane folds increase the STB basal membrane surface area and hence the diffusion capacity by 4.4-fold, further emphasizing the importance of STB at the placental barrier [33]. However, more detailed information about the molecular mechanisms of materno-fetal directed transport of iron and particularly the regulation of ferroptosis in the placenta is required. Thus, the present review is mainly focusing on the STB-mediated materno-fetal transfer of the most abundant trace element iron and the emerging role of ferroptosis pathways in gestational diseases.

2.2 Iron transport across the placenta

In humans, the iron requirement during pregnancy is significantly higher in comparison to the nonpregnant state in order to fulfill fetal and placental iron demands. Although reduced during the first trimester due to the cessation of menstrual bleeding, iron requirements rise to 4-6 mg in the second and third trimester, respectively. Major changes in the red blood cell mass commence in the middle of the second trimester, iron requirements may reach as much as 10 mg/d during the last 6-8 weeks of

pregnancy [34]. Irrespective of the exact value, it is apparent that at the later stage of pregnancy daily iron requirements cannot be solely met by absorption from regular diet. In diets containing large quantities of bioavailable iron, overall iron absorption is usually 3-4 mg/d or 5 mg/d at most [35]. The amount of iron absorbed is much lower when the diet contains only small amounts of bioavailable iron, as is often the case in many developing countries where the staple food is cereal and the intake of meat and ascorbic acid is limited [36]. As pregnancy progresses, iron requirements for fetal growth rise steadily in proportion to the weight of the fetus. Although the requirements of iron are even lower in the first trimester as compared to the nongravid state, approximately 80% of the iron needed for pregnancy is acquired during the third trimester upon term [36]. In the following chapters, the principles of materno-fetal iron transfer across the placental barrier are described. Hereby it is distinguished between uptake/efflux of transferrin (Tf)-bound iron and transport mechanisms of non- Tf -bound iron. The currently known iron homeostasis genes expressed in human placenta are listed in Table 1.

Table 1: Iron homeostasis genes expressed in human placental tissue

Accession No.	Protein	Name
Iron endocytosis		
P02787	Tf	Serotransferrin
P02786	TFR1	Transferrin receptor protein 1
Q07954	LRP1	Lipoprotein receptor-related protein 1
Q6ZMJ2	SCARA5	Scavenger receptor class A member 5
Iron transmembrane transporters		
P49281	DMT1	Divalent metal transporter 1
Q9C0K1	ZIP8	Zrt- and Irt-like protein 8
Q15043	ZIP14	Zrt- and Irt-like protein 14
Q9NP59	FPN1	Ferroportin-1
Q9NYZ2	MFRN1	Mitoferrin-1
Q9Y5Y0	FLVCR1	Feline leuk. virus subg. C receptor 1
Q9UPI3	FLVCR2	Feline leuk. virus subg. C receptor 2
Q96NT5	HCP1	Heme carrier protein 1
Q6P1K1	HRG1	Heme transporter 1

Fe-storage		
P02794	FTH	Ferritin heavy chain
Oxidoreductases		
P00450	CP	Ceruloplasmin
Q9BQS7	HEPH	Hephaestin
Q6MZM0	ZP	Zyklopen
P09601	HO1	Heme oxygenase 1
P30519	HO2	Heme oxygenase 2
Q658P3	STEAP3	Metalloreductase STEAP3
Regulators		
P81172	HEPC	Hepcidin
Q30201	HFE	Hereditary hemochromatosis protein
Iron sensing, mRNA binding		
P21399	IRP1	Iron regulatory protein 1
P48200	IRP2	Iron regulatory protein 2

Abbreviations: Zrt, Zinc-regulated transporter; Irt, Iron-regulated transporter; STEAP3, six-transmembrane epithelial antigen of the prostate 3

2.2.1 Transferin-mediated iron uptake across the microvillous membrane and intracellular storage

The primary route of transport across the placental barrier for nutrients and important ions such as iron is in most cases conveyed by specific solute carrier-mediated transport mechanisms [37]. The most common pathway for regulated nutrient transport in the placental barrier is via transporter proteins in the STB plasma membrane. [38][37]Of note, there is evidence of major clinical relevance indicating that the expression of placental transport systems is responsive to nutritional and hormonal stimuli. This flexibility allows the placenta to adapt functionally by insertion of transporter proteins into the appropriate membrane [39,40].

Materno-fetal iron transfer is initiated by receptor-mediated intracellular vesicle formation that occurs by invagination of the apical plasma membrane of STB. In general, receptor-mediated endocytosis involves the binding of a specific ligand to a receptor in the plasma membrane, which initiates the clathrin-mediated invagination of

the plasma membrane. Iron binds to the transferrin receptor (TfR) at the MVM of the STB surface, concentrates in clathrin-coated pits and is delivered to early endosomes [41].

Since the placenta is not able to secrete iron back to the maternal circulation, fetal iron homeostasis is controlled exclusively by regulating iron uptake into the placenta and iron efflux towards the fetal circulation as outlined in **Error! Reference source not found.**. Figure 2 depicts the pathways of transplacental iron transport in humans. Tf-bound iron is the major, if not the only maternal iron source for placental transfer (Figure 2A). Tf receptor 1 (TFR1) is a homodimeric transmembrane protein with high affinity for diferric Tf ($2\,Fe^{3+}$-Tf). Expressed in virtually all cells except mature erythrocytes, TFR1 is found at extraordinarily high levels in STB. This is probably also one of the reasons, why TFR1 was first isolated from placental tissue [42]. Despite its importance, the mechanism of iron transport in the placenta remains poorly characterized, partly due to the lack of *in vitro* models that mimic the physiology of the polarized placental STB layer [15]. Immunohistochemical studies have localized TFR1 and Tf to the apical membrane of STB in clathrin-coated endosomes [43]. The disruption of endosomal acidification after endocytosis by the weak base chloroquine inhibits placental accumulation and fetal transfer of Tf-bound iron, demonstrating the requirement of acidification in Tf-bound iron utilization in the classic Tf cycle [44].

In endosomes of erythrocytes, ferric iron (Fe^{3+}) is reduced to ferrous iron (Fe^{2+}) by the ferrireductase called six-transmembrane epithelial antigen of the prostate 3 (STEAP3) and is subsequently transported across the endosomal membrane by divalent metal transporter 1 (DMT1/*SLC11A2*). Although there is still a lack of experimental proof, STEAP3 and DMT1 probably play important roles in the placenta [15]. Of note, the

alternative ferrireductase candidate STEAP4, a homolog of STEAP3, is also highly expressed in the human placenta [45].

Besides DMT1, there are also alternative ferrous iron transporters that mediate endosomal iron export from the endosome into the cytosol. DMT1 is critical for endosomal iron release in erythrocytes and enterocytes [46]. *In vivo* studies suggest DMT1 is not an essential iron transporter in placenta, as demonstrated in the two DMT1 mutant animal models microcytic anemia (mk) -mouse and Belgrade-rat bearing the same missense mutation (G185R) in DMT1 giving rise to iron-deficient but viable animals. [46–48]. Due to viable but severely anemic birth of Dmt1-null mice, the role of DMT1 in placental iron transport requires further experimental confirmation [49]. Other potential endosomal iron transporters with high expression in the human placenta are Zrt- and Irt-like protein 8 (ZIP8/*SLC39A8*) and Zrt- and Irt-like protein 14 (ZIP14/*SLC39A14*), both members of the SLC39 metal-ion transporter family that transport several divalent metals like zinc (Zn^{2+}), ferrous iron (Fe^{2+}), manganese (Mn^{2+}) and cadmium (Cd^{2+}) [50,51]. ZIP14 has been shown to mediate plasma membrane uptake of non-Tf-bound iron (Figure 2A) [52] as well as Tf-iron from endosomes (Figure 2B) [53]. Targeted Zip14 mutants have no abnormal birth phenotype except lower birth weight [54], while deletion of Zip8 in mice leads to complete mortality before birth [55]. Despite different pH-dependence, ZIP14 and ZIP8 together with DMT1 probably play redundant roles in placental endosomal iron export into the cytosol. So far none of these knockout experiments could exclude the possibility of non-Tf-bound iron across the placental barrier (see Figure 2).

After entering the intracellular space iron can be stored by ferritin. Of note, there is still a debate regarding ferritin expression in STB (Figure 2A). There are reports showing a lack of expression [56,57], while others demonstrate some staining for a placental-

specific ferritin heavy chain (FTH) homolog [58,59]. This discrepancy may be due to the use of antibodies showing differential reactivity towards single ferritin isoforms. However, fetal villous stromal cells consistently show pronounced ferritin staining in all studies cited above [56–59]. This raises the possibility that the villous stroma may serve as a buffer between the STB and the fetal circulation to ensure adequate, but not excessive, iron supply. Whether synthesis and degradation of ferritin in the stromal cells respond to fetal iron demand is currently unknown [15]. The function of the villous macrophages, known as Hofbauer cells (see Figure 1), is not well defined but may include the support of trophoblast differentiation, stromal development, angiogenesis and erythroid cell maturation [60,61]. Interestingly, Hofbauer cells express most of the major heme and nonheme iron transporters and storage proteins [56,62], suggesting a role in early iron transport, storage and/or regulation [61].

Although it can be inferred that TFR1STEAP3, DMT1, ZIP8 and ZIP14 are essential for iron trafficking and processing in the placenta, experiments designed to exactly localize and functionally test this hypothesis are still lacking.

