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Studies on the regulation of iron homeostasis during pregnancy using mouse (*Mus musculus*) and pigs (*Sus scrofa*) models.

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życzliwą i przyjacielską atmosferę w godzinach pracy i po za nią.

Wspomnienia, które zostaną na całe życie.

za bycie „**Doktorem**”

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za niezapomniane wrażenia z wyjazdów na doświadczenia,

za bycie „**Profesorem**”

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List of publications constituting a doctoral dissertation

1. Effect of oral supplementation of healthy pregnant sows with sucrosomial ferric pyrophosphate on maternal iron status and hepatic iron stores in newborn piglets.
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2. Marginally reduced maternal hepatic and splenic ferroportin under severe nutritional iron deficiency in pregnancy maintains systemic iron supply.
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Summary

Iron homeostasis plays a pivotal role throughout pregnancy, serving as a cornerstone for sustaining the heightened requirements essential for optimal foetal development and maintaining maternal physiological equilibrium. The dynamic interplay between iron uptake, utilization, and storage undergoes intricate regulation during this critical period to ensure an adequate supply of this essential micronutrient for both the developing foetus and the maternal organism. The regulatory mechanisms orchestrating iron balance during pregnancy are multifaceted, encompassing a myriad of molecular players and signalling pathways. At the forefront of this regulatory network is the peptide hormone hepcidin, which acts as a master regulator of systemic iron homeostasis by modulating the activity of the iron exporter ferroportin (FPN), thereby controlling iron absorption from the diet and its release from cellular stores. Moreover, the expression and activity of other key iron-handling proteins, such as transferrin receptor 1 (TfR1) and ferritin, are finely tuned to accommodate the increased demands imposed by the growing foetus while maintaining maternal iron reserves. Within the intricate landscape of maternal-foetal iron transfer, the placenta emerges as a pivotal site of exchange, facilitating the transport of iron from the maternal circulation to the developing foetus. This process involves a delicate interplay between various transporters, including transferrin (Tf), transferrin receptor 1(TfR1), and divalent metal transporter 1 (DMT1), ensuring the efficient delivery of iron to foetal tissues while safeguarding maternal iron homeostasis. The use of animal models, such as mice (*Mus musculus*) and pig (*Sus scrofa*), provides invaluable insights into the regulation of iron homeostasis during pregnancy.

The aim of the study on the regulation of iron homeostasis during pregnancy using mouse (*Mus musculus*) and pig (*Sus scrofa*) models was to comprehensively investigate and understand the intricate mechanisms governing iron balance in maternal-foetal physiology. The doctoral dissertation includes three publications in indexed scientific journals.

First publication aimed to investigate the effects of a new form of iron supplement, called sucrosomial ferric (SFP), on iron status of pregnant sows and their offspring. Pigs are often used in research because they share similarities with humans in terms of

physiology and genetics, making them useful models for studying nutrition in pregnancy. During the last trimester, both humans and pigs experience a rapid increase in the need for iron, mainly due to the increase of red blood cells pool. Iron deficiency during pregnancy can lead to health disturbances for both the mother and the newborn. The study compared the effects of SFP with a traditional iron supplement, ferrous sulphate, on pregnant sows. We found that neither supplement affected the iron levels or overall health of the pregnant pigs. Additionally, there were no differences in iron levels in the milk or colostrum of supplemented pigs compared to those not given supplements, the iron status of the newborn piglets did not show improvement with maternal supplementation, and there were no significant differences in the weight or number of piglets born between the groups. Furthermore, the study examined the expression of iron transporters in the placenta, which are crucial for delivering iron to the developing foetus. The expression of these transporters varied depending on the type of iron supplement given to the pregnant pigs. Overall, the study suggests that while SFP and ferrous sulphate did not have adverse effects on the pregnant pigs, they also did not significantly improve the iron status of their offspring.

The second study focused on understanding the mechanisms of iron transfer in pregnant mice with iron deficiency. In anaemic pregnant individuals, the available iron is prioritized for the foetus and placenta, leading to potential health risks for both the mother and the developing offspring. Using a mouse model of iron deficiency during pregnancy, the aim was to understand how iron is sourced and transported in this condition. It was found that iron recycled from aging red blood cells becomes a primary source of this essential element, particularly in pregnant individuals with depleted iron stores. Ferroportin, a key protein involved in iron release from cells, was significantly reduced in the duodenum, placenta, and foetal liver under conditions of iron deficiency during pregnancy. However, this reduction was not observed in maternal liver macrophages or in the spleen. Despite low levels of hepcidin, which would typically predict an increase in ferroportin expression, the final expression level of ferroportin was likely influenced by tissue iron levels. This suggests that iron released into the bloodstream of anaemic pregnant individuals is taken up by the placenta, as indicated by increased expression of iron importers. However, a significant decrease in ferroportin

levels on the basolateral side of placental cells, may lead to reduced iron transfer to the foetus. As a result, some iron is retained in the placenta, indicating a complex regulation of iron turnover in iron-deficient pregnant mice and their foetuses. These findings underscore the critical role of ferroportin in modulating iron dynamics during pregnancy, particularly in conditions of iron deficiency, and provide insights into potential mechanisms underlying maternal-foetal iron transfer.

The last article aimed to critically assess the effectiveness of iron supplementation in pregnant pigs to prevent iron deficiency anaemia (IDA) in their offspring. IDA is a common condition in piglets during the early postnatal period, leading to poor growth and increased mortality. The main reason for IDA in piglets is insufficient iron stores in their livers, which are crucial for meeting their iron needs in the first weeks of life. This deficiency often stems from inadequate iron transfer from the pregnant sow to the foetuses through the placenta. To address this issue, various methods of iron supplementation in pregnant sows have been attempted to enhance placental iron transfer and increase iron levels in foetal livers. Both oral and injectable approaches have been explored over the years, aiming to improve the red blood cell indices of piglets. However, there is ongoing debate about the effectiveness of such supplementation in preventing IDA in newborn piglets. Moreover, caution must be exercised to avoid over-supplementation, which can lead to iron toxicity. In essence, this article serves as a critical review and evaluation of the practice of iron supplementation in pregnant sows as a means to prevent IDA in piglets. It weights the evidence surrounding the efficacy of supplementation, considering factors such as the method of administration, dosage, and potential risks associated with over-supplementation. Ultimately, the goal is to provide insights into optimizing iron supplementation strategies in pregnant sows to ensure the health and well-being of their offspring.

The dissertation provides valuable insights into the complex mechanisms of iron homeostasis during pregnancy, highlighting the challenges of optimizing iron supplementation to support both maternal health and foetal development. While the studies contribute significantly to understanding iron dynamics, they also underscore the need for further research to refine supplementation strategies, ensuring effective prevention of iron deficiency without risking adverse effects.

Streszczenie

Homeostaza żelaza odgrywa kluczową rolę w trakcie ciąży, stanowiąc fundament dla utrzymania zwiększonych wymagań niezbędnych dla optymalnego rozwoju płodu oraz zachowania równowagi fizjologicznej. Dynamiczna interakcja między pobieraniem, wykorzystaniem i przechowywaniem żelaza podlega skomplikowanej regulacji w tym krytycznym okresie, aby zapewnić odpowiednie zaopatrzenie w ten niezbędny mikroelement zarówno rozwijającego się płodu, jak i organizmu matki. Mechanizmy regulacyjne równowagi żelaza w ciąży są wielopłaszczyznowe, obejmując rozległą gamę cząsteczek i szlaków sygnałowych. Na czele tej sieci regulacyjnej znajduje się peptydowy hormon hepcydyna, który działa jako główny regulator homeostazy żelaza poprzez modulowanie eksportera żelaza, ferroportyny, kontrolując w ten sposób uwalnianie żelaza z komórek w tym z enterocytów odpowiedzialnych za wchłanianie żelaza z diety. Ponadto, ekspresja i aktywność innych kluczowych białek zarządzających metabolizmem żelaza, takich jak receptor transferyny 1 (TfR1) i ferrytyna (Ft), są precyzyjnie kontrolowane, aby sprostać zwiększonym wymaganiom narzuconym przez rosnący płód, jednocześnie utrzymując rezerwy żelaza u matki. W złożonym mechanizmie transferu żelaza między matką a płodem, łożysko wyłania się jako kluczowe miejsce wymiany, ułatwiając transport żelaza z krążenia matki do rozwijającego się płodu. Proces ten obejmuje złożoną interakcję między transporterami, w tym transferyną, TfR1 i transporterem metali dwuwartościowych1 (DMT1), zapewniając efektywne dostarczenie żelaza do tkanek płodu, jednocześnie podtrzymując homeostazę żelaza u matki. Wykorzystanie modeli zwierzęcych, takich jak mysz (*Mus musculus*) i świnia (*Sus scrofa*), dostarcza cennych wskazówek dotyczących regulacji homeostazy żelaza w trakcie ciąży.
Celem badania nad regulacją homeostazy żelaza w trakcie ciąży, przy użyciu modeli myszy i świni, było wszechstronne zbadanie i zrozumienie skomplikowanych mechanizmów rządzących równowagą żelaza w fizjologii matki i płodu.
Rozprawę doktorska stanowi zbiór trzech spójnych tematycznie artykułów opublikowanych w indeksowanych czasopismach naukowych.

Pierwsza publikacja miała na celu zbadanie efektów nowej formy suplementu żelaza, zwanego żelazem sukrozomialnym (SFP), na poprawę statusu żelaza ciężarnych loch i

ich potomstwa. Świnie są często wykorzystywane w badaniach, ponieważ dzielą podobieństwa z ludźmi pod względem fizjologii i genetyki, co czyni je użytecznymi modelami do badania żywienia w ciąży. Podczas ostatniego trymestru ciąży zarówno ludzie, jak i świnie doświadczają szybkiego wzrostu zapotrzebowania na żelazo, głównie ze względu na wzrost liczby czerwonych krwinek. Niedobór żelaza w ciąży może prowadzić do problemów zdrowotnych zarówno u matki, jak i noworodka. Porównano efekty SFP z tradycyjnym suplementem żelaza, siarczanem żelaza, u ciężarnych loch. Stwierdzono, że żaden z suplementów nie wpłynął na poziom żelaza ani na ogólny stan zdrowia ciężarnych świń. Ponadto nie było różnic w poziomach żelaza w mleku ani siarze u świń suplementowanych w porównaniu z tymi, którym nie podawano suplementów, status żelaza noworodków nie uległ poprawie w wyniku suplementacji matek, nie było istotnych różnic w masie ciała ani w liczbie urodzonych prosiąt między grupami. Ponadto porównano ekspresję transporterów żelaza w łożysku, które są kluczowe dla dostarczania żelaza do rozwijającego się płodu. Ekspresja tych transporterów różniła się w zależności od rodzaju suplementu żelaza podawanego ciężarnym świniom. Wyniki sugerują, że choć SFP i siarczan żelaza nie wywołały negatywnych skutków u ciężarnych świń, to również nie poprawiły istotnie stanu żelaza ich potomstwa.

Druga publikacja skupiła się na zrozumieniu mechanizmów transferu żelaza u ciężarnych myszy z niedoborem żelaza. U ciężarnych anemicznych samic dostępne żelazo jest priorytetowo przekazywane dla płodu i łożyska, co prowadzi do potencjalnych zagrożeń zdrowotnych zarówno dla matki, jak i rozwijającego się potomstwa. Celem badań przy wykorzystaniu mysiego modelu niedoboru żelaza w ciąży, było zrozumienie mechanizmów pozyskiwania i transportowania żelaza. Stwierdzono, że żelazo odzyskane z starzejących się czerwonych krwinek przez makrofagi i przekierowane przez te komórki do ponownego użycia staje się głównym źródłem tego istotnego pierwiastka, zwłaszcza u ciężarnych z niedoborem żelaza. Ekspresja ferroportyny, kluczowego białka zaangażowanego w uwalnianie żelaza z komórek, była istotnie zmniejszona w dwunastnicy, łożysku i wątrobie płodowej w warunkach niedoboru żelaza w ciąży. Jednakże to zmniejszenie nie zostało zaobserwowane w makrofagach wątroby matki ani w śledzionie. Pomimo niskich

poziomów hepcydyny, hormonu regulującego poziomy żelaza, co powinno wskazywac na zwiększenie ekspresji ferroportyny, ostateczny poziom ekspresji ferroportyny był prawdopodobnie regulowany przez poziom żelaza w tkankach. Sugeruje to, że żelazo uwalniane do krwiobiegu u ciężarnych myszy z niedoborem żelaza jest pobierane przez łożysko, co wskazuje na zwiększoną ekspresję importerów żelaza. Jednakże istotne zmniejszenie poziomu ferroportyny po stronie bazolateralnej komórek łożyska może prowadzić do zmniejszonego transferu żelaza do płodu. W rezultacie część żelaza zatrzymywana jest w łożysku, co wskazuje na złożoną regulację obrotu żelaza u anemicznych ciężarnych myszy i ich płodów. Te wyniki podkreślają kluczową rolę ferroportyny w regulacji dynamiki żelaza w ciąży, zwłaszcza w warunkach niedoboru żelaza, i dostarczają wiedzy o potencjalnych mechanizmach leżących u podstaw transferu żelaza między matką a płodem.

Ostatni artykuł miał na celu krytyczną ocenę skuteczności suplementacji żelaza u ciężarnych świń w celu zapobiegania niedokrwistości z niedoboru żelaza (IDA) u ich potomstwa. IDA jest powszechnym schorzeniem u prosiąt w okresie noworodkowym, prowadzącym do spowolnionego wzrostu i zwiększonej śmiertelności. Główną przyczyną IDA u prosiąt są niewystarczające zapasy żelaza w wątrobie, które mają kluczowe znaczenie dla zaspokojenia ich zapotrzebowania na żelazo w pierwszych tygodniach życia. Ten niedobór często wynika z niewystarczającego transferu żelaza od ciężarnej lochy do płodów poprzez łożysko. Aby zapobiec temu zjawisku, podjęto różne metody suplementacji żelaza u ciężarnych loch w celu zwiększenia transferu żelaza przez łożysko i zwiększenia poziomów żelaza w wątrobie płodów. Na przestrzeni lat, zarówno podanie doustne, jak i iniekcje były badane w celu poprawy wskaźników czerwonokrwinkowych u prosiąt. Ponadto istotne przy suplementacji preparatami żelaza jest to , aby uniknąć przedawkowania, co może prowadzić do toksyczności żelaza. Ten artykuł stanowi krytyczną recenzję i ocenę suplementacji żelaza u ciężarnych loch jako środka zapobiegającego IDA u prosiąt. W tej publikacji zebrano dane dotyczące skuteczności suplementacji, uwzględniając czynniki takie jak sposób podawania, dawkowanie i potencjalne ryzyko związane z nadmierną suplementacją. Ostatecznym celem doświadczenia jest dostarczenie wglądu w optymalizację strategii

suplementacji żelaza u ciężarnych loch w celu zapewnienia zdrowia i dobrostanu ich potomstwa.

Praca doktorska dostarcza cennych informacji na temat złożonych mechanizmów homeostazy żelaza w trakcie ciąży, podkreślając wyzwania związane z optymalizacją suplementacji żelaza w celu wsparcia zarówno zdrowia matki, jak i rozwoju płodu. Choć badania znacząco przyczyniają się do zrozumienia dynamiki żelaza, wskazują również na potrzebę ich kontynuacji w celu udoskonalenia strategii suplementacji, aby skutecznie zapobiegać niedoborom żelaza bez ryzyka efektów ubocznych .

Introduction

Iron homeostasis in pregnancy encompasses a multifaceted process that ensures the optimal supply and utilization of iron to meet the increased demands of maternal physiology and foetal development. Iron is an indispensable micronutrient vital for various physiological functions, including oxygen transport, DNA synthesis, and enzymatic reactions[1]. Throughout pregnancy, the maternal body undergoes dynamic changes to accommodate the growing needs of both the mother and the developing foetus, and iron plays a central role in supporting these changes[2]. One of the primary challenges in maintaining iron homeostasis during pregnancy is the significant increase in iron requirements. This surge in demand stems from several factors, including the expansion of maternal blood volume, the development of the placenta, and the growth of foetal tissues[3]. Additionally, iron is essential for the synthesis of haemoglobin, the oxygen-carrying component of red blood cells, which undergoes a substantial increase to support the growing oxygen needs of both the mother and the foetus[4]. To meet these heightened demands, the maternal body employs a series of adaptive mechanisms to enhance iron absorption, transport, and utilization[5].

Overview of iron metabolism regulation.

Dietary iron is absorbed primarily in the duodenum and upper jejunum of the small intestine. Iron exists in two forms: heme iron, found in animal products, and non-heme iron, present in plant-based foods[6]. Heme iron is more efficiently absorbed than non-heme iron, which is influenced by various dietary factors. The absorption process starts with the reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) by duodenal cytochrome b (Dcytb) on the apical surface of enterocytes[7]. Divalent metal transporter 1 (DMT1) then transports ferrous iron across the apical membrane into the enterocyte[8,9]. Heme iron is absorbed intact by heme carrier protein 1 (HCP1) and subsequently degraded by heme oxygenase 1 (HO1) to release ferrous iron within the enterocyte[10]. Once inside the enterocyte, iron can either be stored in the form of ferritin or exported into the bloodstream. The export of iron is mediated by ferroportin, the only known iron exporter, located on the basolateral membrane of enterocytes[11]. Hephaestin or ceruloplasmin oxidizes the exported ferrous iron back to ferric iron, which then binds to transferrin, the primary iron transport protein in the blood[12]. Schematic duodenal iron

absorption is shown in the Figure 1.

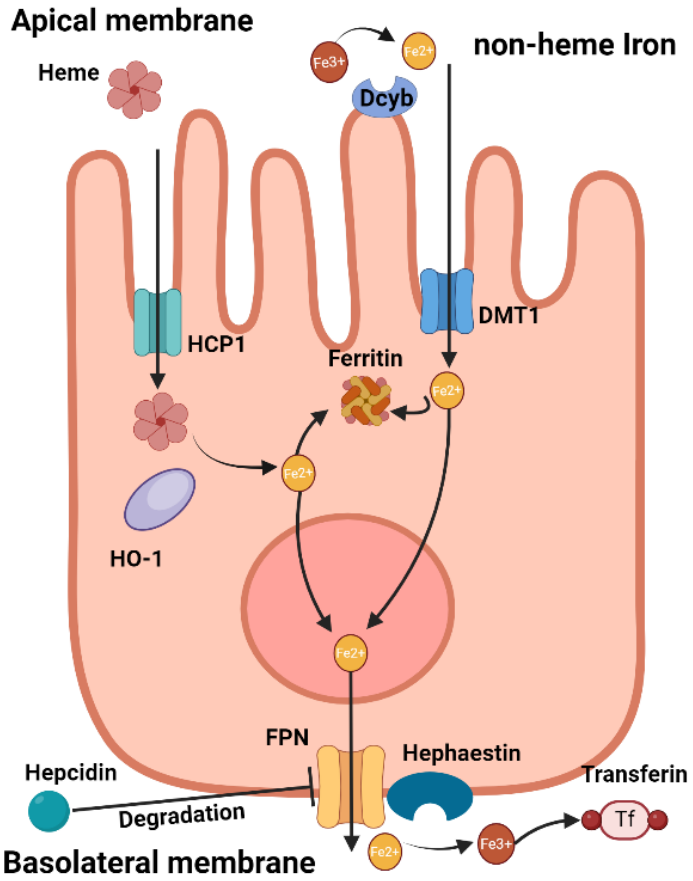

Figure 1. Dietary Iron Absorption.
Ferric iron (Fe3+) is reduced to ferrous iron (Fe2+) in the intestinal lumen by ferrireductase. Ferrous iron (Fe2+) is transported across the enterocyte by divalent metal ion transporter-1 (DMT-1). Dietary heme iron is transported across the enterocyte by heme carrier protein 1 (HCP1). Inside the enterocyte, heme is acted upon degraded ? enzymatically decomposed ? by heme oxygenase, which releases ferrous iron from the protoporphyrin in heme molecule. The released iron enters the same pool as dietary non-heme iron. A portion of this pool is stored as ferritin inside the enterocyte, The remainder of the ferrous iron is transported across the basolateral membrane of the enterocyte by ferroportin. The transported Fe2+ is oxidized back to the Fe3+ form by hephaestin, multicopper ferroxidase. Fe3+ is then incorporated into transferrin in the serum. Hepcidin regulates iron transport into serum by causing internalization of ferroportin with subsequent lysosomal breakdown inside the enterocyte. To nie jest zaznaczone na Ryc moze dodac hepc ? Adapted from Ilyas SI, Schoen RE. Supplementation With Oral vs Intravenous Iron for Anemia With IBD or Gastrointestinal Bleeding: Is Oral Iron Getting a Bad Rap? https://doi.org/10.1038/ajg.2011.232.

Transferrin-bound iron circulates in the bloodstream and is delivered to cells via transferrin receptors (TfR1 and 2) on the cell surface[13]. The iron-transferrin-TfR complex is internalized through receptor-mediated endocytosis. Within the acidic environment of the endosome, iron is released from transferrin and transported into the cytoplasm by DMT1[14]. Excess iron is stored in the liver and spleen in the form of ferritin, a protein complex capable of sequestering up to 4500 iron atoms[15]. Ferritin

serves as a reserve to be mobilized during periods of increased demand or iron deficiency. In cases of iron overload, hemosiderin, a denatured form of ferritin, may accumulate. The body efficiently recycles iron from senescent red blood cells. Macrophages in the spleen and liver phagocytose aged erythrocytes, breaking them down and releasing iron for reuse[16]. This recycled iron is a significant source of iron for erythropoiesis, the production of new red blood cells[16]. The hepcidin-ferroportin axis is central to the regulation of systemic iron homeostasis. Hepcidin, a peptide hormone produced by the hepatocytes, regulates iron absorption and distribution in the body by binding to ferroportin, leading to its internalization and degradation[17]. This process reduces iron efflux from enterocytes, macrophages, and hepatocytes, thereby lowering the amount of iron entering the circulation. Hepcidin expression is regulated by several factors, ensuring that iron homeostasis is maintained. Elevated body iron levels increase hepcidin expression to prevent further iron absorption and promote iron sequestration. Inflammatory cytokines, such as interleukin-6 (IL-6), stimulate hepcidin production, leading to hypoferremia[18].

Conversely, increased erythropoietic activity, as seen in anaemia or hypoxia, suppresses hepcidin expression to enhance iron availability for red blood cell production[14].
The simplified schematic of iron transport and regulation is shown in Figure 2.

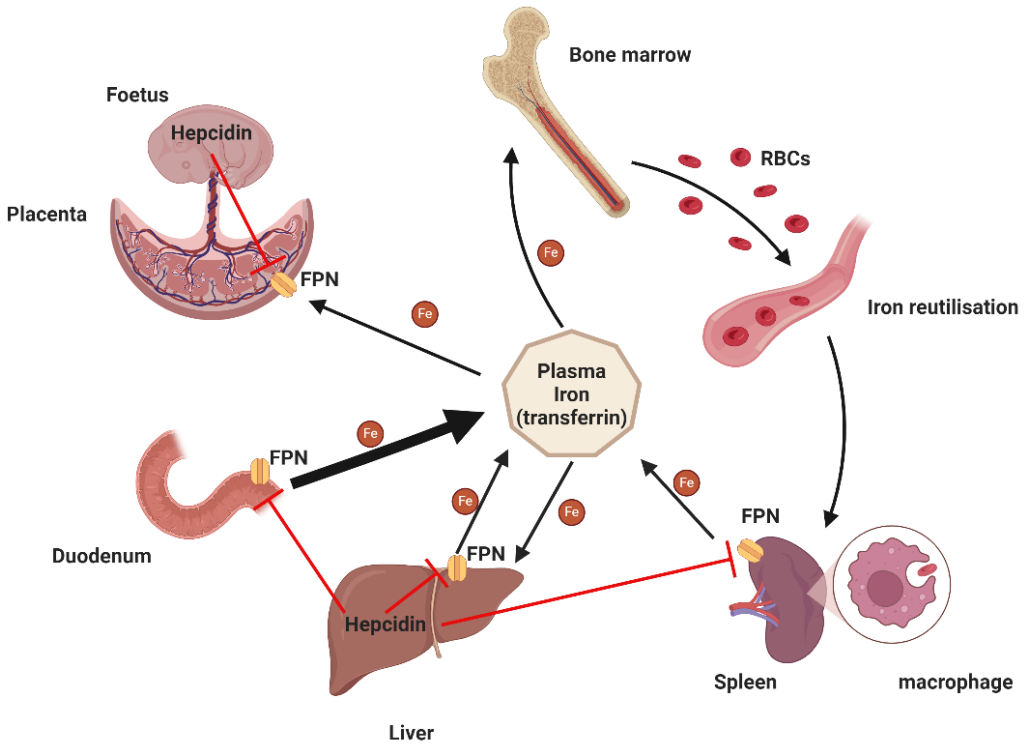

Figure 2 Iron Metabolism Overview.
Figure illustrates the systemic regulation of iron metabolism, highlighting key processes and sites of iron absorption, recycling, storage, and transport. Dietary iron is absorbed in the duodenum via ferroportin (FPN), regulated by hepcidin, reducing iron absorption when body iron levels are sufficient. Hepcidin, produced by the liver, controls iron efflux from enterocytes, macrophages, and hepatocytes by degrading FPN, limiting iron entry into the plasma. Plasma iron is bound to transferrin and distributed to tissues, including the bone marrow for erythropoiesis. Macrophages in the spleen and liver recycle iron from senescent RBCs, releasing it back into circulation via FPN. Excess iron is stored as ferritin in the liver, spleen, and bone marrow. The placenta transfers iron to the foetus via FPN. Foetal hepcidin may regulate foetal iron acquisition. Adapted from Fisher. A, Iron homeostasis during pregnancy, https://doi.org/10.3945/ajcn.117.155812

Placental Iron Regulation.

Iron homeostasis during pregnancy is a finely tuned process, critical to both maternal health and foetal development. As the central organ facilitating nutrient and gas exchange between the mother and the foetus, the placenta plays a pivotal role in regulating iron transport. Iron is an essential micronutrient for cellular processes such as oxygen transport, DNA synthesis, and energy metabolism, making its regulation vital during the rapid growth phases of pregnancy. Placental iron regulation encompasses a series of complex mechanisms and feedback loops involving maternal, placental, and foetal signalling to ensure both mother and foetus to receive adequate amount of iron while preventing toxicity from iron overload.

The majority of iron in maternal circulation is bound to the protein transferrin, which can carry up to two ferric iron ions (Fe^{3+}). The transferrin-iron complex circulates in the maternal bloodstream and binds to transferrin receptor 1[19] (TfR1), which is highly expressed on the surface of the syncytiotrophoblasts, the specialized epithelial cells that form the outer layer of the placenta[20]. This layer acts as the interface for nutrient exchange between maternal and foetal blood[21]. Once the transferrin-iron complex binds to TfR1, the complex is internalized through receptor-mediated endocytosis into vesicles known as endosomes[22]. The acidic environment within the endosome promotes the dissociation of iron from transferrin. This mechanism allows for efficient release of iron, which is subsequently reduced from ferric (Fe^{3+}) to ferrous (Fe^{2+}) iron, the form that is usable by cells[22]. The released iron is then transported across the cytoplasm of the syncytiotrophoblast. Iron export from the syncytiotrophoblast into the foetal bloodstream is mediated by the iron exporter ferroportin[23] (FPN). Ferroportin is the only known iron exporter and plays a crucial role in maintaining iron homeostasis in various tissues, including enterocytes in the duodenum, macrophages (which recycle iron from aged red blood cells), and hepatocytes. In the placenta, ferroportin transports ferrous iron across the basolateral membrane of the syncytiotrophoblast into the foetal capillaries[23]. Once in the foetal circulation, iron must be oxidized back to its ferric form (Fe^{3+}) to bind to foetal transferrin. This oxidation is facilitated by zyklopen, a multi-copper oxidase expressed in the placenta and homologous to hephaestin, which serves a similar function in enterocytes[24,25].

Hepcidin is a peptide hormone produced primarily by the liver and is the master regulator of iron homeostasis. It acts by binding to ferroportin, inducing its internalization and degradation, thereby reducing iron export into the bloodstream. During pregnancy, hepcidin levels are typically suppressed to allow for increased maternal iron absorption from the diet and mobilization of stored iron to meet the increased demands of pregnancy[26]. Maternal hepcidin levels are typically downregulated during pregnancy, allowing for increased iron absorption and mobilization from stores. The suppression of maternal hepcidin is crucial because elevated hepcidin would reduce iron availability, potentially impairing foetal development due to insufficient iron transfer across the placenta[20]. The placenta also

produces hepcidin, which appears to regulate placental iron transport[27]. Placental hepcidin can modulate ferroportin activity in the syncytiotrophoblast, acting as a gatekeeper to control the amount of iron exported to the foetus[5]. The balance between maternal, placental, and foetal hepcidin ensures that iron levels are tightly regulated, preventing iron overload in the foetus, which could lead to oxidative stress and tissue damage[21,28]. The foetus itself also produces hepcidin, primarily in the foetal liver. This foetal hepcidin is thought to play a role in regulating iron uptake into foetus. The regulation of foetal hepcidin is particularly important in late pregnancy when iron needs are highest for rapid foetal growth and red blood cell production[29]. When maternal iron stores are depleted or dietary iron intake is insufficient, the placenta adapts by upregulating key iron transport proteins. Increased TfR1 expression on syncytiotrophoblasts enhances iron uptake from the maternal circulation. Additionally, increased ferroportin expression facilitates more efficient iron export into the foetal bloodstream[5]. These adaptive responses help prioritize foetal iron needs during maternal iron deficiency, ensuring that the developing foetus receives iron necessary for proper growth and development, even under suboptimal maternal conditions[5]. Additionally, the placenta can store iron in the form of ferritin, a protein complex capable of sequestering up to 4500 iron atoms. Ferritin-bound iron in the liver provides a buffer against fluctuations in maternal iron levels and can be mobilized during periods of high foetal demand or maternal iron deficiency[30]. In addition to dietary iron absorption, a significant portion of maternal and foetal iron comes from recycled iron. Macrophages in the spleen and liver phagocytose aged erythrocytes, breaking them down and releasing iron, which is then transported back into circulation. This process, known as recycling, becomes a predominant source of iron in iron-deficient states[31]. Studies in animal models have demonstrated that recycled iron from senescent red blood cells contributes significantly to placental iron transfer, especially in conditions of maternal iron deficiency[16]. In mice, iron from phagocytosed erythrocytes becomes the primary source of iron supplied to the foetus during late pregnancy, when maternal iron stores are severely depleted[32]. Placental iron regulation is a highly coordinated process that balances the iron needs of the mother and the growing foetus. Through the interaction of maternal, placental, and foetal mechanisms, including the roles of transferrin,

ferroportin, hepcidin, and iron recycling, the foetus receives sufficient iron for critical developmental processes such as erythropoiesis and cellular respiration.

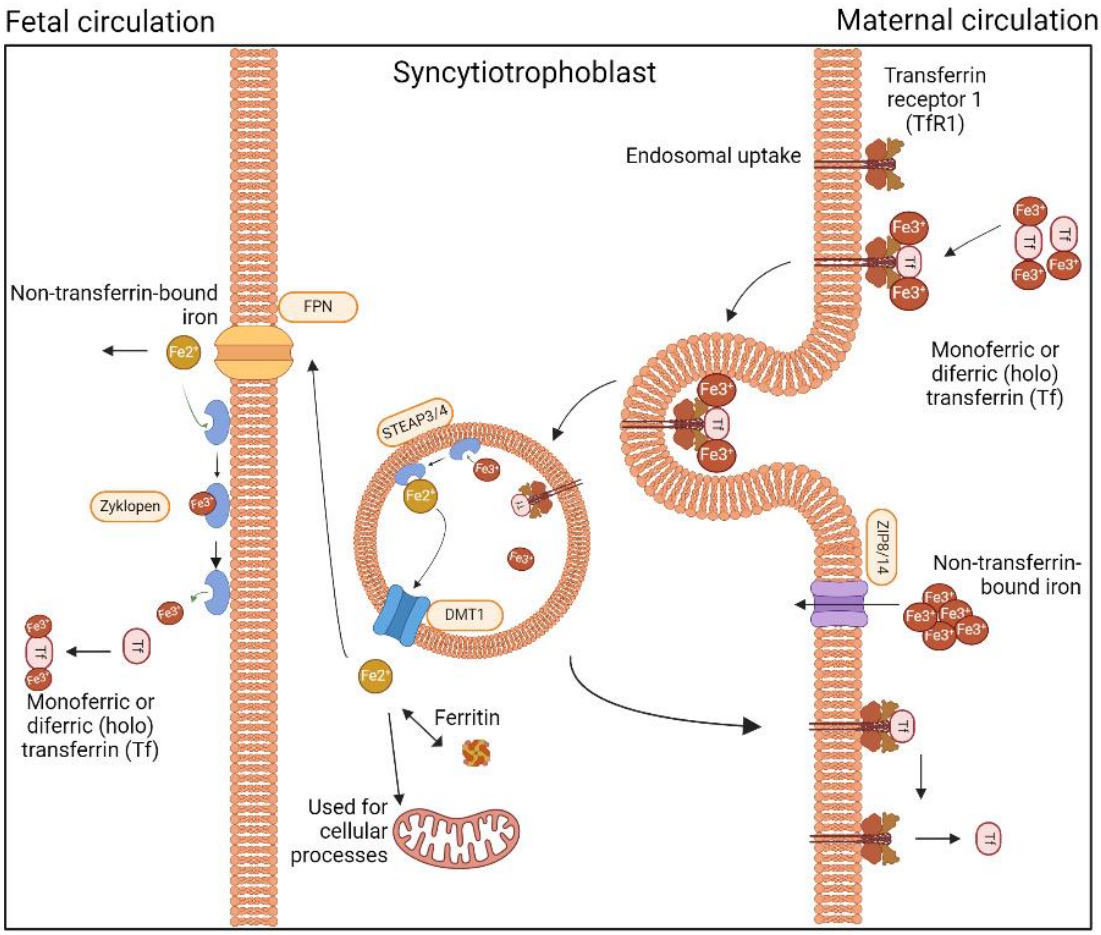

Figure 2. Pathways of iron transfer across the placenta.
Iron is delivered to the placenta by the maternal circulation, where it is mainly bound to transferrin (Tf), forming a monomeric or diferric (holo) Tf-$Fe3^{+}$ complex. The primary route of iron uptake by the placenta involves the uptake of Tf-bound iron from the maternal circulation through transferrin receptor 1 (TfR1) on the apical membrane of the placental syncytiotrophoblast facing the maternal circulation. The Tf-$Fe3^{+}$ complex binds to TfR1 and then is internalized into the cell by clathrin-mediated endocytosis. In the acidic environment of the endosome, ferric ions dissociate from Tf and are then reduced to the ferrous (Fe^{2+}) state by ferrireductases, possibly by STEAP3 (six-transmembrane epithelial of prostate) or STEAP4. Following the release of iron, the apo-Tf-TfR1 complex is recycled back to the membrane. The neutral pH of the extracellular space facilitates the dissociation of apo-Tf from TfR1 and its release back to the maternal circulation. TfR1 is then again available for the uptake of the Tf iron complex. Within the syncytiotrophoblast, ferrous iron is transported out of the endosome into the cytoplasm by divalent metal transporter 1 (DMT1). Other potential iron transporters expressed in the placenta include the Zrt/Irt-like proteins ZIP8 and ZIP14, members of the solute carrier family 39A (SLC39A). Once in the cytoplasm, iron can be stored as ferritin (Ft), used for cellular processes, or exported to the fetal circulation via ferroportin (Fpn), the only known mammalian iron exporter. In this process, Fpn cooperates with zyklopen, a copper-dependent ferroxidase, which oxidases ferrous to ferric iron that can be bound by fetal Tf. Zyklopen is not essential for iron transport to the fetus in mice. Adapted from Mazgaj, R.; Lipiński, P.;Starzyński, R.R. Iron Supplementation of Pregnant Sows to Prevent Iron Deficiency Anemia in Piglets: A Procedure of Questionable Effectiveness. https://doi.org/10.3390/ijms25074106

Hypotheses.