2.2.2 Iron efflux at the fetal side across the basal membrane

Iron export from the STB to the fetal stroma is mediated by FPN1, the only currently known iron exporter. A null mutation of the Fpn1 gene is lethal before gastrulation and hence Fpn1 seems to be essential for early embryonic development[63]. FPN1 is abundantly expressed along the BM of human STB [56]. Mouse embryos with a hypomorphic mutation causing a reduced expression of Fpn1, are severely iron-deficient at embryonic day 12.5 (E12.5) 12.5 and exhibit defects in neural tube closure and forebrain patterning [64]. These results strongly suggest a prominent role of Fpn1 in materno-fetal iron transport (Figure 2A/B). Additionally, the deletion of an iron-responsive element (IRE) in the Fpn1 untranslated region (UTR) results in

dysregulation of Fpn1 in organogenesis of multiple tissues and markedly reduced Fpn1 protein expression in the mouse placenta. As a consequence, severe anemia and iron deficiency in embryonic tissue at birth was detected [65]. These results strongly suggest that Fpn1 expression and function at the materno-fetal interface is essential for normal embryonic development. Although all mouse studies suggest an essential role of Fpn1 in placental iron transfer, to our knowledge, there are no *in vitro* experiments reporting iron efflux across the BM of STB towards the fetal circulation.

As a prerequisite for placental FPN1-mediated export of ferrous iron (Fe^{2+}) towards the fetus, intracellular iron must be re-oxidized to the ferric state (Fe^{3+}) so that it can be bound by Tf on the fetal side of the placental barrier (Figure 2A/B). The three multicopper ferroxidases ceruloplasmin (CP), hephaestin (HEPH), and zyklopen (ZP/*HEPHL1*) have been detected in human placental tissue (see Table 1). CP, a soluble copper-dependent ferroxidase that facilitates iron efflux, has been detected by immunohistochemical staining in STB and fetal capillaries [66]. Unexpectedly, Cp-null animals exhibit a normal phenotype at birth, suggesting that Cp is not essential for placental iron transfer [67]. HEPH has not been localized in human placenta, but expression of *HEPH* mRNA has been detected and quantified in BeWo cells [68]. In 2010, an international group of scientists identified a placenta-specific ferroxidase in connection with sex-linked anemia (SLA)-mice harboring a mutation in Hephl1. [69]. Zp has approximately 50% protein identity with CP and HEPH. It contains a transmembrane domain as well as an extracellular ferroxidase domain with appropriate topology to interact with FPN1 [69]. Absent in liver and intestine, Zp is abundantly expressed only in the placenta and has been localized to the labyrinth, spongiotrophoblasts and yolk sac in murine placenta [69]. Furthermore, the intracellular iron chaperone protein poly(rC)-binding protein 2 (PCBP2) was suggested

to act as a recipient of iron from DMT1 and as a donor of iron to FPN1 based on localization and binding studies [70]. Iron can have toxic effects because it catalyzes the generation of reactive oxygen species (ROS). Thus, the carry-over of iron by chaperons like PCBP2 may be relevant for protection of fetal tissues from putative oxidative ferrous iron and for iron release at the placental barrier across the BM (Figure 2A) [15]. Recently, PCBP2 silencing has been shown to negatively affect the epithelial mesenchymal transition of extravillous trophoblasts and to reduce their ability to invade the decidua of the mother in both *in vitro* and *ex vivo* experiments. Reduced invasion of extravillous trophoblasts into the maternal decidua leads to aberrant spiral artery remodeling causing insufficient blood flow to the fetus. However, a potential effect on the expression of iron homeostasis gene has not been investigated [71]. There is evidence that all three ferroxidases and the chaperone PCBP2 are expressed in the human placenta, but experimental evidence regarding their functions in the iron transport mechanism across the placenta is mostly lacking.

2.2.3 Materno-fetal non-transferrin-bound iron transport

As indicated before, it is not yet clear whether Tf-mediated iron uptake is the exclusive pathway for placental iron acquisition. Hence, other circulating forms of iron such as free non-Tf-bound iron and heme iron may be taken up by the placenta as well. Although global deletion of Tfr1 in mice leads to embryonic lethality by E12.5, some Tfr1$^{-/-}$ embryos were still able to develop hemoglobin-containing erythrocytes upon E10.5 [72], suggesting that the Tf cycle may not be essential for erythropoiesis during early development. Furthermore, it is unclear whether insufficient materno-fetal iron transport or defects in erythroid iron uptake or both caused anemia in Tfr1$^{-/-}$ embryos. To clarify these aspects, tissue-specific knockout (KO) of Tfr1 in the placenta should be investigated.

Electron microscopy of placental villi from radio-labeled ferritin-injected animals showed ferritin-containing endosomes [73] and ferritin accumulation in the basement membranes [74] suggesting ferritin endocytosis and transport in the placenta. Li et al. found that scavenger receptor class A member 5 (SCARA5) conveys serum ferritin binding and then stimulates its endocytosis from the cell surface with consequent iron delivery into ureteric bud tips of the kidney (Figure 2B) [75]. However, the molecular mechanism of ferritin endocytosis by STB and the relevance of this process for maternal delivery of iron to the fetus is still unknown.

Finally, the placenta may be able to utilize heme iron sources from macrophages or dietary heme intake, as suggested by the high expression of heme iron transporters and heme catabolic enzymes in placental tissue. Recent findings that the placenta represents a major hematopoietic organ supporting hematopoietic stem cell development, shed light on placental heme pathways (Figure 2B) [76]. Free heme may be taken up by the placental STB via lipoprotein receptor-related protein 1 (LRP1)-mediated endocytosis [77] or potential heme transporter like heme carrier protein 1 (HCP1)[78], feline leukemia virus subgroup C receptor 1 (FLVCR1) [79] and heme-responsive gene 1 (HRG1) [80]. Furthermore, heme iron could be either exported into the maternal circulation by FLVCR1 or converted to free ferrous iron by heme oxygenase 1 and 2 (HO1 / HO2) in the endoplasmic reticulum and exported to the fetal circulation by FPN1 [81]. Additionally, it was reported that HO1 overexpression in human placenta-derived mesenchymal stem cell (hPMSC) decreased apoptosis and enhanced migration properties by improving the balance of angiogenic factors *in vitro* [82]. The improved placental vascularization induced by HO1-hPMSC could bear therapeutic potential as an alternative treatment for PE. Recently, a Japanese group found HO1 exclusively expressed in trophoblasts at various periods of pregnancy and

upregulated in the placenta after miscarriage (spontaneous loss of a pregnancy within the first and second trimester) [78]. At present, there is no evidence showing a direct relationship between heme transporters and heme oxygenases and placental iron transport. However, few studies have reported a direct relationship of HO1 and ferroptosis. Kwon et al. demonstrated significant effects on erastin-induced ferroptotic cell death by HO1 knockout and overexpression in HT-1080 fibrosarcoma cells [84]. Moreover, the over-activation of HO1 by IκBα inhibitor BAY11-7085 has been shown to increase cellular redox regulation and iron accumulation, which induces ferroptosis [85].

As depicted in Figure 2B and labeled with question marks, experimental evidence of placental heme-iron transfer, the relevance of chaperones, ferritin uptake, endosomal or MVM localization of several iron transporters and the putative redundancy of ferroxidases is still lacking. Furthermore, mechanisms such as ferritin capturing, heme transport and expression of alternative transporters like ZIP8 / ZIP14 might explain the relative late lethality of the Tfr1 and Dmt1 knockout in mouse at mid or end gestation [49,72]. Intense further research is needed to characterize alternative iron transport pathways across the placenta.

2.3 Regulation of placental iron transport

The relative resilience of fetal hemoglobin levels to maternal anemia highlights the ability of the placenta to respond to altered maternal iron supply. Stable isotope data in human pregnancies have demonstrated that more iron from the maternal diet is transferred to the fetus when maternal stores are low. In contrast, materno-fetal iron transfer after intravenously administered radioactive iron was not significantly depending on the maternal iron status [86]. This is probably a consequence of intestinal and placental iron transporter upregulation [87,88]. The mechanisms underlying this

regulation are not well characterized and may involve placental iron regulatory proteins 1 and 2 (IRP1 and IRP2). In brief, during cellular iron deficiency, IRPs bind to the stem-loop structure of IREs within the UTR of iron-regulated genes including *TFR1*, *ferritin*, *FPN1* and *DMT1* (Figure 2C). The binding of IRPs to 3'-IREs promotes stabilization of mRNAs mediating increased iron uptake mainly via TFR1 and DMT1, whereas binding to 5'-IREs prevents translation of mRNAs involved in iron storage and export via ferritin and FPN1 (Figure 2C) [89]. Both IRP1 and IRP2 activity have been detected in human placenta [90]. IRP regulation of placental FPN1 is less clear [15].

The iron regulatory hormone hepcidin (HEPC) is expressed in human liver and placental tissue [91,92], but also in mouse embryos in the second trimester [93]. HEPC binding results in rapid ubiquitination of FPN1 causing its internalization and degradation [94]. Thus, HEPC might negatively regulate cellular iron export towards the fetal circulation via degradation of FPN1 at the BM (Figure 2C). Furthermore, HEPC may play a role in fetal sensing of the placental iron status and hence in signaling fetal demand to the placenta or even to the mother [15]. The anemic, iron-deficient phenotype of transgenic mouse embryos overexpressing Hepc further supports this notion [95]. Both the post-transcriptional inhibition of FPN1 synthesis through the IRP/IRE system [96] and hepcidin-dependent inhibition of its expression at the post-translational level [97], highlights the crucial role of FPN1 in the tight regulation of fetal iron bioavailability. A recent study, however, questioned the contribution of barely detectable fetal liver HEPC to the regulation of placental FPN1 in both normal and iron-deficient conditions [98]. In contrast, the importance of a concerted action of the post-transcriptional intracellular IRP/IRE system for placental iron homeostasis has been clearly demonstrated in human studies [92,99,100]. Of note, several reports of the same research group showed a profound decrease in maternal HEPC expression

during pregnancy even for iron-loaded mothers compared with nonpregnant females [101,102]. However, the relative upregulation of HEPC in mothers with high iron status probably prevents the transfer from excess iron into the maternal circulation and protects the fetus by regulation of FPN1-mediated iron transport in the maternal gut and by indirectly affecting placental iron status via IRP/IRES at the placental barrier, respectively (Figure 2C) [103]. Furthermore, the effect of maternal iron status on the Irp1-mediated regulation of TFR1 and Fpn1 in Hepc- and Irp-KO mice has been studied [103]. Since Irp1-KO, but not Hepc-KO, mice failed to decrease Fpn1 protein levels during maternal iron deficiency, it has been concluded that placental Irp1, but not Hepc plays a critical role in controlling placental iron levels. [103]. Strikingly, in contrast to iron overload, strong FPN1 downregulation and adaption of placental iron homeostasis in iron deficiency was observed, seemingly prioritizing placental iron status against fetal supply. Another protein expressed in the placenta regulating iron metabolism is the hemochromatosis factor HFE [104]. HFE is an MHC class I-like glycoprotein that assembles with β2-microglobulin to form a heterodimeric complex. A mutation in the HFE gene results in the autosomal iron-overload disease hereditary hemochromatosis [105]. HFE is associated with TFR1 [105–107] and DMT1 [108] (Figure 2C).

The placenta-controlled iron handling described above as well as the incapability of the fetus to compensate for maternal iron deficiency, but also the limited placental protection of iron excess emphasizes the need for a tightly controlled iron supplementation during pregnancy.

2.4 Adverse effects of imbalanced iron homeostasis for gestation

Altered nutrition during fetal development has a fundamental impact on lifelong health and wellbeing and may contribute by fetal programming to later prevalence for cardiovascular disease, diabetes and adiposity [109–112]. Appropriate *in utero* fetal

growth is largely dictated by the availability of nutrients in the maternal circulation, which is ensured during pregnancy by the capacity of the placenta to transfer these nutrients into the fetal circulation. A human retrospective cohort study including more than 57'000 singleton pregnancies compared placental weight with high or low maternal hemoglobin concentrations. The authors found a strong association between decreasing placental weight and increasing maternal hemoglobin concentrations [113]. Furthermore, a high placenta/newborn weight ratio in maternal iron deficient anemia was reported, in agreement with studies in mice [103]. It has been demonstrated that the reduced amount of iron that the fetus acquires under maternal iron deficiency is preferentially used for hemoglobin synthesis by red blood cells to compensate for chronic fetal hypoxia [114]. The prioritization of iron for fetal red cell synthesis together with the preference of iron acquisition within the placenta under iron-deficient conditions may be the main reasons for the reported 33% decrease in neonatal brain iron concentration in IUGR infants at autopsy [115]. Already in the 1990s autopsies of seven infants revealed that the iron concentration in brains of infants born to mothers with diabetes mellitus and uncontrolled hyperglycemia was significantly reduced. Although the placenta apparently attempts to increase iron transport by upregulating TfR density, full compensation does not occur, and the fetus develops a relative iron deficiency. At autopsy, brain iron concentration in newborn infants of diabetic mothers was decreased by 40% [116]. Moreover, such fetal and newborn anemic periods due to maternal iron deficiency earlier in pregnancy were reported to be associated with a significantly increased risk of autism spectrum disorder, attention-deficit/hyperactivity disorder and intellectual disability in the offspring [117].

2.4.1 Adverse effects of dysregulated maternal iron status on outcomes after birth

According to a very recent systematic review on maternal iron status during pregnancy in relation to child health outcomes after birth, 91% of the studies until January 2021 focused on reduced maternal iron status or iron deficiency, while iron status above normal or iron excess was significantly under-investigated [118]. The considered studies suggested deleterious effects on infant growth, cognition [119,120], neonatal sepsis after premature rupture of membranes [121] and childhood type 1 diabetes [122]. There seems to be a segmented U-shape relation between maternal iron status and the risk of adverse birth outcomes like stillbirth, preterm- or small for gestational age, especially in early pregnancy [12]. While already clear explanations for the anemic complications in iron-deficient pregnancies exist (see above), more research is needed to understand the effects of high iron status on adverse birth outcomes.

Although increasing relative HEPC levels may be sufficient to counteract high maternal iron levels under physiological/untreated conditions, a substantial amount of iron may be absorbed after iron supplementation in iron-replete mothers, thereby increasing the hemoglobin concentration [101]. There are at least four indications for putative negative effects of high serum iron levels on gestation and hence on birth outcomes. Excess iron supplementation of iron-replete women may i) enhance oxidative stress via increases in circulating unabsorbed iron, which can lead to lipid peroxidation and DNA damage in placental cells [123], ii) impair the systemic HEPC-dependent innate response to inflammation and infection [124], iii) alter the maternal gut microbiome due to increased iron levels in the colon as demonstrated by two double-blinded randomized controlled trials in Kenyan infants consuming iron supplemented porridge

for 4 months [125] and iv) increase the risk of copper and zinc deficiency in pregnant women [126].

2.4.2 Link between GDM and iron homeostasis

GDM, defined as any degree of glucose intolerance with onset or first recognition during pregnancy, is one of the most common pregnancy complications and has a prevalence of 4.8% in Switzerland [127] and 9.2% in the US [128], respectively. Within a Swiss cohort study (n=1042), GDM was strongly associated with short-term complications of fetal development and metabolic diseases early in life [129]. The authors found a high birth complication rate, e.g. 6.3% neonatal hypoglycemia, 9.4% respiratory distress syndrome and 9.4% jaundice, which was far above the incidence in the control group and the general population. Despite considerable progress in the diagnosis and treatment of GDM, it still has substantial adverse health effects on the mother and her offspring. The etiology of GDM is multifactorial. Well-known modifiable risk factors for GDM include obesity, high fat diet, high blood pressure, excess weight gain in pregnancy, endocrine dysfunction and sedentary lifestyle [130]. Despite the fact that ferritin and HEPC are changing in the acute phase response to infection and inflammation [11], both parameters served in several clinical studies to ascertain a positive association between serum ferritin levels (as a measure for maternal iron status) and the risk to develop GDM [131–138]. Only a few studies measured other parameters, such as serum iron [139] or heme iron intake [140,141], but confirmed the positive relationship between increased maternal iron status and the risk for GDM. Zein et al. found ferritin levels at early pregnancy correlating positively with glucose levels in oral glucose tolerance tests (oGTTs) [132]. More recently, Rawal et al. associated in a longitudinal and prospective study elevated ferritin levels already in gestational week 10-14 with an increased risk to develop GDM [133]. The authors further tested

plasma levels of hepcidin, ferritin and soluble TfR (sTfR) for diagnostic purposes and recommended to assess the sTfR:ferritin ratio as inversely related diagnostic marker to predict the risk to develop GDM. Very recently, a Finnish multicenter case-control study found a strong association between maternal hemoglobin levels and GDM and a dose-dependency with gestational hypertension [142]. All these findings raise potential concerns for the recommendation of routine iron supplementation among iron-replete pregnant women especially for those with a high risk to develop GDM. However, the pathophysiological association of GDM and elevated maternal iron plasma levels reported in clinical studies as well as the underlying mechanism are still unclear.

2.4.3 Association of GDM-mediated hyperglycemia and placental iron homeostasis

Both the hyperglycemic environment and increased levels of iron may play a role in the generation of oxidative stress during pregnancy [143]. Oxidative stress occurs when the delicate balance between the generation of ROS and the production of antioxidant neutralizing species, such as nicotinamide adenine dinucleotide phosphate (NADPH) and glutathione (GSH), is disturbed. Hyperglycemia raises oxidative stress levels through different metabolic pathways. On the one hand, glucose is converted under intracellular hyperglycemic conditions to the polyalcohol sorbitol by utilizing intracellular NADPH and GSH. Consequently, hyperglycemia induces an excess of ROS by antioxidant depletion via the hyperglycemia-induced process of overproduction of superoxide by the mitochondrial electron-transport chain [144]. On the other side, excessive iron supplementation might expose women to increased lipid peroxidation and protein carbonylation by intracellular generation of ROS [145,146]. Recently, it has been shown that hyperglycemia alters placental iron uptake and that

the treatment with the antioxidant sodium selenite (NaSe) can rescue the alterations in iron homeostasis gene expression [92]. However, whether antioxidant supplementation is able to reduce the complications in placental iron transfer caused by GDM in both the mother and the fetus has not been explored yet.

3 Placental Ferroptosis

3.1 Mechanistic hallmarks of placental ferroptosis

Ferroptosis has been associated with a wide variety of disorders, including cardiovascular, neurodegenerative, renal, and hepatic diseases, as well as cancer [147].

Three hallmarks have been attributed to ferroptosis by Dixon and Stockwell [150]: 1) the availability of redox-active iron, 2) the oxidation of phospholipids containing PUFA and 3) the loss of lipid peroxide repair capacity by glutathione peroxidase 4 (GPX4). Those hallmarks and the interaction of the corresponding ferroptosis pathways are discussed in a placenta oriented manner and illustrated in Figure 3.

3.1.1 Availability of redox-active iron

In brief, the first hallmark is the availability of redox-active iron (see Figure 3A). As already discussed in the context of placental iron uptake (see 2.2.1), mainly Tf-bound ferric iron (2 Fe^{3+}-Tf) is imported from the maternal circulation by endocytosis into trophoblasts through TFR1. There it is reduced to ferrous iron and released from the endosome by a divalent iron transporter like DMT1 to contribute to a labile iron pool in the cytosol or stored in ferritin before exported towards the fetal circulation by FPN1 (see Figure 2) [151]. Redox-active iron from the labile iron pool in the cytoplasm can oxidize PUFA-containing membrane phospholipids through the Fenton reaction [19].

In the Fenton reaction, divalent iron ions react with hydrogen peroxide to hydroxyl radicals, which can seize hydrogen atoms from phospholipids. This results in the generation and accumulation of phospholipid peroxides, exemplarily shown as destabilization of the MVM in Figure 3A/B, which leads to ferroptotic cell death [152,153]. Furthermore, iron is involved in the enzyme-mediated peroxidation of PUFA containing a *cis,cis*-pentadiene structure by the nonheme iron-containing dioxygenase called lipoxygenases (LOX) [154].