- Adaptation of Iron Homeostasis Mediated by Hepcidin During Pregnancy:
  Through the reduction of circulating hepcidin levels maternal iron homeostasis is adapted, to facilitate increased iron absorption and mobilization of stored iron to meet the increased demands of the developing foetus.
- Efficiency of Placental Iron Transport Mechanisms:
  Placenta optimizes iron transfer to the foetus by regulating the expression and activity of key iron transport proteins. These mechanisms ensure efficient iron acquisition by the foetus, particularly in response to maternal iron deficiency.

Objectives.

- To investigate the mechanisms of iron absorption and distribution in pregnant mice and pigs.
- To examine the role of ferroportin-hepcidin regulatory axis in iron homeostasis during pregnancy.

Materials and methods.

Experiment 1. Effect of Oral Supplementation of Healthy Pregnant Sows with Sucrosomial Ferric Pyrophosphate on Maternal Iron Status and Hepatic Iron Stores in Newborn Piglets.

Animal Model and Housing Conditions.

The experiment was conducted at the Pig Hybridization Centre in Pawłowice, part of the National Research Institute of Animal Production (Balice, Poland). The study involved a total of 15 healthy, non-anaemic pregnant 990 line sows, as confirmed by veterinary examinations and haematological indices. These sows were housed in standard conditions, maintaining 70% humidity and a temperature of 22°C in cages with straw bedding. Gestation crates were utilized to prevent fighting for food, biting, and miscarriages.

Feeding Protocol.

They were randomly allotted to one control group and two iron-supplemented groups.

- **Control Group (n=5):** The control group received a standard fodder for pregnant sows, containing 80 mg Fe/kg, designed to meet the National Research Council (NRC) iron requirements for pregnant sows.
- **SFP Group (n=5) :** The first experimental group received the standard fodder, supplemented from day 80 of pregnancy up to delivery with 60 mg of iron daily in the form of sucrosomial ferric pyrophosphate (SFP) (SiderAL®, PharmaNutra, Pisa, Italy).
- **$FeSO_4$ Group (n=5) :** The second experimental group was supplemented with 60 mg of iron daily in the form of ferrous sulfate ($FeSO_4$, Gambit, Kutno, Poland), added to the standard fodder with the same timing and dosage as the SFP group.

Sample collection.

Blood from the sows was drawn on days 80 and 115 of gestation by venipuncture of the jugular vein (Vena jugularis externa). Blood and tissue samples from piglets were collected 24 hours after birth. Piglets were euthanized by intracardiac injection of 0.5 mL/kg body weight of Morbital® (133.3 mg/mL of sodium pentobarbital + 26.7 mg/mL of pentobarbital; Biowet, Puławy, Poland). Blood samples from sows and piglets were collected into tubes coated with heparin as an anticoagulant and centrifuged (1200× g, 10 min, 4°C) to separate the plasma. Plasma samples were immediately aliquoted and stored at −80°C. Tissue samples collected from piglets for molecular analyses were rinsed with PBS, snap frozen in liquid nitrogen, and stored at −80°C. Placenta samples were collected immediately after parturition, snap frozen in liquid nitrogen for molecular analyses, and other placenta samples were fixed with paraformaldehyde for immunofluorescence analyses. Colostrum samples were collected immediately after delivery, and milk samples were collected 48 hours after delivery. Both colostrum and milk samples were stored at −20°C until further analysis.

Red Blood Cell indices and plasma iron measurement.

Hematological indices were measured using an automated ADVIA 2010 analyzer (Siemens, Erlangen, Germany). Plasma iron concentration was determined via

colorimetric measurement of an iron-chromazurol complex, following the manufacturer's protocol (Biomaxima S.A., Lublin, Poland).

Measurement of Non-Heme Iron Content in Tissues.

The non-heme iron content in liver, spleen (100 mg wet tissue), milk, and colostrum (1 mL) was determined by acid digestion of the samples at 100°C for 10 minutes. This was followed by a colorimetric measurement of the absorbance of the iron-ferrozine complex at 560 nm.

Plasma Hepcidin-25 Measurement.

Plasma hepcidin-25 levels in piglets were measured using a combination of weak cation exchange chromatography and time-of-flight mass spectrometry (WCX-TOF MS).

Real-Time Quantitative RT-PCR.

Total cellular RNA was extracted from liver and placental tissue (20 mg) using Trizol reagent (Invitrogen) according to the manufacturer's protocol. Two micrograms of DNase-treated RNA were reverse transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany). Real-time quantitative polymerase chain reaction (PCR) analysis was performed on a Light Cycler U96 (Roche Diagnostics, Mannheim, Germany) with gene-specific primer pairs. The amplified products were detected using SYBR Green I (Roche Diagnostics, Mannheim, Germany) as previously described. Amplification specificity was confirmed through melting curve analysis and agarose gel electrophoresis. Transcript levels were normalized relative to the control reference gene, selected using NormFinder software (Aarhus, Denmark) (https://moma.dk/normfinder-software).

Immunofluorescence (IF) Analysis and Confocal Microscopy of Placental Sections.

Following delivery, pig placentas were immediately dissected and fixed in 4% paraformaldehyde (Sigma-Aldrich, Poznan, Poland) in phosphate-buffered saline (PBS) at 4°C for 24 hours. After two 30-minute washes in PBS, the tissues were successively soaked in 12.5% and 25% sucrose (Bioshop, Burlington, Ontario, ON, Canada) for 24 hours and 7 days, respectively, at 4°C. The placenta was embedded in Cryomatrix medium (Thermo Fisher Scientific, Warsaw, Poland), frozen in liquid nitrogen, and sectioned into 10-µm slices using a cryomicrotome (Shandon, London, UK). The

sections were washed in PBS for 10 minutes and permeabilized by bathing in PBS/0.1% Triton X-100 (Sigma-Aldrich, Poznan, Poland) for 20 minutes. Non-specific antibody binding was blocked by incubating the tissue sections in PBS/5% Bovine Serum Albumin (BSA) (Bioshop, Burlington, Ontario, ON, Canada) for 1 hour. For protein detection, sections were incubated overnight at room temperature with primary antibodies diluted in PBS/5% BSA. Negative control sections were incubated without primary antibody. Next, the sections were washed for 5-6 minutes with PBS/0.1% Triton X-100 and incubated for 1 hour with secondary antibody diluted in PBS/5% BSA at room temperature. Finally, sections were washed for 5-6 minutes in PBS and additionally stained with Hoechst (Thermo Fisher Scientific, Warsaw, Poland) for 2 minutes, then washed twice with PBS and mounted in a glycerol-based medium.

Placental Samples and Fixation for Transmission Electron Microscopy (TEM).

Five placentas per experimental group were obtained from sows. Small pieces of the basal plate (10 x 10 x 2 mm) were excised within minutes of delivery (expelled placentas) and fixed for 1 hour in a solution containing 4% paraformaldehyde and 0.4% glutaraldehyde in sodium cacodylate trihydrate (Acros Organics, Geel, Belgium) at pH 7.2. This was followed by fixation in 1% OsO_4 (Thermo Fisher, Kandel, Germany) for 16 hours at 4°C. Subsequently, samples were dehydrated through an ethanol series (30%, 50%, 70%) and dried using a Leica EM CPD300 Critical Point Dryer (Leica, Wetzlar, Germany). They were then sputtered with gold using a Low Vacuum Coater Leica EM ACE200 (Leica, Wetzlar, Germany). Transmission electron microscopy was conducted using the COXEM EM-30AXplus SEM microscope (Yuseong-gu, Daejeon, Korea).

Ethical Statement.

The experimental procedures conducted in this study adhered to the European Union (EU) guidelines for the care and handling of research animals (EU Directive 2010/63/EU for animal experiments). The Second Local Ethical Committee on Animal Testing at the Warsaw University of Life Sciences in Warsaw (Poland) granted a formal waiver of ethical approval because the study involved routine veterinary procedures, namely piglet euthanasia and blood collection from pregnant sows. Furthermore, the

administration of a dietary supplement such as sucrosomial ferric pyrophosphate (SFP) was not classified as a research procedure.

Statistical Analysis.

For the analysis of iron supplementation in one-day-old piglets, statistical analysis was conducted using one-way analysis of variance (ANOVA), with the type of supplementation as the factor. This was followed by Tukey's multiple comparisons test to assess differences between groups. The results obtained from sows with iron supplementation were subjected to two-way ANOVA, utilizing the type of supplementation and time as factors. Sidak's multiple comparisons test was then applied to evaluate differences between groups over time. Statistical analysis and figures were generated using GraphPad Prism version 8.00 for Windows (GraphPad Software, La Jolla, CA, USA). A p-value of less than or equal to 0.05 (*) and 0.01 (**) were considered significant and are indicated accordingly.

Experiment 2. Marginally reduced maternal hepatic and splenic ferroportin under severe nutritional iron deficiency in pregnancy maintains systemic iron supply.

Experimental animals and tissue collection.

The experiments utilized 5-month-old pregnant female mice (B6; 129S7 strain) bred at the Department of Molecular Biology, Institute of Genetics and Animal Biotechnology of the Polish Academy of Sciences. The mice were housed under constant conditions of 22°C and a 12-hour photoperiod. Two weeks prior to mating with males of the same strain, females were placed on either control or iron-deficient diets (Harlan Laboratories, Madison, WI, USA) containing 48 ppm and 4 ppm of iron, respectively. These dietary conditions were maintained throughout pregnancy. Timed mattings were conducted, with the identification of the morning plug marking E0.5. Plugged females were euthanized at E18.5 using isoflurane overdose. In some instances, non-pregnant females under the same dietary iron conditions were utilized. Blood samples were obtained from the inferior vena cava using heparin-coated syringes and collected in heparin-coated tubes. After centrifugation at 800 g for 10 minutes at 4°C, plasma samples were frozen in liquid nitrogen and stored at −80°C. Foetal livers were collected

and frozen after dissecting them from the uterus and sacrificing the foetuses by decapitation. Placentas from randomly selected foetuses of each mother were rapidly dissected and frozen in liquid nitrogen, then stored at −80°C. Tissue samples (maternal and foetal livers, maternal duodenum, and placenta) for immunofluorescence analysis were fixed in 4% paraformaldehyde in PBS. Bone marrow cells for total cellular RNA extraction were flushed from the femur of females using ice-cold Hanks balanced salt solution. All procedures were conducted in accordance with the guidelines of the 2nd Local Ethical Commission (permission number WAW2/53/2016).

Red blood cell indices, blood plasma iron parameters and non-heme iron content in tissues.

Red blood cell (RBC) count, hemoglobin concentration (HGB), hematocrit (HCT), and mean cell volume (MCV) were assessed using an automated hematology analyzer (Abacus Junior Vet 5; Diatron, Budapest, Hungary). Plasma iron concentration and total iron-binding capacity (TIBC) were measured using a colorimetric assay based on the formation of an iron-chromazurol complex (absorbance at 630 nm), following the manufacturer's protocol (Alpha Diagnostic, Poland). The percentage transferrin saturation (TSAT) was then calculated from these values. The non-heme iron content of liver and placenta fragments (100 mg) and spleen fragments (40 mg) was determined by digesting the samples in acid at 100°C for 10 minutes, followed by colorimetric measurement of an iron-ferrozine complex (absorbance at 560 nm).

Real-time quantitative PCR (RT-qPCR).

Total cellular RNA was extracted from liver, bone marrow, kidney, and placental tissue (20 mg) using Trizol reagent (Invitrogen) following the manufacturer's instructions. Two micrograms of DNase-treated total RNA underwent reverse transcription using a Transcriptor First-Strand cDNA Synthesis Kit (Roche, Switzerland). The resulting cDNA served as the template for real-time quantitative PCR analysis using gene-specific primer pairs on a Light Cycler U96 (Roche Diagnostics, Mannheim, Germany). Amplified products were detected using SYBR Green I (Roche Diagnostics). To verify amplification specificity, PCR products underwent melting curve analysis and agarose gel electrophoresis. Data analysis was performed using Light Cycler U96 Software.

Transcript levels were normalized relative to control reference genes identified using NormFinder software v0.953 (https://moma.dk/normfinder-software).

Western blot analysis.

For the detection of ferroportin (Fpn) and L-ferritin (L-Ft) protein levels, membrane and cytosolic extracts from maternal liver, spleen, duodenum, fetal liver, and placenta were prepared according to previously described methods. These extracts were separated by electrophoresis on 10% and 14% SDS-PAGE gels, respectively. After electrophoresis, proteins (100 µg) were electroblotted onto polyvinylidene difluoride membranes (Millipore), followed by blocking and incubation with primary antibodies. Primary antibodies included a rabbit antiserum against recombinant mouse L-Ft (provided by Dr. P. Santambrogio, Department of BioTechnology, San Raffaele Scientific Institute, Milan, Italy), a rabbit polyclonal anti-Fpn antibody (Alpha Diagnostic), and an affinity-purified rabbit polyclonal anti-mouse Fpn antibody (kindly provided by Dr. François Canonne-Hergaux, INSERM UMR 1043, Centre de Physiopathologie de Toulouse Purpan, Toulouse, France). Additionally, transferrin receptor 1 monoclonal antibody (Thermo Fisher) and anti-human Natural Resistance-Associated Protein 2 (DMT1/NRAMP2) antibody (Alpha Diagnostic Intl. Inc.) were used. Following primary antibody incubation, membranes were washed and incubated with peroxidase-conjugated anti-rabbit secondary antibody (Santa Cruz Biotechnology) for 1 hour at room temperature. Immunoreactive bands were visualized using the ECL Plus Western blotting detection system (Amersham Life Sciences). Band intensities were quantified via densitometric analysis using Quantity One software, version 4.6.6 (Bio-Rad).

Immunofluorescence (IF) analysis and confocal microscopy of liver, placenta and duodenum sections.

After performing a laparotomy, livers and placentas from pregnant mothers at E18.5 were promptly dissected and fixed in 4% paraformaldehyde (Sigma) in phosphate-buffered saline (PBS) at 4°C for 24 hours. Following two 30-minute washes in PBS, the tissues underwent successive soaking in 12.5% and 25% sucrose (Bioshop) at 4°C for 2 hours and 7 days, respectively. Subsequently, hepatic and placental samples were embedded in Cryomatrix medium (Thermo Scientific), frozen in liquid nitrogen, and sectioned into 20-µm slices using a cryomicrotome (Shandon). The sections were then

washed in PBS for 10 minutes and permeabilized by immersion in PBS containing 0.1% Triton X-100 (Sigma) for 20 minutes. Non-specific antibody binding was blocked by incubating the tissue sections in PBS containing 3% BSA (Bioshop) at room temperature for 1.5 hours. For protein detection, sections were incubated overnight at room temperature with primary antibodies diluted in PBS containing 3% BSA. As a negative control, some sections were incubated without the primary antibody. Next, the sections were washed five times for 6 minutes each in PBS containing 0.1% Triton X-100 and incubated for 1.5 hours at room temperature with secondary antibodies diluted in PBS containing 3% BSA. Finally, the sections were washed for 10 minutes in PBS and mounted in Vectashield medium with DAPI (Vector Labs). The presence of ferroportin (Fpn) in Browicz-Kupffer cells (liver macrophages) was determined by double immunofluorescence staining of the investigated protein and the macrophage marker F4/80. For this, liver sections were first incubated with anti-Fpn followed by anti-F4/80 primary antibody, and then incubated with a mixture of secondary antibodies conjugated with different fluorochromes: Cy3 for Fpn and Alexa488 for F4/80. Immunofluorescence was analysed using a Zeiss LSM 710 confocal microscope (Carl Zeiss) equipped with a ×40 objective and Zeiss ZEN software. Confocal microscopy was utilized to examine liver and placenta sections from three different females per experimental group. The same acquisition parameters were consistently applied to capture images within each experimental set, ensuring uniformity. Subsequently, ImageJ software (NIH, Bethesda) facilitated the measurement of mean fluorescence in each tubule section. The signal intensity was manually quantified to derive a Mean Gray Value, calculated as the sum of grey values in the selected area divided by the number of pixels within that area. A total of 30 measurements for livers and placentas were conducted per experimental group, equating to 10 measurements per female.