Reactive iron is essential for ferroptosis and represents a target for inhibition. Iron chelators, such as deferoxamine (DFO), desferrioxamine mesilatec (DFOM), ciclopirox olamine (CPX) or 2,2-bipyridyl (2,2-BP) seem to block the peroxidation of phospholipids by preventing electron donation of solubilized and highly reactive iron ions [19,155,156]. Moreover, higher expression of the iron importer TFR1 is an effective biomarker for ferroptosis and its silencing prevents cells to undergo ferroptotic cell death [157,158]. These findings are also supported by in vitro studies showing that the supplementation of iron in growth medium directly promoted ferroptosis [19,157]. In addition, the reduction of the iron-storage protein ferritin increases free iron availability and thus promotes ferroptosis [26,159]. Of note, there is a mechanism for the degradation of ferritin in the cytosol called "ferritinophagy". In brief, the cargo receptor nuclear receptor coactivator-4 (NCOA4) binds ferritin and this leads to a lysosomal release of free Fe^{2+} and consequently results in the generation of lysosomal ROS [160]. Enhanced iron export due to increased FPN1 expression, however, reduces ferroptotic activity due to lower cellular iron availability [161,162]. All these factors emphasize the important role of iron availability in ferroptosis and underline that tightly controlling iron homeostasis is crucial for cell viability [147].

3.1.2 Oxidation of phospholipids

The oxidation of phospholipids through the Fenton reaction is the second hallmark of ferroptosis (Figure 3B). The accumulation of lipid peroxides leads to ferroptosis, but the exact mechanisms responsible for the ferroptotic irreversible cell death downstream of phospholipid peroxidation remain poorly understood [147]. The resulting phospholipid peroxides can in turn oxidize new PUFA containing phospholipids, leading to a chain reaction [19]. It is unclear whether the lipid damage triggers cell death only by destruction of the cell membrane or also by covalent modification of intracellular proteins by toxic 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA) production [163,164]. 4-HNE and MDA are secondary products of lipid peroxidation and can be used to detect ferroptotic cell death [165,166] in addition to measuring lipid peroxide content by Liquid chromatography-tandem mass spectrometer (LC-MS/MS). Interestingly, excessive ferroptosis and inflammasome activation has been reported in oxidative stress models suggesting an association between the nucleotide-binding domain (NBD) and leucine-rich repeat containing protein (NLR) family, pyrin domain containing 1 (NLRP1)-inflammasome expression and ferroptosis. Inflammasomes are protein complexes that can activate the formation of the pro-inflammatory molecule interleukin as well as the cleavage of gasdermin-D that finally leads to a highly inflammatory form of programmed cell death known as pyroptosis (Figure 3B). In the gravid uterus of oxidative stress abortion rat models, the expression of NLRP1, NLRP3, TFR1 and ACSL4 were elevated, whereas GPX4 mRNA levels were decreased [167]. This study revealed reduced ferroptosis levels in trophoblasts, when NLRP1 was silenced which returned to normal levels, when silencing was removed. Hence, ferroptosis was elevated after treatment with an NLRP1 activator. On the other hand, the application of both the ferroptosis activator

erastin and the inhibitor ferrostatin-1 affected the expression of NLRP1 [167]. These results indicate the importance of NLRP1 in oxidative stress trophoblast cell models and underline the involvement of NLRP1 in the development of ferroptosis. Therefore, the interactive relationship between ferroptosis and NLRP1 inflammasomes could play a role in the pathogenesis of pregnancy disorders. However, more pre-clinical investigations are needed to support these assumptions.

3.1.3 Loss of antioxidant generation and repair

The third hallmark of ferroptosis is the loss of the lipid peroxide repair capacity by GPX4 (Figure 3C). The cysteine-GSH-GPX4 axis is the main opposing system of ferroptosis [168]. Reactive products of lipid peroxidation are reduced to their corresponding alcohols by the oxidation of GSH catalyzed by the selenoprotein glutathione peroxidase 4 (GPX4). This process impedes the chain reaction and thus GPX4 is the major enzyme preventing ferroptosis [19,169]. GSH, the most abundant reductant in mammalian cells, is built from cysteine, which is its key limiting substrate [170]. Cysteine is imported as cystine into the cell by the cystine/glutamate antiporter system x_c^-, a transmembrane protein consisting of two subunits, xCT/SLC7A11 and 4F2hc/SLC3A2 (Figure 3C) [171]. If any element of the cysteine-GSH-GPX4 axis is dysfunctional, the vital capability to oppose ferroptosis is significantly impaired. Hence, ferroptosis can be induced by preventing the biosynthesis of GSH or by inhibiting GPX4. In this context high extracellular concentrations of glutamate or blocking the xCT/SLC7A11 transporter (e.g. by erastin, sulfasalazine and sorafenib) can inhibit the import of cystine, thus decreasing the biosynthesis of GSH and inducing ferroptosis (Figure 3C) [19,148,172,173]. These system x_c^- inhibitors were classified as "class 1" ferroptosis-inducing compounds (FINs), inactivating GPX4 indirectly [148,155]. Furthermore, the tumor suppressor protein p53 contributes at the transcriptional level

to ferroptosis by upregulating glutamate production as well as by decreasing the expression of xCT (Figure 3C) [174]. An alternative import pathway for cystine can be achieved by treatment with β-mercaptoethanol (β-ME), which suspends the effect of system x_c^- inhibitors *in vitro* (Figure 3C) [173]. Ras-selective lethal molecule 3 (RSL3) and nine other synthetic small molecules can inactivate GPX4 directly and were classified as "class 2" FINs [19,148,155]. Notably, the identification of the lethal mechanisms of erastin and RSL3 initiated the discoverey of ferroptosis as novel cell death concept [19,175]. Furthermore, glutamate-cysteine ligase (GCL), which plays an important role in GSH biosynthesis, can be inhibited by buthionine sulfoximine (BSO) thus inducing ferroptosis [168,176]. Compared to class 1 or 2 FINs, BSO is a far less potent inducer of ferroptosis [163].

Besides the cysteine-GSH-GPX4 axis, there are GPX4-independent lipophilic antioxidants, which can inhibit ferroptosis by preventing the generation of phospholipid peroxides [177]. For example, synthetic ferrostatin-1 (Fer-1), liproxstatin-1, α-tocopherol (vitamin E, Trolox), butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) have been shown to reduce or even prevent ferroptosis (Figure 3C) [19,163,168,178,179]. As liproxstatin-1 and ferrostatin-1 selectively inhibit ferroptosis, they are also used to detect ferroptosis [166]. Further downstream in the ferroptosis process, the activation and integration of PUFA-containing phospholipids into membranes by the acyl-CoA synthetase long-chain family member 4 (ACSL4) or lysophophatidylcholine acyltransferase 3 (LPCAT3) are required and drive the ferroptotic process. Consequently, their deletion results in the prevention of ferroptosis [173–176].

In contrast to these pro-ferroptotic genes, ferroptosis suppressor protein 1 (FSP1 or AIFM2) can prevent a *GPX4*-knockout cell from undergoing ferroptosis (Figure 3C)

[180]. FSP1 catalyzes the reduction of ubiquinone (Coenzyme Q10) to ubiquinol, which then reduces the production of phospholipid radicals [180,181]. Moreover, phospholipase PLA2G6 can hydrolyze phospholipid peroxides and thereby directly mitigate ferroptosis [182]. The latter study demonstrated that deficiency of PLA2G6 in BeWo trophoblasts increases the levels of phospholipid peroxides resulting in an increased sensitivity to RSL3-induced ferroptosis (Figure 3C). However, PLA2G6 deficiency only induced ferroptosis if GPX4 function was impaired, indicating a secondary defense role of PLA2G6 against ferroptosis [182,183]. Thus, several small GPX4-independent molecules can contribute to the prevention of ferroptosis.

3.1.4 Signaling pathways involved in ferroptosis

Ferroptosis is controlled by specific signaling pathways such as the mitogen-activated protein kinase pathways (MAPK). The latter can be grouped into three families, namely extracellular signal regulated kinases (ERK), p38-kinases and c-Jun N-terminal kinases (JNK), all of which appear to play an important and cell-type specific role in ferroptosis [148]. Blocking the ERK pathway inhibits erastin-induced ferroptosis in Ras-mutated cancer cells, while blocking p38 or JNK, but not ERK, inhibits erastin-induced ferroptosis in leukemia cells [148,179,184]. In the placenta MAPK signaling pathways play a central role in villous trophoblasts differentiation and have been associated with preeclampsia and hemolysis, elevated liver enzymes and low platelet count (HELLP) syndrome [185]. Therefore, trophoblast-specific dysregulation of MAPK pathways due to the activation of ferroptosis may contribute, aggravate or even cause severe pregnancy complications by dysregulating proliferation, differentiation, inflammation and programmed cell death.

Moreover, energy stress-dependent upregulation of AMP-activated protein kinase (AMPK) signaling as known sensor of cellular energy status was protecting against

ferroptosis, while this AMPK-related protection was antagonized by glucose excess [186]. This study suggests that glucose levels contribute to the induction or regulation of ferroptosis and raises the question if AMPK-mediated regulation of ferroptosis is disturbed in hyperglycemic conditions such as GDM.

Another signaling pathway, E-cadherin-NF2-Hippo-YAP has been associated with conferring resistance to ferroptosis in epithelial cells with cell density induced E-cadherin expression and RNA-interference of the tumor suppressor neurofibromin 2 (NF2) [187]. Recently, a study in primary cells, three-dimensional organoids and KO cell lines supported the hypothesis of Hippo signaling being a main driver of placentation and demonstrated upregulation of nuclear yes-associated protein (YAP)-TEAD4 complexes stimulating the expression of stemness and proliferation genes, while inhibiting hCG-dependent cell fusion and trophoblast differentiation [188]. Indeed, the YAP signaling pathway modifies the expression of several ferroptosis regulatory genes, such as ACSL4 and TFR1 through the co-transcription factor YAP [187]. However, further mechanistic investigation of E-cadherin-mediated inhibition of ferroptosis via Hippo signaling is needed to evaluate the physiological and pathophysiological relevance in placental development.

Beside the signaling processes outlined above, nuclear factor erythroid 2-related factor 2 (NRF2)-dependent signaling seems to play a role in controlling placental ferroptosis. NRF2 is an oxidative-stress-responsive transcription factor and ROS sensor. It mitigates ferroptotic lipid peroxidation through regulation of antioxidant and iron signaling gene expression, such as xCT, GCL, ferritin, FPN1. Increased levels of ROS lead to the dissociation of NRF2 from Kelch-like ECH-associated protein 1 (KEAP1), followed by its translocation into the nucleus and induced transcription of genes containing antioxidant response elements, which helps to restore the oxidative balance

[189]. Indeed, inhibition of NRF2 increased the sensitivity of FINs to induce ferroptosis [150,190,191]. Previous studies indicate that activators of NRF2, as well as its downstream targets, could be viable targets to reduce oxidative imbalance in various gestational diseases [192,193]. Thus maintaining a balanced NRF2-KEAP1 activity could represent a future strategy to combat severe pregnancy disorders.

3.2 Placental ferroptosis in pregnancy related complications

The pathogenesis of several gestational diseases, such as fetal growth restriction, fetal demise, GDM, gestational hypertension disorders (e.g. PE, eclampsia and HELLP syndrome), preterm birth or invasive placenta, is not fully understood. Since longtime oxidative stress has been associated with pregnancy related complications [194]. On the other side, the role of ferroptosis in placental dysfunction, trophoblast injury and in the pathogenesis of PE, fetal growth restriction and preterm birth has been established only recently [166]. Trophoblasts are rich in iron and highly express ferroptosis key players such as LPCAT3, ACSL4 and arachidonic acid, which are involved in the production of PUFA [160,189]. Trophoblasts are particularly susceptible to ferroptosis due to their high iron content and hypoxia/reperfusion events, both commonly occurring in placental physiology [166] and potent triggers of oxidative stress [190]. However, also external agents can induce ferroptosis in trophoblasts. Benzo[a]pyrene-7,8-diol-9,10-epoxide (BPDE) is a metabolite of Benzo[a]pyrene, which belongs to polycyclic aromatic hydrocarbons, a group of carcinogenic environmental contaminants. BPDE exposure to human trophoblast cells has been identified to up-regulate iron metabolism, increase free iron levels, produce ROS, down-regulate GPX4 and thus induce ferroptosis [191].

Recently, Kajiwara et al. revealed that human trophoblasts are sensitive to ferroptosis by showing macro-blebbing and vesiculation after the induction of ferroptosis by the

GPX4 inhibitor RSL3. Those macro-blebs could reach cell size and were stained positive for F-actin, but did not contain any other organelles. Hence, membrane blebbing in placental trophoblasts could be used as a characteristic biomarker for ferroptosis. According to the authors, macro blebbing could be a passive process triggered by hydroperoxyl-phosphatidylethanolamine facilitated stretching and osmotic pressure during cell death [192]. In contrast to apoptotic vesicles, detached and transferred macrovesicles or blebbing cells did not stimulate ferroptosis in target cells [192]. Interestingly, it was detected that sublethal ferroptosis activation impaired trophoblast differentiation to syncytiotrophoblasts and that syncytiotrophoblasts were more susceptible to ferroptosis than undifferentiated cytotrophoblasts. Hence, reduced differentiation of trophoblasts could represent a mechanism to reduce vulnerability; however, the consequences of trophoblastic macro blebbing and its pathophysiological relevance must be further clarified [181,193].

The following paragraphs summarize studies that focus on ferroptosis in PE, preterm delivery and GDM.

3.2.1 Ferroptosis and preeclampsia

PE is a complication in 2-8% of all pregnancies, causing high blood pressure and intrauterine growth restriction. In addition, it has been associated with later-life cardiovascular disease [194]. Whereas the etiology of PE is not yet fully understood, oxidative stress due to inadequate trophoblast invasion of spiral arteries leading to endothelial damage is considered as the main cause [190,195]. In a recent study, ferroptosis was identified to be involved in the pathogenesis of PE [196]. Ng, Norwitz, and Norwitz proposed a model in which reproductive complications exert a ferroptosis overreaction to hypoxia reperfusion that occured between 8 and 10 weeks of gestation, resulting in damage to maternal spiral arteries and thereby in the pathogenesis of PE

[189]. Changes in various ferroptosis markers that provided evidence for a link between PE and ferroptosis are discussed below.

Concerning the presence of oxidative iron (defined as the first hallmark of ferroptosis), serum iron levels, serum ferritin, Tf-saturation and labile divalent iron levels in placental tissue are higher in PE compared to healthy pregnancies. On the other side, for hepcidin serum levels conflicting results exist regarding an increase or decrease in PE [202–213]. In the clinical context, iron levels positively correlate with an elevation of blood pressure and the severity of the disease [202,205,214]. H. Zhang et al. explained the elevated iron levels by the downregulation of the iron exporter FPN1 in placental tissues of patients with PE compared to healthy pregnancies. In contrast, the iron importer TFR1 and the intracellular iron storage protein ferritin were not altered in PE patients [196]. It has been assumed that the decreased expression of FPN1 in PE placental tissues may be related to the downregulation of PAX3, a transcription factor of FPN1 [196]. However, the increase in iron serum parameters could also be explained by a lower blood volume, as occurring in PE and fetal growth restriction [200,209]. In hypoxia experiments with human trophoblast cell lines, intracellular labile iron levels were lowered by the iron chelator and ferroptosis inhibitor DFO, but not by the TFR1 inhibitor ferrostatin-1 [196]. Erlandsson et al. concluded that excessive iron and heme could promote oxidative stress and ferroptosis and thus amplify the underlying pathology of PE [200]. Other investigations, however, suggested ferroptosis to be part of the pathology [189,196]. A recent study found a higher number of differentially expressed ferroptosis-related genes in early-onset compared to late-onset PE patients (20 vs. 3) versus controls [216]. The identified genes were mainly involved in hypoxia and iron metabolism (e.g. FTH1, HIF1A, FTL, MAPK8 and PLIN2).

In summary, these studies indicate that an imbalance of iron homeostasis leading to ferroptosis could play a role in PE.

Several studies have found elevated levels of MDA, as end product of lipid peroxidation (second hallmark of ferroptosis), in preeclamptic sera and placental tissue compared to healthy controls. MDA levels were positively correlated with the severity of the disease and negatively with ferroptosis inhibitor concentrations [196,211–217]. Basbug et al. even suggested serum MDA as a predictor for PE [215].

Referring to the third ferroptosis hallmark, the loss of antioxidant generation and turnover, various studies suggested an important role of GPX4 in preeclamptic pregnancies. Imai et al. showed that GPX4-knockout mouse embryos did not survive beyond day 8.5 and thereby demonstrated its vital importance [218]. Beharier et al. have demonstrated, that genetic or pharmacological inhibition of GPX4 activity causes ferroptotic damage in primary human trophoblasts and mouse pregnancies [183]. In PE pregnancies, GSH levels, GPX4 activity and GPX4 expression are lower compared to healthy controls and correlate with the severity of the disease [196,216,219,220]. Other studies also found decreased expression of xCT in PE patients, which consequently may have contributed to decreased GSH levels [196]. Genetically, a higher risk of PE was shown in women with GPX4 mutations [221]. Moreover, ferroptotic damage in primary human trophoblasts and BeWo cells induced by GPX4-inhibitor RSL3 or hypoxia was reduced by ferrostatin-1 or DFO [181,196]. Furthermore, the activation of the critical antioxidant gene regulator NRF2 reduced ferroptosis in a hypoxia induced human trophoblast PE cell model and hence indicates a potential protective role of NRF2 in PE. From a mechanistic point of view, it has been shown that NRF2 translocates under hypoxia into the nucleus and initiates NRF2/ HO1 signaling; this process was enhanced by ferric ammonium citrate and lowered by DFO

[222]. These studies also demonstrated that overexpression of NRF2 mitigates oxidative stress-induced changes, e.g., GSH, MDA, ROS and divalent iron levels were decreased while xCT, GPX4 and FPN1 expression were transcriptionally elevated, indicating a relief of ferroptosis through NRF2 activity in this PE cell model [222]. By investigating the post-transcriptional regulation of the placental redox homeostasis, H. Zhang et al. found 273 miRNAs upregulated and 235 miRNAs downregulated in PE tissues. The authors identified an increase in miR-30b-5p in PE patient sera and placental tissue, which was presumably responsible for the reduced expression of xCT and paired box protein Pax-3 (PAX3)-mediated downregulation of FPN1 leading to ferroptosis *in vitro* [196]. In contrast, the prevention of miR-30b-5p upregulation in a hypoxia PE model increased the expression of xCT, PAX3 and consequently FPN1. Furthermore, PE symptoms were mitigated in a rat model by inhibiting the expression of miR-30b-5p and using the ferroptosis inhibitor ferrostatin-1. These results support an involvement of ferroptosis in the pathogenesis of PE and suggest miR-30b-5p as a potential therapeutic target [196].

The numerous observational and experimental associations between typical ferroptosis parameters and characteristic PE markers, highlight the need for deeper investigation into placental ferroptosis, its role in obstetrical diseases and emphasize the deployment of targeted ferroptosis therapeutics in the future.

3.2.2 Possible role of ferroptosis in spontaneous preterm birth

Preterm birth or the delivery before 37 completed gestational weeks, is the leading cause of childhood mortality. Since oxidative stress has been associated with preterm birth [223,224] this disorder was also investigated in the context of ferroptosis. Beharier et al. found increased levels of phospholipid peroxides and 4-HNE in human spontaneous preterm birth placentas, supporting an association with ferroptosis [183].

Likewise, a low dose of the GPX4 inhibitor RSL3 showed no maternal toxicity in pregnant mice, but increased placental ferroptosis and resulted in a higher incidence of fetal demise in PLA2G6-knockout mice. In addition, hypoxia-reperfusion experiments in PLA2G6-knockout mice showed higher phospholipid peroxide levels and a higher incidence of fetal demise than wild-type mice [183]. These results suggest that PLA2G6 may represent another potential therapeutic target against ferroptotic injury.