Measurement of blood plasma erythroferrone (ERFE) level.

Serum samples, standards, and controls were mixed with sample diluent (100 µL/well) and then incubated for 1 hour with immobilized polyclonal antibody in the precoated plate, using an indirect ERFE ELISA kit (Intrinsic Lifesciences, The BioIron Company, catalog no. SKU# ERF-200). Subsequently, the plate was washed to remove unbound mouse ERFE. A second anti-mouse ERFE antibody, conjugated with biotin, was added

for 1 hour, followed by washing and incubation with HRP-SA conjugate for 30 minutes. This conjugate binds to the biotinylated anti-mouse erythroferrone. Upon completion of the incubation steps, the enzyme-TMB reaction was initiated, resulting in the formation of a blue-coloured complex within 15 minutes. The reaction was then halted by adding a stop solution, which changed the color of the mixture to yellow. A microplate reader (ELx800 BIO-TEK INSTRUMENTS, Inc., Winooski, VT, USA) was employed to measure the absorbance at 450 nm. The intensity of the yellow colour directly correlated with the concentration of ERFE in the samples. A standard curve was constructed by plotting the log concentration of the standard curve against its corresponding OD (450).

Statistical analysis.

Statistical analysis was conducted employing ANOVA and Kruskal-Wallis ANOVA tests for parametric and non-parametric data distributions, respectively. Post-hoc tests, including Tukey and Dunn tests, were applied accordingly. Statistical significance was defined as $p \leq .05$ (*), $p \leq .01$ (**), and $p \leq .001$ (***), denoting significance, high significance, and very high significance, respectively.

Results.

Experiment 1. Effect of Oral Supplementation of Healthy Pregnant Sows with Sucrosomial Ferric Pyrophosphate on Maternal Iron Status and Hepatic Iron Stores in Newborn Piglets.

Haematological and Iron Status in Pregnant Sows.

The study initiated with the measurement of red blood cell (RBC) indices and plasma iron parameters in pregnant sows on day 80 of pregnancy. All experimental groups exhibited similar baseline values, with no significant differences detected in RBC indices or plasma iron parameters at this stage. By day 114 of pregnancy, RBC indices largely remained stable, with the exception of minor fluctuations observed in RBC count and haemoglobin levels among sows supplemented with ferrous sulphate ($FeSO_4$). A notable, statistically significant decline in plasma iron, ferritin, and hepcidin levels was recorded across all groups, regardless of iron supplementation, indicating a depletion of iron stores as pregnancy progressed. There were no significant differences in plasma iron status between control and iron-supplemented sows at day 114,

highlighting that iron supplementation did not significantly impact maternal haematological and iron parameters during late pregnancy .

Iron Content in Colostrum and Milk.

To determine if oral iron supplementation would enhance iron concentration in colostrum and milk, iron levels in these fluids were measured from all experimental groups. The results showed that iron supplementation with either sucrosomial ferric pyrophosphate (SFP) or FeSO4 did not lead to a significant increase in iron content in colostrum and milk compared to the control group. Although there was a slight elevation in iron concentration in the supplemented groups, the increase was not statistically significant. This indicates that oral iron supplements administered to pregnant sows did not substantially affect the iron content of colostrum and milk.

Neonatal Iron Status.

The investigation extended to the neonatal piglets to assess the impact of maternal iron supplementation on their iron status. The red blood cell indices and plasma iron status of piglets born to sows supplemented with SFP or $FeSO_4$ were evaluated. The findings clearly demonstrated that maternal iron supplementation did not improve the RBC indices or plasma iron status in the newborn piglets. Haemoglobin levels in all piglets, measured one day post-birth, were above the anaemia threshold of 8 g/dL, indicating that none of the piglets were anaemic. There were no significant differences in haematological parameters or plasma iron levels between piglets from iron-supplemented sows and those from control sows, suggesting that maternal supplementation had no beneficial effect on neonatal iron status.

Table 1. Red blood cell indices and plasma iron status in 1-day old piglets from control and SFP and FeSO4-supplemented sows.

Blood Parameters	Control	SFP	$FeSO_4$	*p*-Value	
				Control *vs.* ….	
Hb (g/dL)	9.4 ± 1.4	9.8 ± 1	9 ± 1.3	SFP 0.6906	$FeSO_4$ 0.7161
RBC mln/mm^3	4.9 ± 0.7	5.1 ± 0.9	4.69 ± 0.6	SFP 0.8252	$FeSO_4$ 0.7332
MCV (μm^3)	60 ± 2.7	63.1 ± 3.7	61.6 ± 4	SFP 0.1301	$FeSO_4$ 0.5538
Serum Iron (μmol/L)	14.8 ± 9.9	10.5 ± 8.4	18 ± 8.1	SFP 0.2284	$FeSO_4$ 0.9994
Serum Ferritin (ng/mL)	823.5 ± 161.4	906.2 ± 75.1	772.7 ± 137.6	SFP 0.3656	$FeSO_4$ 0.6631

Table 1. Effect of ferrous iron sulfate ($FeSO_4$) and sucrosomial ferric pyrophosphate (SFP) oral supplementation on blood parameters in one day old piglets. RBC – red blood cell count, Hb – hemoglobin level, MCV—mean corpuscular volume. All parameters were determined for 5 sows from each experimental group. n = 9 piglets per experimental group.

Pregnancy Outcomes.

The study also assessed the impact of iron supplementation on pregnancy outcomes such as average piglet weight and litter size. No significant differences were observed among the experimental groups in terms of these parameters. This suggests that iron supplementation during pregnancy did not influence the overall pregnancy outcomes, including birth weight and litter size of the piglets.

Placental Iron Transport

Further analysis was conducted to understand the effect of iron supplementation on the expression of iron transporters in the placenta. The type of iron supplement administered influenced the expression levels of placental iron transporters. Despite these variations in expression, there were no corresponding changes in systemic iron homeostasis in either the sows or their offspring. This finding indicates that while the type of iron supplement may affect placental iron transporter mechanisms, it does not necessarily translate into measurable changes in maternal or neonatal iron status.

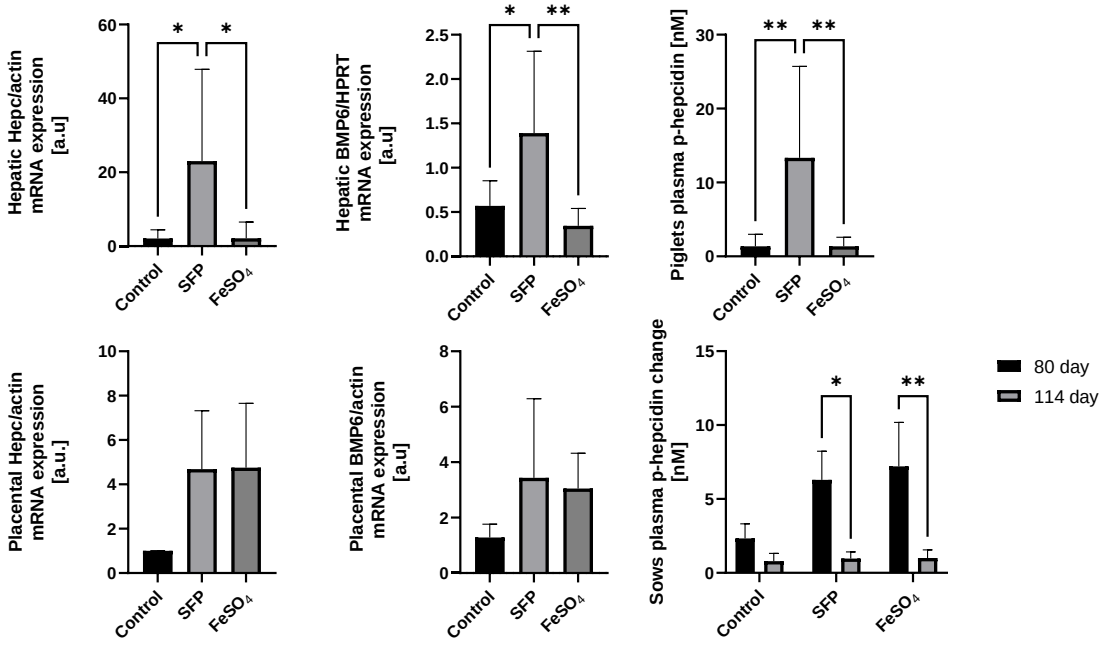

*Figure 3. Changes in placental and foetal hepatic and plasma hepcidin and Bone Morphogenetic Protein 6 (BMP6) levels under deferent oral iron supplementations. Foetal hepatic and placental hepcidin and BMP6 mRNAs levels were measured using Real Time polymerase chain reaction (PCR) and normalized to actin/Hypoxanthine Phosphoribosyltransferase (HPRT) mRNA. Hepcidin-25 (p-hepcidin) measurements in sows and piglets plasma were performed by peptide enrichment through weak cation exchange chromatography coupled to time-of-flight mass spectrometry (WCX-TOF MS) (Bruker Daltonics, Billerica, Massachusetts, United States) [29,30]. * and ** asterisks denote statistically significant dierences between parameters in control and ferrous iron sulfate (FeSO4) or sucrosomial ferric pyrophosphate (SFP) supplemented group at $p < 0.05$ and $p < 0.01$ respectively.*

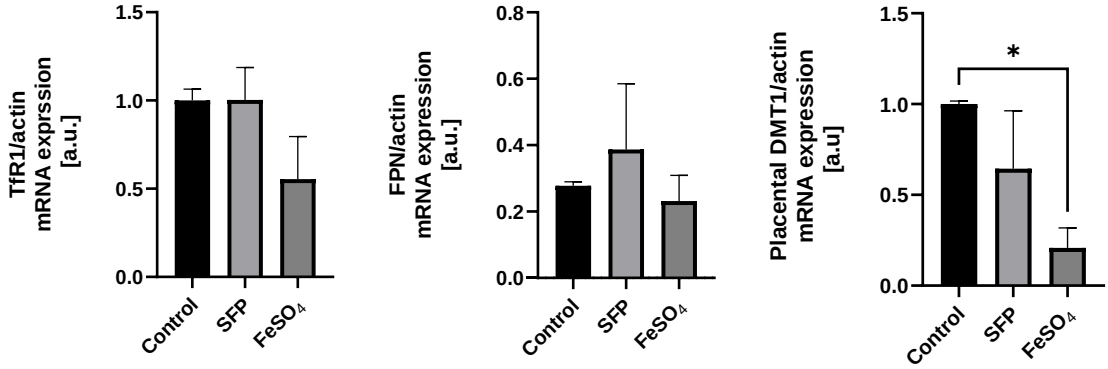

*Figure 4. Regulation of placental iron transporters after oral administration of ferrous iron sulphate ($FeSO_4$) or sucrosomial ferric pyrophosphate (SFP) to pregnant sows. Placental iron transporters mRNA expression measured using Real time PCR normalized to actin mRNA. * asterisk denote statistically significant differences between parameters in control and SFP or FeSO4 supplemented group at $p < 0.05$.*

Experiment 2. Marginally reduced maternal hepatic and splenic ferroportin under severe nutritional iron deficiency in pregnancy maintains systemic iron supply.

Iron Deficiency Anaemia (IDA) in Pregnant Females Fed a Low-Iron Diet

Exposure of pregnant rodent females to a low-iron diet is a well-established method for inducing iron deficiency and iron-deficiency anaemia during pregnancy. In this study, pregnant female mice subjected to dietary iron restriction exhibited significantly decreased iron parameters in blood plasma, such as iron concentration and transferrin saturation, compared to those fed a control diet. Consequently, these iron-deficient pregnant females displayed symptoms of microcytic anaemia, characterized by significantly reduced RBC count, haemoglobin concentration, haematocrit, and MCV values. This deterioration of RBC status was more pronounced in iron-deficient mothers compared to non-pregnant iron-deficient females and pregnant females on a control diet.

Decreased Hepatic Iron Stores in Iron-Deficient Pregnant Females and Their Foetuses.

Measurement of hepatic non-heme iron content revealed a five-fold reduction in anaemic pregnant females compared to iron-replete controls. This reduction was also observed in the livers of foetuses from anaemic mothers, though the decrease was only three-fold. Correspondingly, levels of hepatic L-ferritin, a major iron-storage protein, were significantly diminished in both iron-deficient mothers and their foetuses. However, the reduction in L-ferritin levels was more pronounced in mothers than in foetuses.

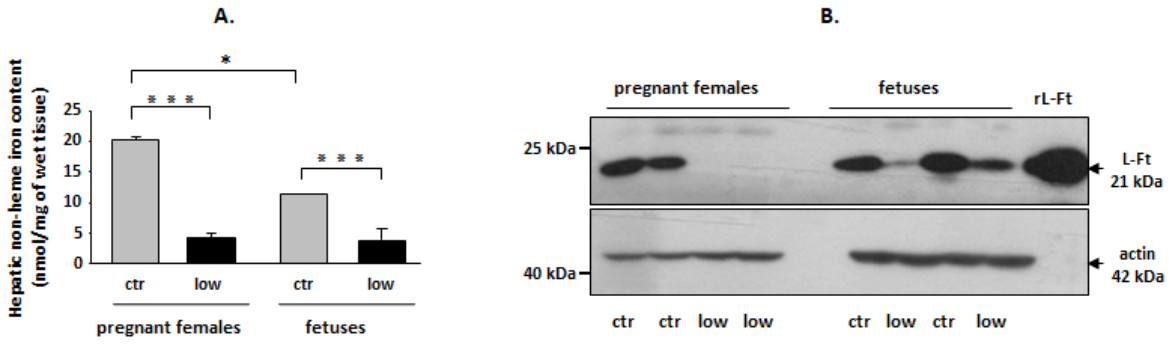

Figure 5. Decreased hepatic iron status of pregnant females fed a low-iron diet and their E18.5 fetuses. (A) Non-heme iron content in the livers of pregnant females and fetuses (means ± S.D.). Liver samples were obtained from 6 pregnant females of both the iron-replete (ctr) and iron-deficient (low) groups. L-ferritin (L-Ft) levels in liver cytosolic extracts (100 µg/lane) were assessed by Western blotting.

Hepatic Hepcidin and BMP6 mRNA Levels in Iron-Deficient Females and Foetuses.

Hepcidin, a small peptide hormone produced by hepatocytes, regulates body iron fluxes by inducing degradation of ferroportin (Fpn), thereby inhibiting iron release from cells. The expression of hepcidin is controlled by iron levels, erythropoietic activity, and inflammatory cues. During pregnancy, hepcidin levels are regulated by both maternal and foetal iron conditions. In this study, hepcidin mRNA abundance was assessed in maternal and foetal livers to examine the hepcidin-ferroportin regulatory axis under iron deficiency. The findings indicated a significant decrease in hepatic hepcidin mRNA levels in both iron-deficient mothers and their foetuses.

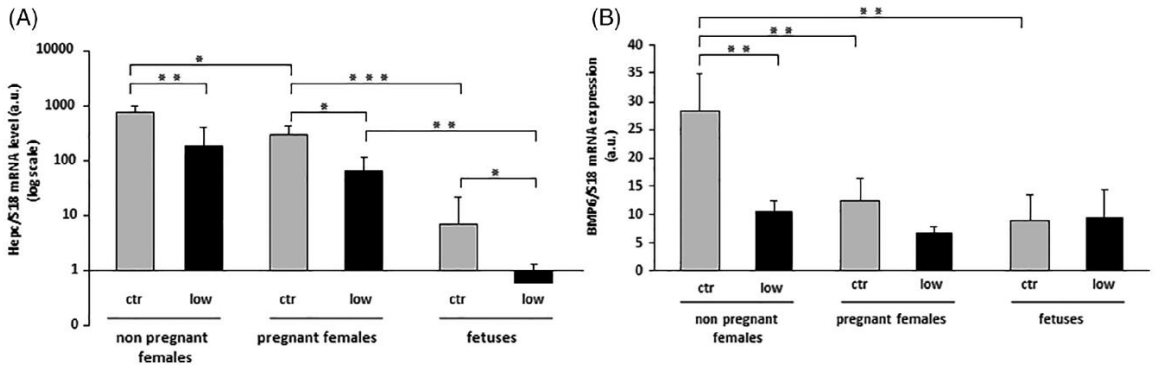

Figure 6. Decrease in hepatic hepcidin and BMP6 mRNA levels in iron-deficient non-pregnant females, pregnant females and their E18 foetuses. RT-qPCR analysis of hepcidin (A) and BMP6 (B) transcript levels in livers.

Increased Expression of Erythroid Inhibitors of Hepcidin in Iron-Deficient Pregnant Females.

Iron-deficient pregnant females showed increased expression of erythroid regulators of hepcidin, such as erythroferrone (ERFE), in their blood plasma. This upregulation is likely a compensatory response to increased erythropoietic demand under iron-deficient conditions. The elevated levels of ERFE contribute to the suppression of hepcidin expression, facilitating increased iron availability for erythropoiesis.