3.2.3 Ferroptosis in fetal demise

Pregnancy loss, miscarriage or spontaneous abortion represent fetal loss up to 20 weeks of gestation and has an incidence between 10-20%, with the highest risk in the early weeks of pregnancy [225]. In placentas from fetal loss, elevated HO1 levels in the first trimester and an increased HO-1/FPN1 ratio in the first and second trimester were found [226]. The authors related those findings to ferroptosis, as it has been shown that HO1 overexpression enhances erastin-induced ferroptosis, probably because of higher intracellular iron levels [226,227]. Moreover, decreased GPX4 expression in spontaneous preterm abortion was reported, while long non-coding RNA H19 expression was increased [228]. In lncRNA H19 silenced human trophoblast cell lines, the expression of GPX4 was also decreased supporting the assumption that ferroptosis may play a role in spontaneous abortions [228].

In a rat model it has been shown that ferroptosis is one of the potential mechanisms activated by hyperandrogenism and insulin resistance in polycystic ovary syndrome (PCOS) leading to placental and uterine dysfunction and further to fetal loss [229,230]. However, the partially contrasting expression patterns of pro- and anti-ferroptosis genes, as well as mitochondrial aberrations suggested an enhanced ferroptotic activity in the PCOS rat model mainly in the gravid uterus rather than in the placenta [229].

Indeed, in the investigated rat model decreased levels of GPX4 were detected only in certain placental cells, but no changes in GSH levels, iron accumulation and TFR1 expression were found. In contrast, in the gravid uterus an array of alterations occurred, among them decreased GPX4 and GSH levels, increased iron and MDA levels, aberrant expression of ACSL4, TFR1, xCT, and GCL, and mitochondria with typical ferroptotic changes [229,231,232]. However, treatment with antioxidants improved fetal survival [231]. In another study using the PCOS rat model, the antioxidant N-acetylcysteine partly reversed the uterine and placental ferroptosis through increased expression of anti-ferroptosis genes and increased GPX protein level proposing N-acetylcysteine as potential therapy for PCOS [233]. In addition, in the PCOS model organ-specific differences in the signaling pathways were detected: the MAPK signaling pathway was activated by ERK and JNK in the gravid uterus and by p38 in the gravid uterus and the placenta [229]. Moreover, chronic exposure to 5α-dihydrotestosterone (DHT) alone or in combination with insulin resulted in increased ferroptosis and decreased apoptosis activity in the gravid uterus while increased necroptosis was detected in the placenta. Therefore, apoptosis and necroptosis may play a role in compensating or coordinating activated ferroptosis in PCOS-like rats [229,232,234].

3.2.4 Emerging role of ferroptosis in gestational diabetes

While there is usually an increased insulin tolerance in healthy pregnancies, gestational diabetes mellitus (GDM) describes a pathological state that exceeds defined blood glucose values exclusively during pregnancy. GDM is a common pregnancy complication associated with decreased infant iron stores at birth (see section 2.4.2).

Unlike in IUGR, the expression of placental TFR1 in GDM shows a significant relationship between fetal iron stores and placental IRP1 [235]. Studies performed in our laboratory revealed an association between placental tissues from GDM patients, hyperglycemic conditions *in vitro* and functional changes in placental iron homeostasis [236]. Furthermore, own preliminary investigations of diet-induced GDM in wildtype mice indicate a dysregulation of iron homeostasis genes in placental tissues, aberrant iron storage and the involvement of ferroptosis as an underlying mechanism [237]. Altered iron homeostasis in BeWo cells grown under hyperglycemic and hyperlipidemic conditions could be induced by exposure to additional oxidative stress (tert-BOOH, tert-Butylhydroperoxid) and was rescued by subsequent treatment with the antioxidant sodium selenite NaSe [236]. Interestingly, decreased selenoenzyme activity has been associated with preterm birth, miscarriage, PE, and intrauterine growth retardation [238]. Several studies also observed significantly decreased serum selenium levels in pregnant women with GDM [239–243], but there are also investigations lacking this relationship [244–246]. Furthermore, reduced GSH levels, impaired iron transport and increased lipid peroxidation in trophoblast cells under hyperglycemic conditions suggest the involvement of hyperglycemia in the induction of ferroptosis [236]. These findings are in line with recent studies from Han et al., who showed that high glucose exposure greatly increased ROS content, causing ferroptosis and reducing the viability of human and porcine trophoblast cell lines [247]. Furthermore, upregulation of Sirtuin 3 (SIRT3) was found in trophoblast cell lines under hyperglycemic conditions. SIRT3 deacetylates ROS-regulated mitochondrial proteins and positively affects the AMPK/mTOR pathway, which plays a role in cellular redox homeostasis [248]. The depletion or silencing of SIRT3 resulted in an increase of GPX4 and suppression of the AMPK/mTOR pathway and thus prevented glucose- and/or erastin-induced

ferroptosis as well as autophagy [247]. Therefore, the authors concluded that SIRT3 may play a role in trophoblast ferroptosis and autophagy under hyperglycemia and may thus represent a potential therapeutic target for GDM. In a two years prospective case-control study with age-matched non-anemic pregnant women from the Diabetes in Pregnancy Study Group India (DIPSI), pre-pregnancy BMI, oral glucose tolerance test (oGTT) values and HbA1c concentrations were positively correlated with ferritin concentrations as well as with the ratio between antioxidants and the total amount of oxidants in the serum samples [249]. In a recent study using a GDM mouse model, adiponectin treatment led to an inhibition of oxidation/peroxide imbalance-induced ferroptosis putatively due to the restoration of carnitine palmitoyltransferase I (CPT-1) activity, a β-oxidation rate limiting enzyme [250]. Moreover, it has been experimentally shown that pharmacologically induced ferroptosis resulted in impaired viability and function of isolated human pancreatic islets [251]. Another pregnancy-independent study demonstrated that FPN1 mediates ferroptosis in the hippocampus of rats with streptozotocin-induced type 1 diabetes inducing cognitive dysfunction [252]. In summary these studies indicate that diabetes type 1 is associated with iron homeostasis, lipid peroxidation and ferroptosis in rats. In addition, they suggest a synergistic relationship of gestational hyperglycemia and altered placental iron homeostasis as the mechanistic basis of ferroptosis.

4 Conclusions and Perspectives

Nutrients are transferred across the materno-fetal barrier by diffusion, endocytosis/exocytosis and transporter-, channel- or facilitator-mediated mechanisms depending on their molecular properties, relative distribution and on the presence of

co-substrate or electrochemical gradients. The tight syncytium of syncytiotophoblast cells represents the limiting barrier for charged nutrients such as iron. However, there are still open questions concerning the exact cellular mechanism of iron transfer across the placental barrier. A systematic approach to localize all proteins potentially involved in placental iron uptake and transfer across the placental barrier including alternative heme- and non-heme-iron transporter, receptors, chaperones, storage proteins, reductases and oxidases, could inspire the entire field of placental nutrient transport research. Further, there is urgent need for experimental characterization of placental iron and heme iron transfer to foster novel insights into the pathophysiology of tightly nutrition-related and highly prevalent pregnancy diseases, such as PE and GDM.

There are undeniable advantages of gestational iron supplementation that improves a high number of pregnancies or even rescues many lives all over the world. A general recommendation of iron supplementation for all women throughout pregnancy seems plausible, because gestational iron deficiency has been found to sustainedly impact the developing brain by metabolic programming. The problem is even amplified by socio-economic issues due to low dietary iron absorption and limited intake of meat and ascorbic acid, while iron requirements are rising especially in the second and third trimesters. Nevertheless, the improvement of maternal and fetal iron parameters through iron supplementation depends on an evident and manifested iron deficiency below a certain threshold and is only efficient in a narrow window of vulnerability during fetal development. Furthermore, the placenta's own iron demands and the incapability of the fetus to compensate for maternal iron deficiency, but also the limited protection of the placenta against fetal iron excess emphasizes the need for a tightly controlled gestational iron supplementation. So far, most studies focused on reduced maternal iron status or iron deficiency, while effects of iron excess are seriously under-

investigated. Various mostly observational studies using different diagnostic parameters confirmed the positive relationship between increased maternal iron status and an increased risk to develop GDM. However, the pathophysiological association of GDM and elevated maternal iron plasma levels, as well as the underlying mechanisms are still unclear. Hence, the exact mechanism of transplacental iron transport in highly prevalent gestational disorders has been largely ignored so far. The recently identified potential associations between aberrant placental iron homeostasis and ferroptosis could develop into a strong approach to uncover new promising therapeutic targets for PE and GDM, but also against fetal loss.

Although ferroptosis has been associated with various diseases, the investigation of a potential relationship between ferroptosis and gestational diseases has commenced only recently. Apparently, trophoblasts are particularly vulnerable to oxidative stress due to the high iron content and hypoxia/reperfusion events. Interestingly, ferroptosis is controlled by signaling pathways known to be crucial for placental development, successful pregnancies and healthy fetal development. Therefore, ferroptotic mechanisms have the potential to modulate or even aggravate the progression of prevalent gestational diseases. There is substantial evidence that ferroptosis plays a role in PE. In this context, there are already promising therapeutical targets, such as the balanced expression of NRF2 leading to a reduction of ferroptosis in a PE hypoxia cell model and the inhibition of miR-30b-5p reducing PE symptoms in a rat model. Focusing on other gestational diseases, hypoxia-reperfusion experiments in PLA2G6-knockout mice gave a hint on the involvement of ferroptosis in preterm birth or fetal demise. There are further indications of ferroptosis activation in PCOS rat models and in placental tissue from spontaneous fetal loss. Despite the rather well investigated association between ferroptosis and PE, only few recent studies emphasize a potential

role of ferroptosis in GDM. These, however, confirm the numerous observations relating increased maternal iron levels to the risk to develop GDM. Within the past few years, various studies emerged describing an interrelation between hyperglycemia, placental lipid metabolism, maternal iron status and oxidative stress, which could be due to effects or consequences of ferroptosis. These observations further suggest a synergistic mechanism of pre-pregnancy and gestational obesity that increases sub-clinical inflammation, lipid peroxidation in trophoblasts and hence initiates a ferroptotic impairment of human pancreatic islet function leading to gestational hyperglycemia. Furthermore, there is experimental evidence that therapeutical balancing of gestational redox homeostasis by application of antioxidants, such as selenium, or targeting SIRT3 may represent a potential therapeutic target for GDM.

Assessing the severity of the diseases and a subsequent systematic correlation analysis of already existing clinical cohorts would allow to better estimate the risk of gestational iron supplementation. Furthermore, a definition and standardization of biomarkers to measure ferroptosis, could act as fundamental prerequisite for more targeted experimental studies to subsequently prove causality. Finally, there is still the open question, whether and under which circumstances iron supplementation in pregnancy could trigger ferroptosis and hence may contribute to PE, GDM, preterm birth or even fetal demise. Given the broad application of iron supplementation in women independent of diagnostic evidence of iron deficiency and the herein described putative ferroptosis-driven relationship between maternal iron status and frequent pregnancy diseases, it is recommended to carefully prescribe iron supplementation especially to pregnant women at high risk for PE or GDM.

5   CRediT authorship contribution statement

Jonas Zaugg: Conceptualization, visualization, writing – original draft. **Fabia Solenthaler**: Conceptualization, writing – original draft. **Christiane Albrecht**: Supervision, resources, conceptualization & editing.

6   Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

7   Acknowledgements

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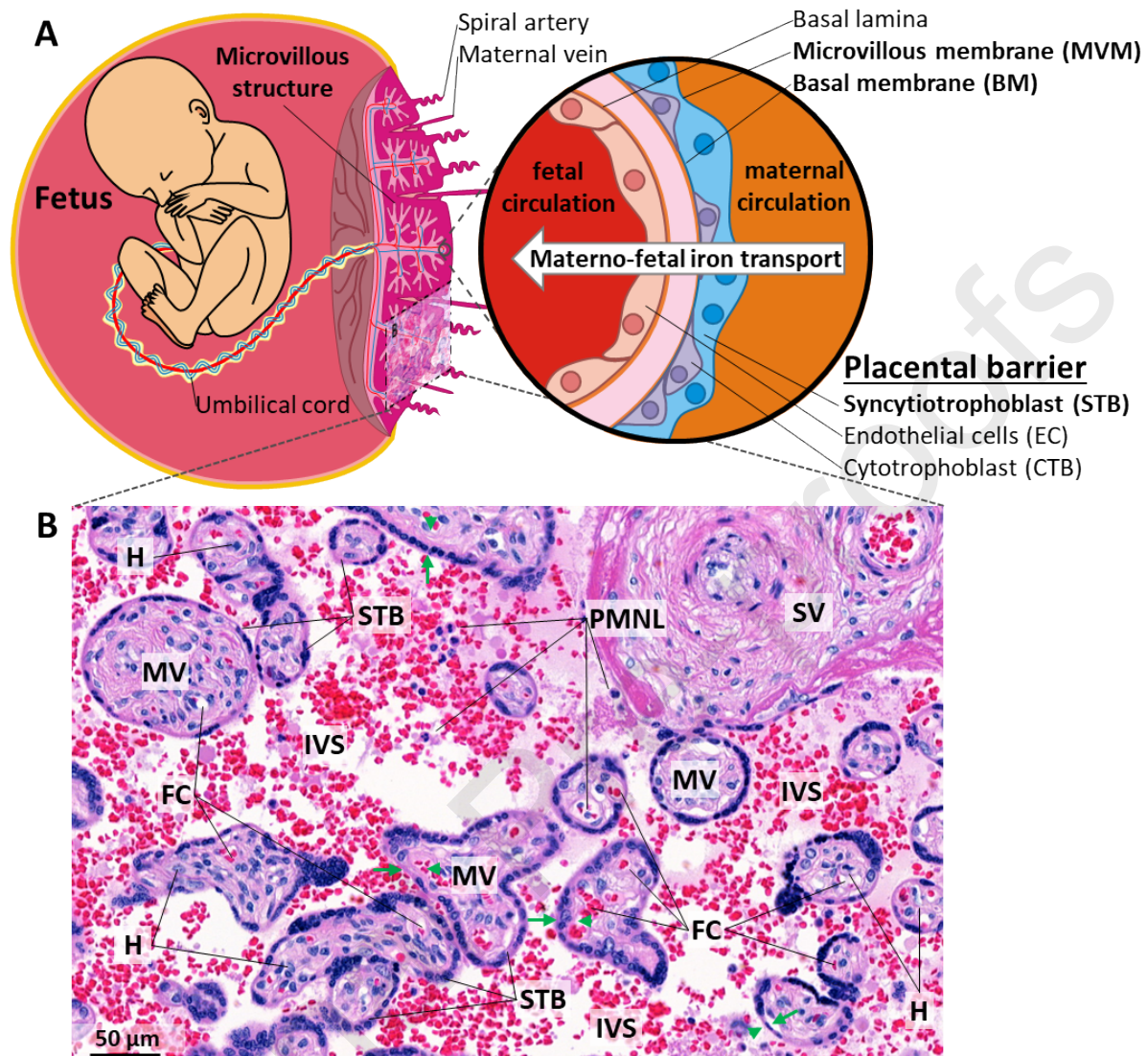
9 Figures

Figure 1: Schematic and histological representation of the human placenta as interface between the developing fetus and the maternal circulation. A) *left panel*, scheme of a developing fetus connected via the umbilical cord to the placenta. Cross section of the placental disk revealing fetal villous structures and the maternal interface connecting the intervillous space via spiral arteries and maternal veins to the uterine blood supply. **A)** *right panel*, closeup of the human placental barrier in a chorionic villous as interface between the fetal capillaries (fetal blood compartment in red) and the maternal circulation (maternal blood compartment in orange). The human placental barrier involves three different cell types: endothelial cells (EC, bright orange) lining the fetal capillaries, single-nucleated cytotrophoblasts (CTB, violet) and syncytiotrophoblasts (STB, blue) forming a multi-nucleated

monolayer. The STB are in direct contact with maternal blood and mainly responsible for materno-fetal iron/nutrient transport and feto-maternal CO_2 and waste product exchange. Iron is transported unidirectional only in materno-fetal direction (white arrow). **B)** Histological evaluation of a haematoxylin and eosin (HE) stained placenta cross-section at term (20 x magnification) as indicated by the dashed box in panel A. The section through the chorionic plate depicts large villi with condensed collagen fibers (fibers in magenta) in the stroma called stem villi (upper right structure), but also small and intermediate villi that are long and slender with numerous capillaries. The circular haematoxylin-positive structures are cross-sections of the finger-like villi, which are growing into the maternal endometrial wall of the uterus. There are small and intermediate villi with multinucleated STB at the borders and fetal capillaries (haematoxylin-positive nuclei in dark blue), stromal and Hofbauer cells in the microvillous core, but also numerous erythrocytes (purple cells) in the intervillous space (IVS). Green arrows indicate the apical microvillous membrane (MVM), i.e. maternal blood orientated side; green arrowheads depict the basal membrane (BM) of the STB. Abbreviations: SV, stem villi; MV, microvilli; IVS, intervillous space; STB, syncytiotrophoblast; PMNL, polymorphonuclear leukocytes; H, Hofbauer cell; FC, fetal capillary.
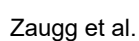
Figure 2: Schematic representation of the mechanisms and regulation of materno-fetal iron transfer across the placenta. Iron (Fe, *ferrum*) is transferred unidirectionally from the mother to the fetus across the blood-placenta barrier (right to left). The placenta is in direct contact with the maternal blood circulation via a tight monolayer of syncytialized trophoblast cells (syncytiotrophoblasts, STB in blue). **A)** Transferrin (Tf)-mediated iron transfer is initiated by transferrin receptor (TFR1)-binding of di-ferric transferrin (2 Fe^{3+}-Tf) followed by clathrin-dependent endocytosis at the maternal side of the STB (microvillous membrane, MVM) into endosomes. Due to endosomal acidification ferric iron (Fe^{3+}, red) is released from Tf, followed by reduction to the ferrous state (Fe^{2+}, yellow) presumably by the metalloreductases STEAP3 or STEAP4. The divalent metal transporter 1 (DMT1) is supposed to transport Fe^{2+} proton (H^+)-dependently from endosomes into the cytosol. Transferrin (Tf) and TFR1 return to the apical microvillous membrane (MVM) to be used for further cycles. Cytosolic iron is transferred to the fetal circulation through the iron exporter ferroportin (FPN1) or stored intracellularly in oxidized form bound to ferritin (FTH). After crossing the the basal membrane (BM), Fe^{2+} is oxidized by either ceruloplasmin (CP), hephaestin (HEPH) or zyklopen (ZP/HEPHL1). Finally, Fe^{3+} can be bound by Tf and further distributed via the fetal circulation towards the fetus. Human poly (rC) binding protein 2 (PCBP2) is an iron chaperone that may modulate iron release across the BM and may protect fetal tissue by delivery of oxidative ferrous iron from DMT1 to FPN1 or ferritin (dotted line). **B)** Tf-independent iron transfer: Although there is apical expression of lipoprotein receptor-related protein 1 (LRP1) mediating hemopexin (HPX)-bound heme endocytosis, heme carrier protein 1 (HCP1) and heme-responsive gene 1 (HRG1), free heme may be exported directly towards the maternal or fetal side by feline leukemia virus subgroup C receptor 1 or 2 (FLVCR1/2) or converted to inorganic Fe by heme oxygenase in the endoplasmic reticulum and exported to the fetal circulation by FPN1. The exact mechanisms as well as the role of other apically expressed Tf-independent transporters such as the Zrt- and Irt-like proteins (ZIP) ZIP8 or ZIP14 in transplacental iron transfer are currently still unclear. The relevance of heme iron transport across the placenta and the possibility of reverse transfer of iron into the maternal circulation need further investigation. Similarly speculative is the

function of scavenger receptor class A member 5 (SCARA5) potentially mediating the binding and endocytosis of serum ferritin. **C)** Placental iron transfer regulation: Placental and/or fetal hepcidin (HEPC) likely acts as sensor of the placental iron status and initiates FPN1 degradation at the BM of STB in the placenta and in maternal enterocytes (dashed-dotted line). The binding of iron regulatory protein 1 (IRP1) to 3'-IRE promotes mRNA expression of TFR1 and DMT1, whereas binding to 5'-IREs prevents mRNA translation of FTH and FPN1 thereby affecting iron storage and export (dashed line).