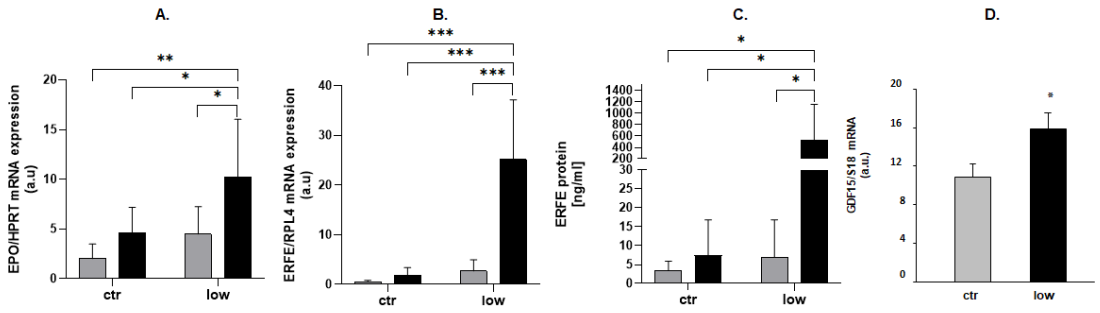

Figure 7. Increase in erythropoietin (Epo), erythroferrone (Erfe) and GDF15 expression in pregnant females fed a low-iron diet. (A) RT-qPCR analysis of Epo transcript levels in the kideny of 5-9 pregnant and nonpregnant females of the iron-replete (ctr) and iron-deficient (low) groups. (B) RT-qPCR analysis of Epo transcript levels in the bone marrow of 5-9 pregnant and nonpregnant females of the iron-replete (ctr) and iron-deficient (low) groups. (C) Erfe levels were measured in the blood plasma of 5-9 pregnant and non-pregnant females of the iron-replete (ctr) and iron-deficient (low) groups using a competitive ELISA. (D) RT-qPCR analysis of GDF15 transcript levels in the bone marrow of 8 pregnant females of the iron-replete (ctr) and iron-deficient (low) groups.

A Slight Decrease in Hepatic and Splenic Ferroportin in Iron-Deficient Mothers vs. a Prominent Decrease in Other Tissues.

Western blot and immunofluorescence analyses revealed only a marginal decrease in ferroportin (Fpn) levels in the liver and spleen of anaemic mothers. In contrast, Fpn levels were significantly reduced in the duodenum, placenta, and foetal liver. Specifically, the duodenum of iron-deficient pregnant females exhibited a 46% reduction in Fpn immunofluorescence signal intensity and a 52% decrease in Fpn protein level compared to control diet mothers. Similarly, placental Fpn levels were reduced by approximately 42%, as evidenced by both Western blot and immunofluorescence analyses.

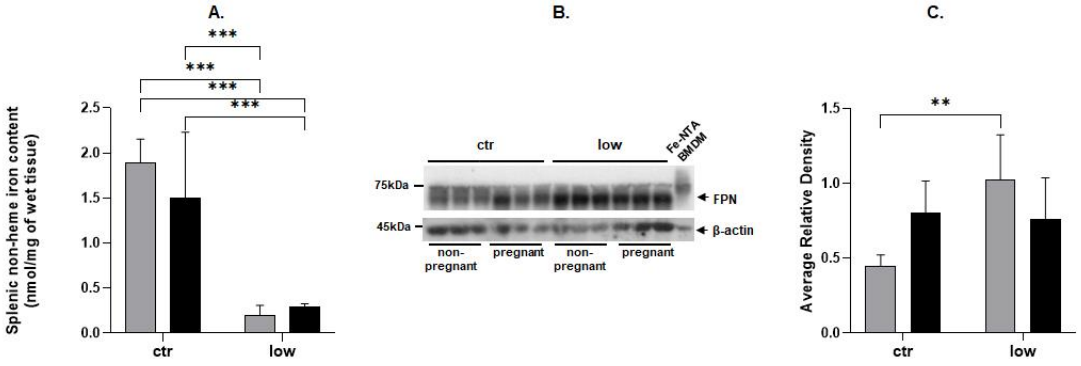

Figure 8. No reduction in ferroportin (Fpn) protein level despite substantially decreased splenic iron content in pregnant females fed a low-iron diet. (A) Non-heme iron content in the spleens of 5-9 pregnant and nonpregnant females of the iron-replete (ctr) and iron-deficient (low). (B) Western blot analysis of Fpn protein levels in spleen membrane fractions prepared from pregnant and non-pregnant females of the iron-replete (ctr) and iron-deficient

(low) groups. Fe-NTA BMDM, ferric nitrilotriacetate (100 μM) treated mouse bone marrow-derived macrophages (C) Immunoreactive Fpn bands from the blot shown in (b) were quantified by densitometry using a Molecular Imager.

Increased Expression of Iron Importers in the Placenta of Iron-Deficient Females.

Despite the overall reduction in ferroportin levels, the placenta of iron-deficient females showed high expression of iron importers on syncytiotrophoblasts, suggesting enhanced iron uptake from the maternal circulation. However, the reduced levels of ferroportin on the basolateral membrane of syncytiotrophoblasts likely contributed to decreased iron transfer to the foetus, with some iron being retained in the placenta.

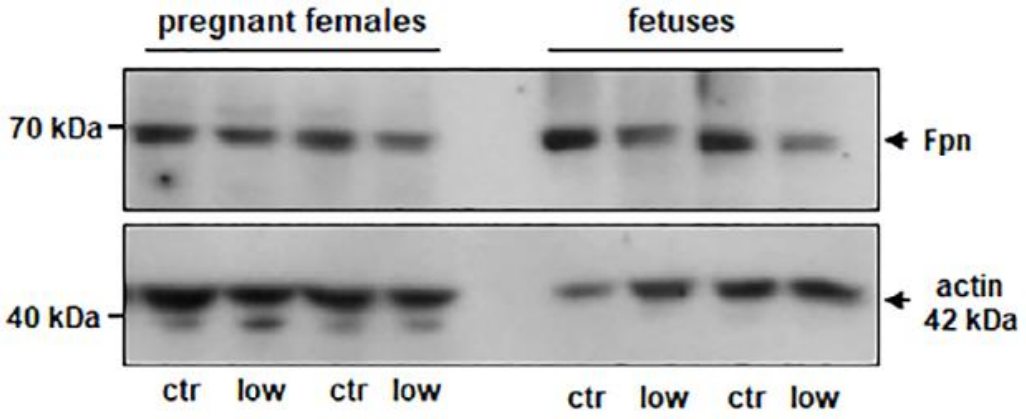

Figure 9. Differential decrease in hepatic ferroportin (Fpn) expression in pregnant females fed a low-iron diet and in their E18 fetuses.(A) Western blot analysis of Fpn protein levels in liver membrane fractions prepared from pregnant females (individuals) of the iron-replete (ctr)and iron-deficient (low) groups, and their fetuses (pooled samples).

Discussion.

Experiment 1. Effect of Oral Supplementation of Healthy Pregnant Sows with Sucrosomial Ferric Pyrophosphate on Maternal Iron Status and Hepatic Iron Stores in Newborn Piglets.

The experiment conducted on pregnant sows, which investigated the effects of oral supplementation with Sucrosomial Ferric Pyrophosphate (SFP) and Ferrous Sulphate ($FeSO_4$), provides a comprehensive look at maternal and neonatal iron status under physiological conditions and adequate nutrition. The lack of significant alterations in maternal haematological and iron parameters, neonatal iron status, and pregnancy outcomes opens an important discussion on the role and utility of iron supplementation of non-anaemic sows for the purpose of increasing iron status in their progeny.

Maternal Iron Homeostasis

One of the critical findings was that despite increasing physiological iron demands as pregnancy advanced, supplementation with SFP or $FeSO_4$ did not result in substantial changes in maternal red blood cell (RBC) indices or iron parameters by the end of gestation. The decline in plasma iron, ferritin, and hepcidin levels across all groups is consistent with known physiological adaptations during pregnancy[33]. These declines reflect the redistribution of iron to support foetal development, maternal erythropoiesis, and the increasing demands of placental function. However, supplementation with SFP and $FeSO_4$ did not counterbalance these natural declines.

This suggests that the bioavailability or dosing of the iron supplements may not have been sufficient to overcome the significant demands of late-stage pregnancy. Alternatively, it may also indicate that in healthy, non-anaemic sows, the body's iron regulatory mechanisms, particularly those mediated by hepcidin, maintain a balance that prioritizes maternal and foetal iron needs, rendering additional supplementation redundant[34]. This finding has important implications for routine iron supplementation in healthy pregnancies, suggesting that it may be more beneficial to reserve such interventions for cases where iron deficiency anaemia (IDA) is present evident , rather than using a blanket approach for all pregnant populations[35].

Iron Content in Colostrum and Milk

The lack of significant increases in iron content in colostrum and milk following supplementation raises further questions about the effectiveness of maternal iron supplementation in improving neonatal iron intake through lactation[36]. While colostrum and milk are vital sources of nutrition for neonates, the study demonstrated that even with supplementation, iron levels in these fluids remained statistically unchanged[37].

This outcome suggests that maternal iron supplementation may not directly translate into increased iron secretion into colostrum or milk. A possible explanation could lie in the regulatory mechanisms governing iron transport, which may prioritize maternal systemic iron homeostasis over excessive iron mobilization into secretions[37]. This raises a point of interest for further studies—whether this limited transfer is an adaptive feature that protects maternal iron stores during pregnancy and lactation, or whether it

reflects a cap on the amount of iron that can be absorbed and transferred to the neonate under non-anaemic conditions[21].

Neonatal Iron Status

Neonatal iron status, as measured by RBC indices and serum iron parameters, did not show significant differences between the control and supplemented groups, suggesting that the maternal iron levels, despite supplementation, were sufficient to meet foetal iron requirements under normal conditions. This is a critical finding as it implies that in non-anaemic pregnancies, the maternal system can provide adequate iron for foetal development without the need for additional supplementation[38].

Interestingly, all piglets exhibited haemoglobin concentrations above the threshold of anaemia, reinforcing the idea that maternal iron supplementation may not be necessary in populations where maternal and foetal iron demands are already being met through balanced nutrition[38]. This finding is particularly relevant when considering populations where over-supplementation might pose risks, such as increased oxidative stress or interference with the absorption of other essential nutrients like zinc or copper[39].

Pregnancy Outcomes

The lack of significant differences in pregnancy outcomes, including average piglet weight and litter size, further supports the conclusion that iron supplementation does not notably influence reproductive performance in non-anaemic sows. These findings suggest that the overall nutritional status of the sows, along with standard prenatal care, was sufficient to support normal pregnancy outcomes.

This challenges the routine use of iron supplementation in populations where iron deficiency is not evident, suggesting that blanket supplementation may not provide additional benefits in terms of reproductive success. It also raises questions about the optimal conditions under which iron supplementation would be effective.

Placental Iron Transport and Gene Expression

The placental analysis in this study provided critical insights into the effects of maternal iron supplementation on iron transport mechanisms. Immunofluorescence analysis showed no significant changes in the localization or expression of key iron

transporters—transferrin receptor 1 (TfR1), divalent metal transporter 1 (DMT1), and ferroportin (Fpn)—suggesting that maternal supplementation with SFP or $FeSO_4$ did not significantly affect placental iron transport[40]. This finding aligns with the overall theme of the study, which suggests that the maternal-foetal iron transfer is tightly regulated, and additional iron intake does not necessarily alter these pathways in non-deficient pregnancies.

However, a notable exception was the increased expression of hepcidin and bone morphogenetic protein 6 (BMP6) in the placentas and livers of piglets from sows supplemented with SFP. This suggests that SFP may have subtle regulatory effects on genes related to iron homeostasis, even if these effects do not translate into immediate changes in iron levels[41]. Hepcidin is a key regulator of iron metabolism, and its upregulation could indicate a feedback mechanism that maintains iron homeostasis in the face of increased maternal iron intake[29]. BMP6, known to down-regulate hepcidin expression, may also play a role in this regulatory feedback loop[28,42,43].

The upregulation of these genes could imply long-term regulatory effects on iron metabolism that are not immediately apparent in neonatal iron parameters. It also suggests that iron supplementation may have more nuanced effects on the regulation of iron homeostasis genes, which could influence iron storage and distribution in offspring as it grows. This finding warrants further investigation to explore whether maternal iron supplementation has lasting impacts on the iron metabolism of offspring, especially as they transition from neonatal stages to later life.

Broader Implications for Iron Supplementation in Pregnancy

The broader implications of this study extend beyond the specific animal model used. In human pregnancy, iron supplementation is widely recommended to prevent iron deficiency anaemia (IDA), which is associated with adverse outcomes such as preterm birth, low birth weight, and impaired cognitive development in infants[44]. However, this study raises questions about whether routine supplementation is necessary or beneficial in non-anaemic sows[44].

Given that no significant improvements were observed in maternal or neonatal iron status, pregnancy outcomes, or placental function in non-anaemic sows, the study

suggests that iron supplementation may need to be more targeted. Specifically, supplementation may be more effective in populations with diagnosed IDA, rather than being applied universally. This finding is consistent with human studies, where iron supplementation has been shown to benefit women with iron deficiency but may offer little advantage in women with adequate iron stores[45].

In conclusion, this study emphasizes the complexity of iron metabolism during pregnancy and suggests that iron supplementation should be carefully considered and tailored to the individual's iron status. The results provide a foundation for further research into the regulation of iron transport in the placenta and the long-term effects of maternal iron intake on offspring health, particularly in cases of iron deficiency anaemia. These findings are highly relevant for optimizing iron supplementation strategies in both animal husbandry and human clinical practice.

Experiment 2. Marginally reduced maternal hepatic and splenic ferroportin under severe nutritional iron deficiency in pregnancy maintains systemic iron supply.

This study investigates the impact of severe nutritional iron deficiency on iron metabolism in pregnant mice, particularly focusing on the role of ferroportin (Fpn) and its regulation across maternal and foetal compartments, including the placenta[11,30]. Iron deficiency anaemia (IDA) is a prevalent complication during pregnancy due to the elevated iron demands necessary for the expansion of maternal red blood cell (RBC) mass and the development of the placenta and foetus[46]. The findings from this study provide critical insights into how iron fluxes are regulated in cases of severe iron deficiency and highlight the physiological adaptations in maternal and foetal iron handling mechanisms.

Maternal Iron Deficiency and Anaemia

One of the key outcomes of this experiment is the confirmation of severe microcytic anaemia in pregnant females subjected to a low-iron diet. The notable decreases in RBC count, haemoglobin concentration, haematocrit, and mean corpuscular volume (MCV) clearly demonstrate the impact of iron deficiency on maternal hepatological status. The severity of anaemia was more pronounced in pregnant mice compared to both non-

pregnant iron-deficient females and those on a control diet, underscoring the heightened iron demands during pregnancy.

Hepatic Iron Stores and Ferritin Levels

The study also demonstrates a marked depletion of hepatic non-heme iron stores in iron-deficient pregnant females, with iron levels reduced by approximately five-fold compared to iron-replete controls. The corresponding reduction in hepatic L-ferritin, a critical iron storage protein[15,38], highlights the severity of iron depletion, as both mothers and their foetuses exhibited substantially diminished ferritin levels. While the decrease in foetal hepatic iron stores was less pronounced than in the mothers, this still underscores the critical reliance of the foetus on maternal iron supply. These findings emphasize the vulnerability of foetal iron status to maternal iron depletion, aligning with human studies that link maternal anaemia to poor foetal outcomes, including impaired neurodevelopment and increased susceptibility to infections.

Hepcidin Regulation in Iron-Deficient Pregnancy

The downregulation of hepcidin, a key hormone regulating iron homeostasis, is a significant finding in this study. Hepcidin suppression allows for increased iron absorption and mobilization by preventing Fpn degradation[17], thus enhancing iron availability. The marked reduction in hepcidin observed in iron-deficient pregnant females is consistent with previous studies and reflects an adaptive response aimed at maximizing iron absorption and mobilization to meet the increased demands of pregnancy[33]. Interestingly, the study also observed decreased hepatic BMP6 mRNA levels in iron-deficient and control diet pregnant females, suggesting that BMP6-dependent signalling pathways are attenuated during pregnancy[43], even when iron is adequate[47]. This provides a new perspective on the regulation of hepcidin during pregnancy, indicating that iron availability is tightly controlled by multiple regulatory pathways.

Erythroid Regulators of Hepcidin

In addition to BMP6, erythroid regulators like erythroferrone (ERFE)[48] and growth differentiation factor 15 (GDF15)[49] play significant roles in suppressing hepcidin to increase iron availability for erythropoiesis. The elevated plasma levels of ERFE in

iron-deficient pregnant females highlight the increased erythropoietic activity that accompanies iron deficiency. ERFE is secreted by erythroblasts in response to erythropoietin (EPO) and acts to suppress hepcidin, facilitating greater iron mobilization from stores[20]. This finding emphasizes the interplay between erythropoiesis and iron metabolism, where the body prioritizes the production of RBCs in response to anaemia. The concurrent upregulation of EPO and ERFE points to a coordinated effort to support erythropoiesis and foetal development under conditions of limited iron availability.

Ferroportin Expression in Maternal and Foetal Tissues

The differential expression of Fpn across maternal and foetal tissues provides key insights into how the body adapts to iron deficiency during pregnancy. The marginal decrease in hepatic and splenic Fpn levels in iron-deficient mothers suggests that these organs preserve Fpn expression to continue facilitating iron export despite the overall iron deficiency. This aligns with the known role of hepatic and splenic macrophages in recycling iron from senescent erythrocytes[31,50], a crucial process in maintaining systemic iron supply during pregnancy.