A **Availability of redox-active iron**

B **Oxidation of polyunsaturated fatty acids**

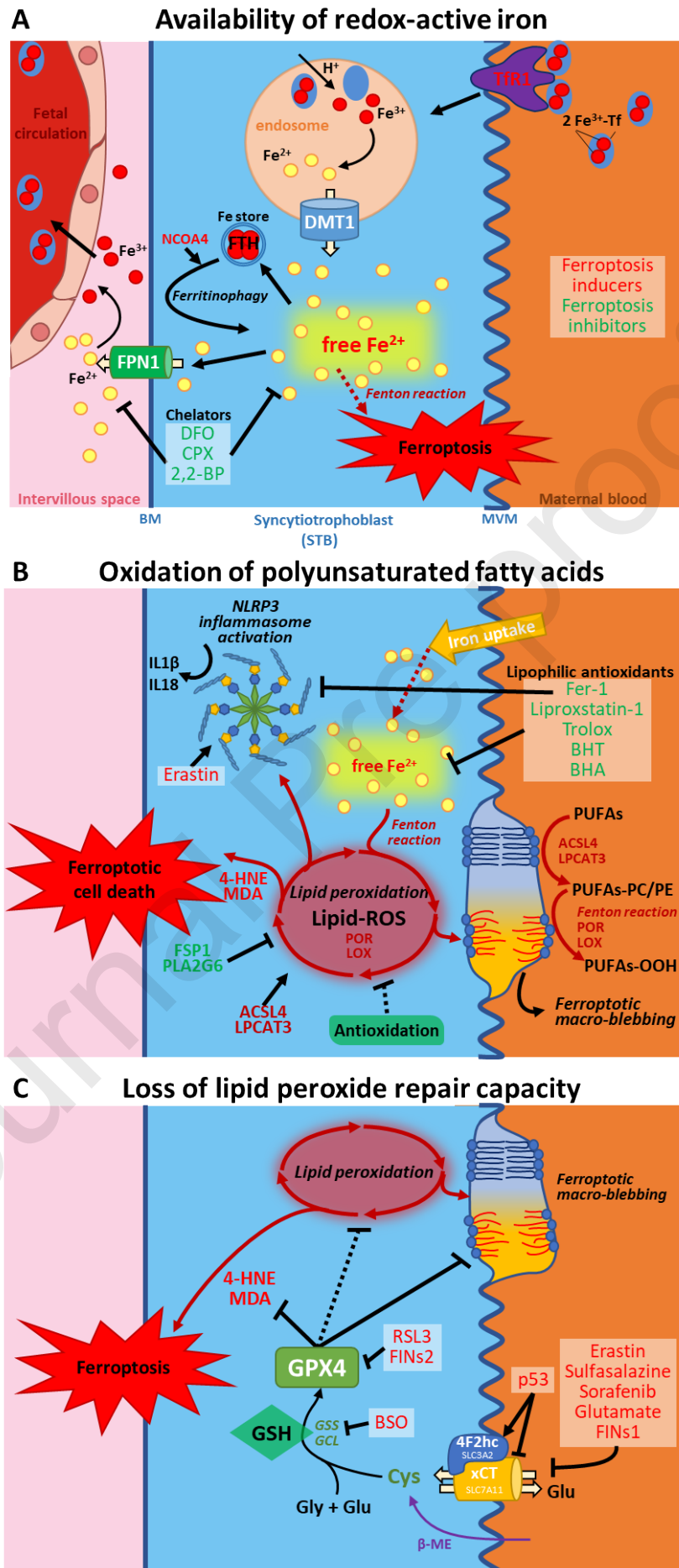**C** **Loss of lipid peroxide repair capacity**

Figure 3: Redox-active iron, oxidation of polyunsaturated fatty acids and loss of lipid peroxide repair capacity as the three hallmarks of placental ferroptosis. A) The availability of redox-active iron is the first hallmark of placental ferroptosis Transferrin (Tf)-bound ferric iron (Fe^{3+}, red circles) is imported into the cell by transferrin receptor (TFR1) via endocytosis across the microvillous membrane (MVM) and released as ferrous iron (Fe^{2+}, yellow) into a labile intracellular iron pool by divalent metal transporter 1 (DMT1) or other alternative transporters (as shown in Figure 2B). Free Fe^{2+} is highly oxidative and hence produces hydroxyl radicals by the Fenton reaction leading to ferroptosis. To counteract this process, cytosolic iron can be stored in ferritin (FTH), exported out of the cell across the basal membrane (BM) by ferroportin 1 (FPN1) or re-oxidized to ferric iron (Fe^{3+}, red circles) by various placental ferroxidases. The highly oxidative Fe^{2+} can be neutralized by iron chelators such as deferoxamine (DFO), ciclopirox (CPX) and 2,2-bipyridyl (2,2-BP). **B)** The self-amplifying oxidation of polyunsaturated fatty acids (PUFA) is defined as second hallmark of placental ferroptosis. PUFA-containing membrane phospholipids undergo lipid peroxidation, which destroys the cellular membrane. Thereby PUFA are activated by acyl-CoA synthetase long-chain family member 4 (ACSL4) and eventually incorporated into phosphatidylethanolamine (PE) and phosphatidylcholine (PC) by lysophophatidylcholine acyltransferase 3 (LPCAT3). Among others, PUFA-PE/PC can be oxidized by free Fe^{2+} through the Fenton reaction, by cytochrome P450 oxidoreductase (POR) or lipoxygenase (LOX) to phospholipid peroxides (PUFA-OOH). The excessive accumulation of hydroperoxidized PUFA-OOH could lead to membrane instability and, in combination with osmotic pressure, to ferroptotic macro-blebbing. PUFA-OOH in turn can oxidize new PUFA starting a self-amplifying chain reaction, which produces 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA) finally leading to ferroptotic cell death. Various peroxides such as PUFA-OOH can be reduced to their corresponding alcohols by lipophilic antioxidants such as ferrostatin-1 (Fer-1), liproxstatin-1, α-tocopherol (Trolox), butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) or further through endogenous ferroptosis suppressor protein 1 (FSP1) and phospholipase PLA2G6. Beside ferroptosis, oxidative stress induces NLRP1-

inflammasome expression activating inflammatory interleukin IL-1β/IL-18 and membrane pore-forming gasdermin-D that finally leads to pyroptosis. **C)** The loss of lipid peroxide repair capacity is described as third hallmark of placental ferroptosis. The main peroxide repair capacity is sustained by glutathione (GSH). Glutathione peroxidase 4 (GPX4) protects cells from ferroptosis through reducing PUFA-OOH by oxidizing GSH. GPX4 can be inhibited by Ras-selective lethal molecule 3 (RSL3) and other class 2 ferroptosis-inducing compounds (FINs2). GSH is built from cysteine by glutamate-cysteine ligase (GCL), which can be inhibited by buthionine sulfoximine (BSO). Cysteine is imported into the cell as cystine through the cystine/glutamate antiporter system x_c^-, a transmembrane protein of two subunits, xCT/SLC7A11 and 4F2hc/SLC3A2. xCT can be inhibited by erastin, sulfasalazine, sorafenib, high extracellular glutamate levels and other class 1 ferroptosis-inducing compounds (FINs1), as well as by protein p53. β-mercaptoethanol (β-ME) can activate an alternative cystine import pathway. Ferroptosis inducers are marked in red, ferroptosis inhibitors in green. Wavy blue lines represent the apical cell membrane, straight blue lines the basal cell membrane at the placental barrier.

Graphical abstract
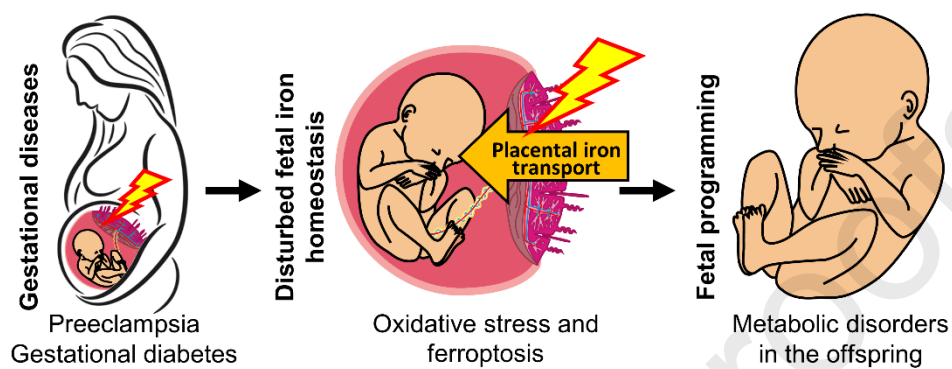
Materno-fetal iron transfer and the emerging role of ferroptosis pathways

By Jonas Zaugg [a,b], Fabia Solenthaler [a,b], & Christiane Albrecht [* a,b]

10 CRediT authorship contribution statement

Jonas Zaugg: Conceptualization, visualization, writing – original draft. **Fabia Solenthaler**: Conceptualization, writing – original draft. **Christiane Albrecht**: Supervision, resources, conceptualization & editing.

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Declaration of interest statement

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Declarations of interest: none