Placental Iron Transport

The placenta serves as the critical interface for maternal-foetal iron transfer[51]. In this study, Fpn expression in the placenta was significantly reduced in iron-deficient females, as evidenced by both Western blot and immunofluorescence analyses. This reduction in placental Fpn likely compromises the efficiency of iron export to the foetal circulation. Interestingly, the expression of iron importers, such as transferrin receptor 1 (TfR1) and divalent metal transporter 1 (DMT1), was enhanced on the apical side of placental syncytiotrophoblast[30], suggesting an adaptive mechanism to increase iron uptake from maternal circulation. However, the reduced Fpn expression on the basolateral membrane likely impairs the efficient transfer of iron from the placenta to the foetus, leading to iron retention in the placenta[29].

This imbalance between iron import and export in the placenta suggests a critical regulatory point in pregnancy where the body attempts to prioritize foetal iron acquisition but faces limitations due to the reduced availability of maternal iron and the impaired function of Fpn in transferring iron across the placental barrier.

Implications for Foetal Iron Status

The decreased hepatic iron stores and L-ferritin levels in foetuses from iron-deficient mothers indicate that foetal iron status is directly impacted by maternal iron deficiency. The three-fold reduction in foetal hepatic iron content, although less severe than the maternal reduction, underscores the foetus's reliance on maternal iron supply. The adaptive increase in placental iron importers may not fully compensate for the decreased Fpn-mediated iron export, raising concerns about potential impacts on foetal iron-dependent processes, such as erythropoiesis and neurodevelopment.

These findings underscore the crucial role of ferroportin in regulating iron turnover in iron-deficient pregnant females and their foetuses, highlighting the complex interplay between maternal and foetal iron homeostasis during pregnancy.

Both experiments provide valuable insights into the complex regulation of iron metabolism during pregnancy, specifically under conditions of iron deficiency or supplementation. These studies highlight the intricate mechanisms through which the maternal system prioritizes iron distribution, particularly to meet the heightened demands of foetal development and placental function. However, the outcomes of these experiments diverge in their approach—one focusing on iron deficiency and its physiological adaptations, and the other on the potential benefits of iron supplementation.

These studies together underscore the complexity of iron regulation during pregnancy and challenge common assumptions regarding iron supplementation in non-anaemic populations. On one hand, the mouse study emphasizes the critical need for iron in pregnancy and the physiological adaptations that occur in response to deficiency, with the body prioritizing placental and foetal iron uptake even at the expense of maternal stores. However, this adaptation may not be enough to prevent foetal iron deficiency under severe nutritional stress.

On the other hand, the study on supplementation in non-anaemic sows raises important questions about the necessity of routine iron supplementation in populations without iron deficiency. The lack of significant benefits from supplementation suggests that additional iron intake may not be essential for healthy pregnancies where maternal iron

reserves are sufficient. This aligns with broader research indicating that over-supplementation can lead to unnecessary iron accumulation and potential health risks.

Moreover, the role of ferroportin and the placenta's iron-handling capacity remain crucial areas for future investigation. Understanding how placental iron transport mechanisms adapt to deficiency or excess iron conditions is key to developing more effective strategies for managing iron metabolism during pregnancy. In conclusion, these studies contribute to a growing body of evidence that iron metabolism during pregnancy is highly regulated and context-dependent, with different outcomes based on the maternal iron status.

Conclusions.

- Critical Role of Iron in Pregnancy:
    - Both studies underscore the critical importance of maintaining adequate iron levels during pregnancy for the health of both the mother and the developing foetus.
    - Iron deficiency in pregnancy can lead to severe haematological and developmental consequences, while iron supplementation can mitigate these risks.
- Mechanisms of Iron Regulation:
    - The regulation of iron homeostasis during pregnancy involves complex interactions between dietary intake, iron transport proteins, and regulatory hormones like hepcidin.
    - Understanding these mechanisms can help develop targeted interventions to optimize maternal and foetal iron status.

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Effect of oral supplementation of healthy pregnant sows with sucrosomial ferric pyrophosphate on maternal iron status and hepatic iron stores in newborn piglets.

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Marginally reduced maternal hepatic and splenic ferroportin under severe nutritional iron deficiency in pregnancy maintains systemic iron supply.

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Iron Supplementation of Pregnant Sows to Prevent Iron Deficiency Anemia in Piglets: A Procedure of Questionable Effectiveness.

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Article

Effect of Oral Supplementation of Healthy Pregnant Sows with Sucrosomial Ferric Pyrophosphate on Maternal Iron Status and Hepatic Iron Stores in Newborn Piglets

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Simple Summary: In most mammals, including humans, the need for iron increases rapidly in the last period of pregnancy. Therefore, in compliance with World Health Organization (WHO) recommendations, iron supplementation has become a standard procedure even in healthy pregnant women although it carries the risk of iron toxicity and dysregulation of systemic iron homeostasis. Due to physiological and genomic similarities between swine and humans, pigs constitute an useful animal model in nutritional studies during pregnancy. Here, healthy pregnant sows were supplemented with sucrosomial ferric pyrophosphate (SFP), a new non-heme iron formulation, to study its effect on their iron metabolism and that of their progeny. In particular, we aimed at verifying whether supplementation of pregnant sows with SFP will increase the level of low hepatic iron stores in newborn piglets. Results of our study show that SFP does not significantly alter neither systemic iron homeostasis in pregnant sows, nor hepatic iron stores in newborn piglets, which can be used during neonatal period for the maintenance of hematological status. We hypothesize that supplemental iron given orally to pregnant sows is poorly transferred across the placenta.

Abstract: Background: The similarities between swine and humans in physiological and genomic patterns, as well as significant correlation in size and anatomy, make pigs an useful animal model in nutritional studies during pregnancy. In humans and pigs iron needs exponentially increase during the last trimester of pregnancy, mainly due to increased red blood cell mass. Insufficient iron supply during gestation may be responsible for the occurrence of maternal iron deficiency anemia and decreased iron status in neonates. On the other hand, preventive iron supplementation of non-anemic mothers may be of potential risk due to iron toxicity. Several different regimens of iron supplementation have been applied during pregnancy. The majority of oral iron supplementations routinely applied to pregnant sows provide inorganic, non-heme iron compounds, which exhibit low

bioavailability and intestinal side effects. The aim of this study was to check, using pig as an animal model, the effect of sucrosomial ferric pyrophosphate (SFP), a new non-heme iron formulation on maternal and neonate iron and hematological status, placental transport and pregnancy outcome; Methods: Fifteen non-anemic pregnant sows were recruited to the experiment at day 80 of pregnancy and randomized into the non-supplemented group (control; n = 5) and two groups receiving oral iron supplementation—sows given sucrosomial ferric pyrophosphate, 60 mg Fe/day (SFP; n = 5) (SiderAL®, Pisa, Italy) and sows given ferrous sulfate 60 mg Fe/day (Gambit, Kutno, Poland) ($FeSO_4$; n = 5) up to delivery (around day 117). Biological samples were collected from maternal and piglet blood, placenta and piglet tissues. In addition, data on pregnancy outcome were recorded.; Results: Results of our study show that both iron supplements do not alter neither systemic iron homeostasis in pregnant sows nor their hematological status at the end of pregnancy. Moreover, we did not detect any changes of iron content in the milk and colostrum of iron supplemented sows in comparison to controls. Neonatal iron status of piglets from iron supplemented sows was not improved compared with the progeny of control females. No statistically significant differences were found in average piglets weight and number of piglets per litter between animals from experimental groups. The placental expression of iron transporters varied depending on the iron supplement.

Keywords: sucrosomial ferric pyrophosphate; iron deficiency anemia; pig; pregnancy; iron supplementation

1. Introduction

In humans, maternal and fetal iron needs exponentially accelerate during the last trimester of pregnancy [1]. Most of gestational iron demand results from the increase in maternal red blood cell (RBC) mass and placental and fetal growth [2]. To meet iron requirements during pregnancy, both absorption of dietary iron and mobilization of this microelement from hepatic stores are enhanced. Iron deficiency during pregnancy is associated with growth retardation, premature birth, low birth weight, muscle dysfunction and low physical capacity [3–6]. Even in developed countries many women enter pregnancy with insufficient iron stores and dietary iron intake during pregnancy remains consistently below nutritional recommendations [7]. Nowadays, the World Health Organization (WHO) recommends daily iron supplementation for all pregnant women [8]. However, it is questionable whether iron supplements given to healthy, non-anemic women may improve maternal iron status (concentration of iron in colostrum, milk and hepatic iron stores), influence fetus development and neonatal Apgar score [9,10].

The pig is being increasingly used in biomedical research for studies of human diseases that are not accurately represented by rodent models [11–13]. For example, the pig model of neonatal iron deficiency anemia (IDA) meticulously reflects the main etiological factor of this defect observed in pre-term human neonates, that is, those having critically low iron content in their livers [14]. Since the molecular potential of iron uptake from the diet in neonates is greatly reduced [15] hepatic iron stores established through maternal-fetal transfer represents the primary source of this microelement to cope with the metabolic demands of developing organisms in the neonatal period. In both, pig term and human preterm neonates insufficient initial iron stores are considered a primary and probably most important etiological factor in the development of neonatal IDA. However, while in preterm human neonates the shortage of stored iron results from shortened period of iron deposition in the fetal liver, in term newborn piglets the main reason is the physiological inability of pregnant sow to meet iron demand for the greater number of fetuses. Several studies have attempted to increase the level of iron hepatic iron stores in fetuses by treating pregnant sows with iron supplements [16–22]. However, supplementation of sows at various stages of pregnancy, using various iron supplements administered

orally or parenterally has no significant impact on the improvement of the iron status of newborn piglets and thus does not prevent suckling animals from becoming anemic (reviewed in Reference [23]).

Recently, liposomal, Sucrosomial® technology became a powerful and promising new formula of sucrosomial ferric pyrophosphate (SFP), non-heme iron characterized by increased bioavailability and reduced toxicity [24,25]. SFP represents an innovative oral iron-containing carrier in which ferric pyrophosphate is protected by a phospholipid bilayer membrane mainly from sunflower lecithin and sucrester matrix [26]. Sucrester is a surfactant derived from the esterification of fatty acids with sucrose (sucrose esters), which has recently been shown to behave as absorption enhancer, because of its ability to reduce intestinal barrier resistance. So far, very promising experiment regarding efficacy of SFP supplementation during human pregnancy has been performed [27].

This study was conducted to determine whether daily oral supplementation of healthy pregnant sows with SFP containing 60 mg Fe/kg of feed during pregnancy is a safe and effective procedure, improving iron status of sows and assuring a rise in the content of iron in hepatic stores in their offspring. Considering that mechanisms of iron transfer across the placenta are far from being fully elucidated in pigs, in our study we also aimed at investigating pathways of iron trafficking across the placenta. Finally, we evaluated pregnancy outcomes in SFP supplemented sows compared with females given ferrous iron sulfate ($FeSO_4$) and non-supplemented controls.

2. Materials and Methods

2.1. Sows and Piglets, Experimental Design and Biological Sample Collection

The experiment was conducted at the Pig Hybridization Centre in Pawłowice belonging to the National Research Institute of Animal Production (Balice, Poland). As shown in Figure 1, total of 15 healthy, non-anemic pregnant 990 line sows (according to veterinary examination and hematological indices shown in Table 1.) and their offspring (sows were mate with the same boar) housed in standard conditions (70% humidity and a temperature of 22 °C in cages with straw bedding) were used. The sows during gestation were in gestation cage (dimensions 2.2 × 0.65 × 1.8 m). Sows were taken to the farrowing cages at 110^{th} day of gestation (dimensions 2.4 × 3.4 m). A gestation crate is used for pregnant sows, which can effectively prevent them from fighting for food, biting and is conducive to sow miscarriage, Sows were fed individually within these cages and at gestation sows were fed 3.0 kg/day of fed given in two portions and water was available ad libitum. Sows used in experiment were in second parity order and had an average weight of 213.90 ± 22.49 kg. Sows were randomly allotted to control and 2 iron supplemented groups. Control females were offered until delivery a standard fodder for pregnant sows routinely used in swine industry (containing 80 mg Fe/1 kg as estimated by flame spectrometry). Fodder was designed to fulfill the National Research Council (NRC) [27] iron requirements for pregnant sows (Supplementary Table S1). In the first experimental group, sows were fed with standard fodder and starting from day 80 of pregnancy up to delivery were orally supplemented with additional iron in the form of sucrosomial ferric pyrophosphate (SFP) (SiderAL®, PharmaNutra, Pisa, Italy) and given in the amount of 60 mg Fe daily. In the second experimental group, pregnant sows were supplemented with iron in the form of ferrous sulfate ($FeSO_4$, Gambit, Kutno, Poland) added to the standard fodder and given to sows according to the same timing and dosage as in the SFP group. Blood from sows was drawn on days 80 and 115 of gestation by venipuncture of the jugular vein (*Vena jugularis externa*). Blood and tissue samples from piglets were collected 24 hours after birth. Piglets were euthanized by intracardiac injection of 0.5 mL/kg body weight of Morbital® (133.3 mg/mL of sodium pentobarbital + 26.7 mg/mL of pentobarbital; Biowet, Puławy, Poland). The blood samples from sows and piglets were collected into tubes coated with heparin as an anticoagulant, centrifuged (1200× *g*, 10 min, 4°C) to separate the plasma. Plasma samples were immediately aliquoted and stored at −80 °C. Tissue samples collected from piglets for molecular analyses were rinsed with PBS and snap frozen in liquid nitrogen then stored at −80 °C. Placenta samples were collected immediately after parturition, snap frozen in liquid nitrogen for molecular

analyses, other placenta samples were fixed with paraformaldehyde for immunofluorescence analyses. Colostrum samples were collected immediately after delivery and milk samples 48h after delivery, both were stored at −20 °C until further analysis.

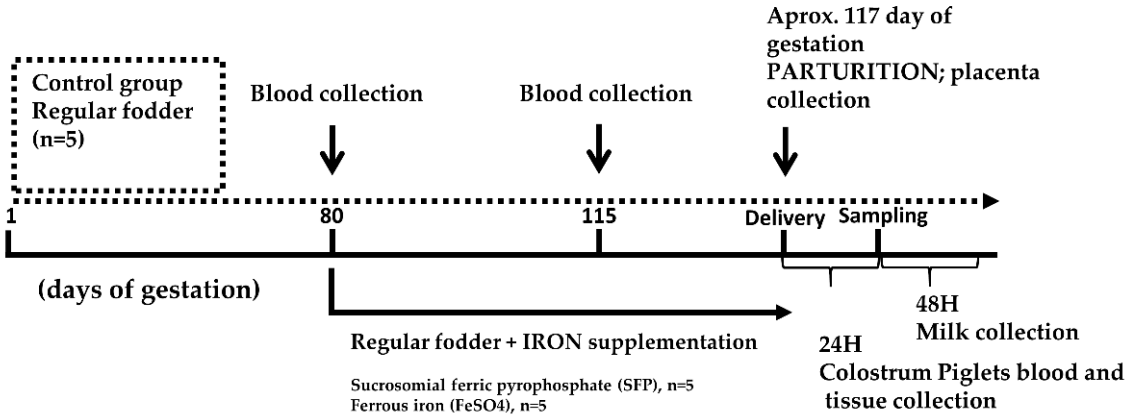


Figure 1. Experimental design scheme. Pregnant sows were allotted to 3 groups: (i) control group receiving a standard fodder; (ii) group supplemented with ferrous iron sulfate ($FeSO_4$); (iii) group supplemented with sucrosomial ferric pyrophosphate (SFP). Both supplementations were applied daily between day 80 of pregnancy and delivery (approximately day 117 of pregnancy). Blood samples were collected from sows on days 80 and 115 of pregnancy. Samples of placenta were collected immediately after delivery. Tissue and blood samples from piglets were collected 24 h after birth.

Table 1. Red blood cell (RBC) indices and plasma iron status in sows at days 80 and 114 of pregnancy.

Experimental Groups	Gestational Days		Supplementation		Time		Supplementation * Time		*p*-Value 80 vs. 114 Day
	Day 80	Day 114	F	*p*-Value	F	*p*-Value	F	*p*-Value	
	Hb (g/dL)								
Control	12.2 ± 0.8	11.7 ± 1.9							0.9519
SFP	9.8 ± 2.9	11.0 ± 0.6	6.539	0.0311*	0.1384	0.7227	0.5869	0.5851	0.9993
$FeSO_4$	11.1 ± 0.88	10.2 ± 2.24							0.7189
	RBC (mln/mm^3)								
Control	6.2 ± 0.6	5.8 ± 1.1							>0.9999
SFP	5.3 ± 1.7	5.6 ± 0.3	6.480	0.0317*	1.504	0.2660	0.5245	0.6167	0.8496
$FeSO_4$	5.7 ± 0.6	6.9 ± 4.6							0.5106
	MCV (μm^3)								
Control	61.2 ± 3.1	63.2 ± 2.7							0.192
SFP	58.4 ± 4.9	62.8 ± 3.5	0.2633	0.7769	38.23	0.0008 **	1.409	0.3150	0.0132*
$FeSO_4$	62.4 ± 4.9	64.5 ± 4.5							0.0198*
	Plasma iron level (μmol/L)								
Control	15.6 ± 3	12.3 ± 4.3							0.8294
SFP	14.4 ± 4.7	12.8 ± 2.8	0.02066	0.9796	4.767	0.0717	0.1594	0.8562	0.4135
$FeSO_4$	15.3 ± 1.8	12.8 ± 1.3							0.5222
	Plasma ferritin concentration (ng/mL)								
Control	514.3 ± 117.4	199.8 ± 57							0.0597
SFP	557.9 ± 67.5	231.1 ± 56.4	0.8294	0.4808	29.20	0.0017**	0.7431	0.5148	0.0218*
$FeSO_4$	402.7 ± 180.4	176.4 ± 85.3							0.1831

Table 1. *Cont.*

Experimental Groups	Gestational Days		Supplementation		Time		Supplementation * Time		*p*-Value 80 vs. 114 Day
	Day 80	Day 114	F	*p*-Value	F	*p*-Value	F	*p*-Value	
	Plasma hepcidin level (nM)								
Control	2.33 ± 0.8	0.8 ± 0.42							0.5571
SFP	6.3 ± 1.58	0.96 ± 0.36	3.979	0.0794	41.62	0.0007 **	4.506	0.0638	0.0115 *
$FeSO_4$	7.2 ± 2.43	1 ± 0.45							0.0055 **

Data are presented as mean values ± SD. Statistical analysis of two factors have been performed by two-way ANOVA for repeated measurement. Two factors analyzed in two-way ANOVA were "Time," "Supplementation" and their interaction. SFP = sucrosomial ferric pyrophosphate, $FeSO_4$ = ferrous iron sulfate RBC = red blood cell count, Hb = hemoglobin level, MCV = mean corpuscular volume, F = variance of the group means/mean of the within group variances. All parameters were determined for 5 sows from each experimental group. * and ** asterisks denote statistically significant differences at $p < 0.05$ and $p < 0.01$.

2.2. Measurement of Red Blood Cell Indices and Plasma Iron Level

Hematological indices were determined using an automated ADVIA 2010 analyzer (Siemens, Erlangen, Germany). The plasma iron concentration was determined by colorimetric measurement of an iron-chromazurol complex according to the manufacturer's protocol (Biomaxima S.A., Lublin, Poland) as previously described [28].

2.3. Measurement of Non-Heme Iron Content in Tissues

The non-heme iron content of liver, spleen (100 mg wet tissue; wt) and milk and colostrum (1 mL) were determined by acid digestion of the samples at 100 °C for 10 min, followed by colorimetric measurement of the absorbance of the iron-ferrozine complex at 560 nm as previously described [29].

2.4. Plasma Hepcidin-25 Measurement

Piglet plasma hepcidin-25 measurements were performed by a combination of weak cation exchange chromatography and time-of-flight mass spectrometry (WCX-TOF MS), as described previously for pig plasma [30] and urine [31]. Peptide spectra were generated on a Microflex LT matrix-enhanced laser desorption/ionization TOF MS platform (Bruker Daltonics, Billerica, MA, USA).

2.5. Real-Time Quantitative RT-PCR

Total cellular RNA was extracted from liver and placental tissue (20 mg) using Trizol reagent (Invitrogen) according to the manufacturer's protocol. Two micrograms of total DNAse-treated RNA were reverse transcribed using a Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany). Real-time quantitative polymerase chain reaction (PCR) analysis was performed in a Light Cycler U96 (Roche Diagnostics, Mannheim, Germany) using gene-specific primer pairs (Supplementary Table S2. The amplified products were detected using SYBR Green I (Roche Diagnostics, Mannheim, Germany) as described previously [32]. To confirm amplification specificity, the PCR products were subjected to melting curve analysis and agarose gel electrophoresis. Light Cycler U96 Software (Roche Diagnostics, Mannheim, Germany) was used for data analysis. Transcript levels were normalized relative to the control reference gene selected using NormFinder software (Aarhus, Denmark) [33] (https://moma.dk/normfinder-software).

2.6. Immunofluorescence (IF) Analysis and Confocal Microscopy of Placental Sections

After delivery, pig placentas were immediately dissected and fixed in 4% paraformaldehyde (Sigma-Aldrich, Poznan, Poland) in phosphate-buffered saline (PBS) at 4 °C for 24 hours. Following two 30 min washes in PBS, the tissues were successively soaked in 12.5 and 25% sucrose (Bioshop, Burlington, Ontario, ON, Canada) for 24 h and 7 days, respectively, at 4 °C. Placenta was embedded

in Cryomatrix medium (Thermo Fisher Scientific, Warsaw, Poland), frozen in liquid nitrogen and sectioned in 10-µm slices using a cryomicrotome (Shandon, London, UK).

The sections were washed in PBS for 10 min and permeabilized by bathing in PBS/0.1% Triton X-100 (Sigma-Aldrich, Poznan, Poland) for 20 min. Non-specific antibody binding was blocked by incubating the tissue sections in PBS/5% Bovine Serum Albumin (BSA) (Bioshop, Burlington, Ontario, ON, Canada) for 1 h. For protein detection, sections were incubated overnight at room temperature with primary antibodies diluted in PBS/5% BSA. As a negative control, some sections were incubated without primary antibody. The primary and fluorochrome-conjugated secondary antibodies used in IF analysis are described in Supplementary Table S2. Next, the sections were washed for 5 × 6 min with PBS/0.1% Triton X-100 and incubated for 1 h with secondary antibody diluted in PBS/5% BSA at RT. Finally, sections were washed for 5 × 6 min in PBS and additionally stained with Hoechst (Thermo Fisher Scientific, Warsaw, Poland) for 2 min then washed 2 times with PBS and mounted in glycerol based medium. The antibodies used can be found in Supplementary Table S3.

2.7. Placental Samples and Fixation for Transmission Electron Microscopy (TEM)

Five placentas per experimental group were obtained from sows following normal, uncomplicated births and pregnancies. Small pieces of basal plate (10 × 10 × 2 mm) were excised within minutes of delivery (expelled placentas) and fixed for 1 h in 4% paraformaldehyde, 0.4% glutaraldehyde in sodium cacodylate trihydrate (Acros organics, Geel, Belgium) pH 7.2, followed by 1% OsO_4 (Thermo Fisher, Kandel, Germany) for 16 h in 4 °C. Samples were then dehydrated through an ethanol series (30; 50; 70%). Samples were dried using Leica EM CPD300 Critical Point Dryer (Leica, Wetzlar, Germany) and sputtered with gold using Low Vacuum Coater Leica EM ACE200 (Leica, Wetzlar, Germany). Transmission electron microscopy was conducted using COXEM EM-30AXplus SEM (Yuseong-gu, Daejeon, Korea) microscope. Transmission Electron Microscopy (TEM) images can be found in supplementary data—Supplementary Figure S1.

2.8. Ethic Statement

The experimental procedures used in this study were in compliance with the European Union (EU) guidelines for the care and handling of research animals (EU Directive2010/63/EU for animal experiments). Second Local Ethical Committee on Animal Testing at the Warsaw University of Life Sciences in Warsaw (Poland) granted a formal waiver of the ethical approval because the only procedure involved in the study was piglets euthanasia and blood collection from pregnant sows. All these procedures are the routine veterinary procedures. Moreover, administration of a dietary supplement such as sucrosomial ferric pyrophosphate (SFP) is not categorized as a research procedure.

2.9. Statistical Analysis

Statistical analysis of iron supplementation in one-day-old piglets was performed using one-way analysis of variance (ANOVA) with type of supplementation as the factor followed by Tukey's multiple comparisons test. The results from sows with iron supplementation were calculated by two-way ANOVA, using type of supplementation and time as the factors followed by Sidak's multiple comparisons test. Statistical analysis and figures were prepared using GraphPad Prism version 8.00 for Windows (GraphPad Software, La Jolla, CA, USA). *p*-value, $p \leq 0.05$ and $p \leq 0.01$ were considered significant and are denoted with asterisks * and ** respectively.

3. Results

3.1. Preganant Sows Supplemented with SFP and $FeSO_4$ Show No Changes in Hematological and Iron Status Compared with Non-Suplemented Controls

At the starting point of the experiment, that is, on day 80 of pregnancy, values of red blood cell (RBC) indices measured in all sows involved in the study were similar (Table 1). Likewise, sows

from different experimental groups showed no differences in plasma iron parameters at this stage of pregnancy (Table 1). As pregnancy progressed (day 114) RBC indices remained unchanged with the exception of some fluctuations in RBC count and hemoglobin level in $FeSO_4$ supplemented sows. In contrast, in advanced pregnancy, a concerted, statistically significant decrease in plasma iron, ferritin and hepcidin levels was observed in sows from all experimental groups. Changes in these parameters indicate decrease in iron stores in sows from all experimental groups (regardless of iron supplementation) at the end of pregnancy. At the same time, on day 114 of pregnancy there were no differences in plasma iron status between control and iron supplemented sows.

3.2. Supplementation of Pregnant Sows with Iron Did Not Influence Iron Content in the Colostrum and Milk

To verify whether supplementation of pregnant sows with iron increases the concentration of this microelement in the milk and colostrum, we evaluated this parameter in sows from all experimental groups. Iron content in these biological fluids was not significantly affected by oral administration of neither SFP nor $FeSO_4$ to pregnant sows compared with non-treated females (Figure 2). Although a slight up regulation of iron content in supplemented groups was observed but it is was not statistically significant.

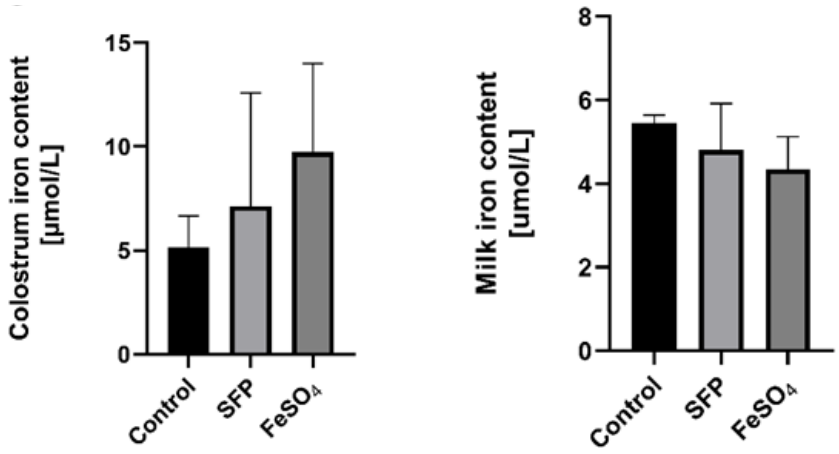


Figure 2. Effect of ferrous iron sulfate ($FeSO_4$)and sucrosomial ferric pyrophosphate (SFP) oral supplementation of pregnant sows on milk and colostrum iron concentrations. The non-heme iron content was measured in colostrum and milk as described in the Materials and Methods section. Values are expressed as the means ± S.D. for 1 mL samples obtained from sows immediately (colostrum) and 24 h after delivery (milk) (n = 15).

3.3. Red Blood Cell Indices and Plasma Iron Status in Piglets from Control and Iron-Supplemented Sows

Results shown in Table 2. clearly demonstrate that supplementation of pregnant sows either with SFP or $FeSO_4$ did not improve RBC indices and plasma iron status of their piglets. Importantly, on day 1 after birth hemoglobin concentration in all newborn piglets was above the threshold value of anemia in this range of age, 8 g/dL) [33].

Table 2. Red blood cell indices and plasma iron status in 1-day old piglets from control and SFP and $FeSO_4$-supplemented sows.

Blood Parameters	Control	SFP	$FeSO_4$	*p*-Value	
				Control *vs.*	
Hb (g/dL)	9.4 ± 1.4	9.8 ± 1	9 ± 1.3	SFP 0.6906	$FeSO_4$ 0.7161
RBC mln/mm^3	4.9 ± 0.7	5.1 ± 0.9	4.69 ± 0.6	SFP 0.8252	$FeSO_4$ 0.7332
MCV (μm^3)	60 ± 2.7	63.1 ± 3.7	61.6 ± 4	SFP 0.1301	$FeSO_4$ 0.5538
Serum Iron (μmol/L)	14.8 ± 9.9	10.5 ± 8.4	18 ± 8.1	SFP 0.2284	$FeSO_4$ 0.9994
Serum Ferritin (ng/mL)	823.5 ± 161.4	906.2 ± 75.1	772.7 ± 137.6	SFP 0.3656	$FeSO_4$ 0.6631

Effect of ferrous iron sulfate ($FeSO_4$) and sucrosomial ferric pyrophosphate (SFP) oral supplementation on blood parameters in one day old piglets. RBC—red blood cell count, Hb—hemoglobin level, MCV—mean corpuscular volume. All parameters were determined for 5 sows from each experimental group. n = 9 piglets per experimental group.

3.4. Supplementation of Pregnant Sows with SFP or $FeSO_4$ Does Not Alter neither Iron Content in the Placenta nor Hepatic and Splenic Iron Status of their Progeny

Importantly in context to pig farming and fattening, the number of piglets in the litter remains unchanged regardless of iron supplementation procedure in comparison to untreated sows. Similarly, piglets from sows receiving different iron supplementations or without iron treatment showed an equal body weight, measured one day after delivery (Supplementary Figure S2). Iron transfer across the placenta is the only pathway providing this microelement from mother to developing fetuses. Although non-heme iron content in the placenta of sows supplemented with SFP or $FeSO_4$ showed a downward trend compared with control females, no statistically significant differences were found (Figure 3). This indicates that despite administration to sows of various iron supplements, iron flux through the placental barrier remained similar in sows from all experimental groups. This is reflected in almost equal iron accumulation in piglets' liver and spleen, key organs for handling systemic iron (Figure 3).

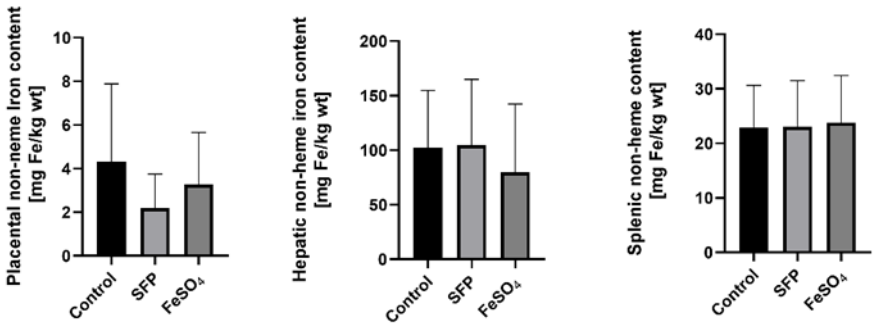


Figure 3. Non-heme iron content in placenta from sows after ferrous iron sulfate ($FeSO_4$) and sucrosomial ferric pyrophosphate (SFP) supplementation and hepatic and splenic non-heme iron content from 1-day old piglets. Non-heme iron content in analyzed tissue was measured as described in Materials and Methods. Values are expressed as the mean and SD (standard deviation) for samples obtained from 9 piglets in each experimental group. Non-heme iron content was expressed as mg Fe/kg of wet tissue.

3.5. Increased Hepcidin and Bone Morphogenetic Protein 6 (BMP6) Expression in the Placenta and in the Liver of Piglets from Sows Supplemented with SFP

Hepcidin, the master regulator of systemic iron metabolism, is mainly synthesized and released by hepatocytes in response to increased body iron concentration [34–41]. Hepcidin expression has been also reported in many other mammalian cells including placenta [42–49]. Although the function of tissue-specific hepcidin remains unknown it has been suggested that local autocrine regulation based on locally produced hepcidin may operate in several tissues [50]. To evaluate the effect

of oral administration of pregnant sows with SFP on hepcidin expression, we measured hepcidin mRNA abundance in the liver of piglets and in the placenta. In tissues collected form the group of SFP-treated sows, hepcidin expression was increased in the liver (statistically significant difference) and in the placenta (a strong upward trend) compared with tissues obtained from control animals (Figure 4). In contrast to the effect of SFP on hepcidin hepatic mRNA expression in piglets, no induction of hepcidin mRNA expression was observed in livers from piglets derived from mothers supplemented with $FeSO_4$ (Figure 4). To check whether hepatic expressional pattern of hepcidin is reflected in the level of hepcidin peptide circulating in the blood, we measured the concentration of active hepcidin-25 in the blood plasma of piglets. We found a similar trend in plasma hepcidin concentration, that is, strong increase in piglets from SFP-treated sows compared with controls and basic level in piglets from pregnant sows supplemented with $FeSO_4$ (Figure 4).

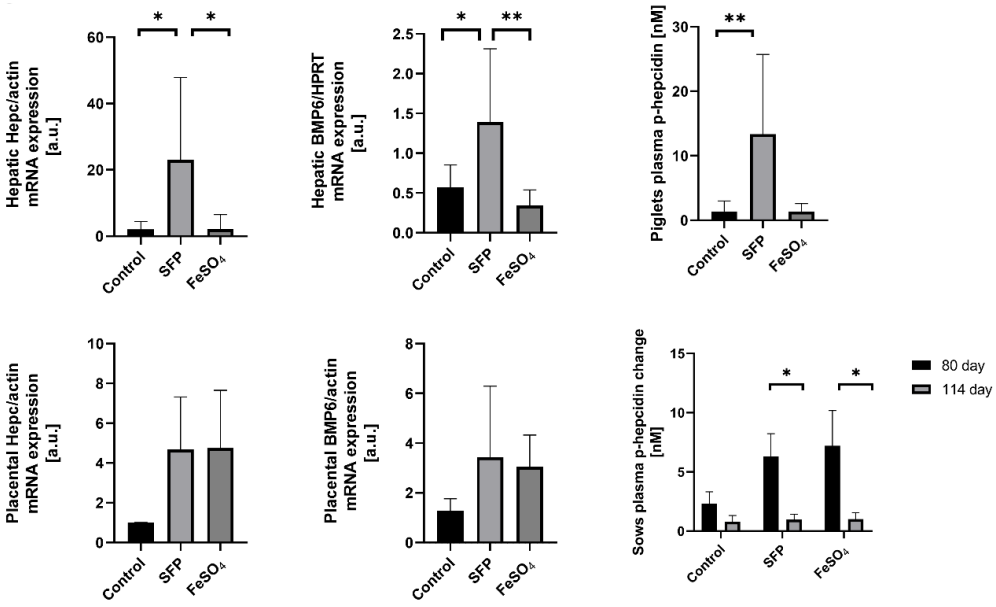


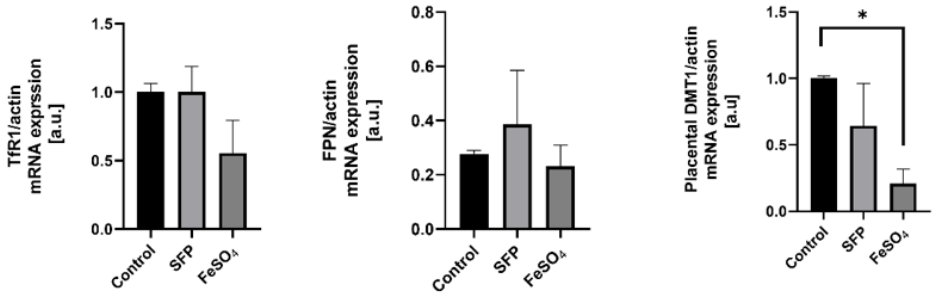
Figure 4. Changes in placental and fetal hepatic and plasma hepcidin and Bone Morphogenetic Protein 6 (BMP6) levels under different oral iron supplementations. Fetal hepatic and placental hepcidin and BMP6 mRNAs levels were measured using Real Time polymerase chain reaction (PCR) and normalized to actin/Hypoxanthine Phosphoribosyltransferase (HPRT) mRNA. Hepcidin-25 (p-hepcidin) measurements in sows and piglets plasma were performed by peptide enrichment through weak cation exchange chromatography coupled to time-of-flight mass spectrometry (WCX-TOF MS) (Bruker Daltonics, Billerica, Massachusetts, United States) [29,30]. * and ** asterisks denote statistically significant differences between parameters in control and ferrous iron sulfate ($FeSO_4$) or sucrosomial ferric pyrophosphate (SFP) supplemented group at $p < 0.05$ and $p < 0.01$ respectively.

Bone morphogenetic protein 6 (BMP6) is a central regulatory factor that increases hepatic hepcidin expression in response to iron. Sinusoidal hepatic endothelial cells are the predominant source of BMP6 in the liver, which acts in a paracrine manner by binding to complex BMP6 receptor on hepatocytes to induce hepcidin transcription [51]. Therefore, we attempted to answer whether BMP6 is involved in hepcidin regulation in the placenta and in the liver of 1-day old piglets after iron supplementation of sows. We found that in both tissues expression pattern of BMP6 perfectly overlaps that of hepcidin in animals from all analyzed groups. This strongly suggests the involvement of BMP6 in the regulation of hepcidin expression under our experimental conditions.

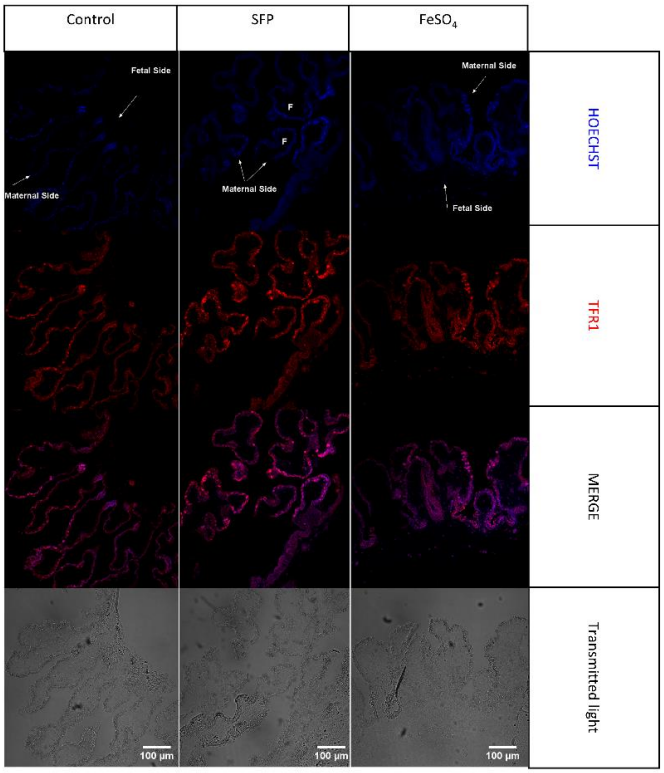
3.6. The Effect of Oral Iron Supplementation on Placental Morphology and Expression of Iron Transporters

The placenta serves as the interface between mother and fetus and it mediates nutrient transport to the fetus including the transport of iron. Uni-directional transfer of iron transported by the placenta

from the maternal to the fetal circulation is effected by transferrin receptor 1 (TfR1), divalent metal transporter 1 (DMT1) and ferroportin (Fpn), located on the apical and basolateral membrane of syncytiotrophoblats, respectively [52]. To determine the influence of supplementation of pregnant sows with SFP on iron transporters expression and localization in the placenta, we analyzed mRNA abundance of TfR1, DMT1 and Fpn as well as localization of these three proteins responsible for iron flow across the placenta. Administration of SFP to pregnant sows had no significant effect on the level of transcripts encoding TfR1, DMT1 and Fpn compared with controls. In contrast, we observed a downward tendency in the expression of analyzed genes in the placenta from sows supplemented with $FeSO_4$. In the case of DMT1, this downregulation was statistically significant compared to controls (Figure 5A). We also showed that in placentas from three experimental groups principal transporters mediating cellular iron uptake and efflux are abundantly and equally expressed in syncytiotrophoblasts (STB) on the maternal and fetal sides, respectively (Figure 5B–D). This indicates that the efficiency of iron transport across the placentas from sows from all groups is similar.

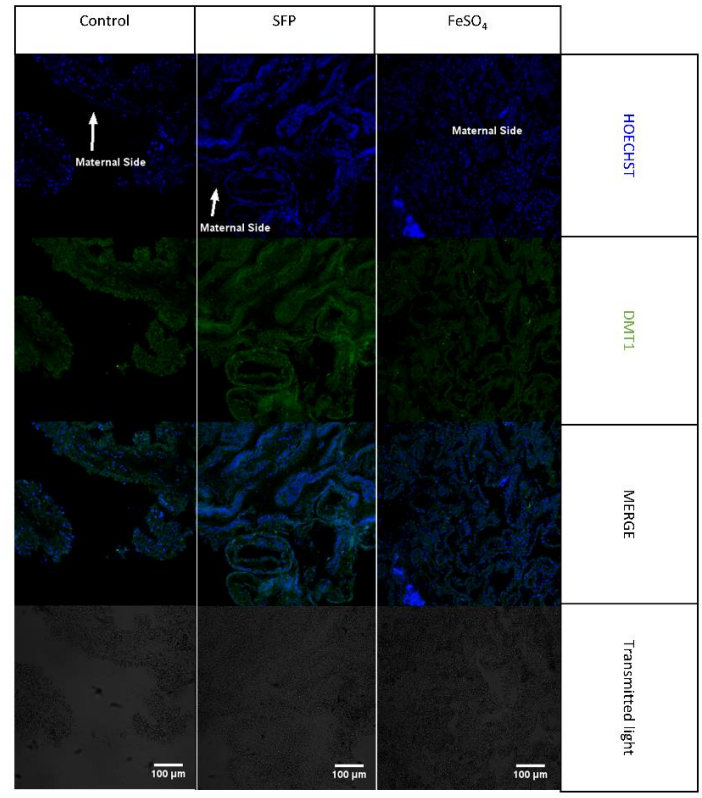


(A)

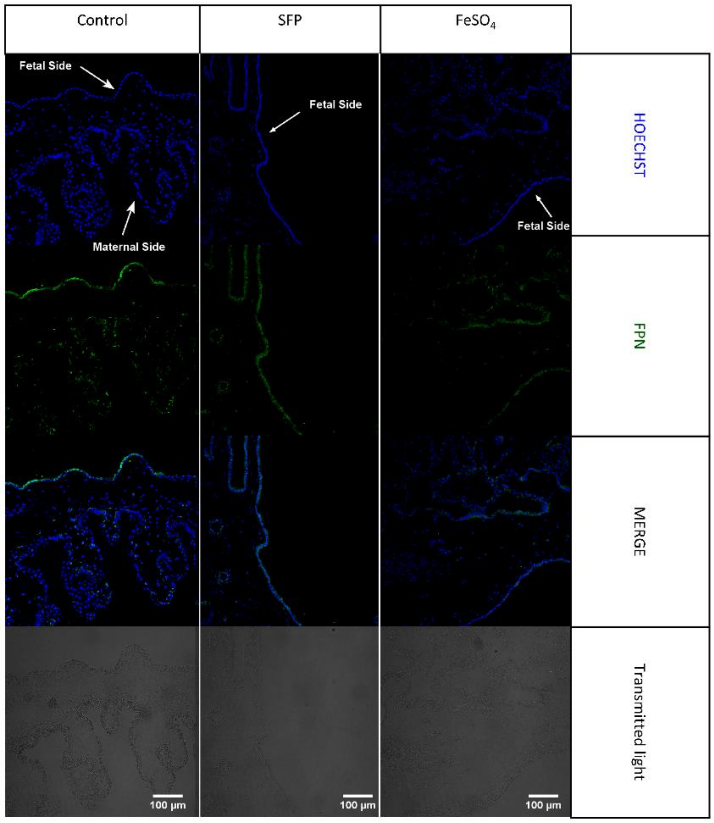


(B)

Figure 5. *Cont.*



(C)



(D)

Figure 5. (**A**). Regulation of placental iron transporters after oral administration of ferrous iron sulfate ($FeSO_4$) or sucrosomial ferric pyrophosphate (SFP) to pregnant sows. Placental iron transporters mRNA

expression measured using Real time PCR normalized to actin mRNA. * asterisk denote statistically significant differences between parameters in control and SFP or $FeSO_4$ supplemented group at $p < 0.05$. (**B**). Localization of placental iron transporters after oral administration of ferrous iron sulfate ($FeSO_4$) or sucrosomial ferric pyrophosphate (SFP) to pregnant sows. Representative immunofluorescence staining for placental iron transporters: TfR1. Scale bars correspond to = 100 µm. Cell nuclei were counterstained with Hoechst (blue). Arrows indicate maternal or fetal site of syncytiotrophoblast (STB). Series sections of placental tissue from 15 sows was analyzed and representative immunofluorescence photos were prepared. (**C**). Localization of placental iron transporters after oral administration of ferrous iron sulfate ($FeSO_4$) or sucrosomial ferric pyrophosphate (SFP) to sows. Representative immunofluorescence staining for placental iron transporters: DMT1. Scale bars correspond to = 100µm. Cell nuclei were counterstained with Hoechst (blue). (**D**). Localization of placental iron transporters after oral administration of ferrous iron sulfate (FeSO4) or sucrosomial ferric pyrophosphate (SFP) to pregnant sows. Representative immunofluorescence staining for placental iron transporters: Fpn. Scale bars correspond to = 100µm. Cell nuclei were counterstained with Hoechst (blue).

To check the potential effect of SFP on the integrity and morphology of placenta we performed an exhaustive analysis of placenta using scanning electron microscopy at different scanning resolutions. We did not observe any visible morphological damages/changes at the surface of syncytiotrophoblasts collected from sows either supplemented with SFP or $FeSO_4$ compared with controls. (Supplementary Table S1). This result is consistent with previous studies indicating low toxicity SFP [24,53,54].

4. Discussion

The pig is an interesting experimental animal model for studying iron supplementation during pregnancy. While pregnant sows of most contemporary pig breeds are usually iron replete and do not manifest symptoms of iron deficiency, their progeny regularly develops IDA approximately 3–4 days after birth [23,55–57]. The fundamental cause of neonatal anemia in pigs is a drastic imbalance between poor iron supply and high iron demand. Poor availability of iron in newborn piglets occurs due to extremely low level of hepatic iron stores (the lowest in mammalian neonates) [14,29,55,58–62] and low iron content in the colostrum/milk [63], accompanied by inefficient absorption of dietary iron [55]. On the other hand, high iron needs are determined by unusually rapid rate growth of piglets (they increase 10-fold their body mass within 6 weeks of birth) [15,55,60,61,64]. The concept of replacing routine, largely non-physiological postnatal parenteral supplementation of piglets with iron dextran [65] by administration of iron to pregnant sows to prevent suckling animals from becoming anemic has emerged in several studies [66]. The rationale behind such a procedure is to increase iron status of pregnant females, intensify iron transfer across the placenta from the mother to the fetuses, consequently increasing hepatic iron content, which can serve as a source of this microelement in piglets during early postnatal development. Among potential benefits of this treatment include reduction of the labor input and improving the welfare of supplemented young animals. However, this treatment is highly challenging considering its possible adverse effects on sow's iron metabolism, the risk of iron toxicity and insufficiency of the molecular machinery involved in transplacental iron transport. Indeed, supplementation of sows at various stages of pregnancy, using various iron supplements (iron salts/chelates, iron dextran) administered orally or parenterally has no significant impact on the improvement of iron status of newly born piglets and has not been proven in prophylaxis of neonatal IDA in piglets [16,19–21,67–72]. Despite these negative results, in this study we attempted to test the efficacy of prenatal oral supplementation with SFP through administration of this compound to pregnant sows. SFP is an innovative preparation of ferric pyrophosphate, covered by phospholipids plus sucrester matrix, with high bioavailability, capacity to overcome gastrointestinal barriers and tolerability [23–26,53,54]. Importantly, the efficacy and no side effects of SFP administration have been reported in pregnant women with iron deficiency [26]. Here, we show that despite supplementation of sows from day 80 of pregnancy with SFP, their iron status few days before delivery was decreased similarly to sows supplemented with $FeSO_4$ or fed with control diet. However, this moderate decrease

did not compromise RBC status of pregnant females. An uniform drop in ferritin plasma levels (in sows from all experimental groups), a marker of hepatic iron stores [73], strongly indicates that to maintain erythropoietic activity of pregnant females, iron is preferentially released from the liver. Similarly, decrease in the concentration of plasma hepcidin observed in sows from all groups on day 114 of pregnancy results from, at least, partial depletion of iron stores. It is known that under conditions of enhanced erythropoiesis observed in late pregnancy [74–76], hepcidin is down-regulated independently from iron deficiency by erythroid factors produced by erythroblasts that act on hepatocytes to suppress hepcidin synthesis [77]. Indeed, it has been reported that not only anemic mothers [77] but also mothers with iron-replete stores [78] show low hepcidin expression at delivery [79]. The possible explanation for hepcidin suppression in pregnant females (even in those supplemented with iron) is the need to increase endogenous maternal iron supply for extra gestational requirements by enhanced iron mobilization from stores, increased iron absorption and accelerated recycling of iron derived from senescent erythrocytes.

Most importantly for this study, supplementation of sows with SFP failed to reinforce iron stores in newborn piglets. All iron indexes such as hepatic and splenic iron content, blood plasma iron parameters attest ineffectiveness of SFP supplementation of sows to improve iron status in piglets. These results collectively with aligned non-anemic RBC pattern of piglets from all experimental groups, confirm previous reports demonstrating that 1-day old piglets born from sows either supplemented with iron preparations or not, do not yet show symptoms of IDA [47]. We hypothesize that the failure in the reinforcement of piglets iron status in response to SFP administration to pregnant sows is associated with the limited molecular potential of the placenta to increase iron transport from the mother to fetuses. Immunofluorescent analysis of 3 main iron transporters such as TfR1, DMT1 and Fpn clearly shows their analogous distribution and similar intensity of fluorescent signal in placentas from all experimental groups. Accordingly, loading pregnant mice with iron showed no influence on the protein level of TfR1 and Fpn in the E18.5 placenta compared with the placenta from females fed standard iron diet [78]. Surprisingly, we noticed that administration of SFP to pregnant sows consequently induced hepcidin expression in the placenta (increase in the mRNA level) and in piglets (increase in the hepatic mRNA level and in the concentration of circulating peptide in the blood) and overlapped the rise in the expression of placental and hepatic BMP6, factor that increases hepatic hepcidin in response to iron [34]. This set of data strongly suggest the involvement of discreetly increased signaling (regulatory) intratissular iron pool, which was not detectable using our analytical methods. Up-regulation of hepcidin may be a part of control mechanisms protecting fetuses and newborns from excessive iron transport through ferroportin from the placenta to the fetal circulation and therefore from exacerbated iron toxicity associated with iron overload [80]. Alternatively, it is likely a consequence of the ancestral regulation of iron transport across the placenta functioning in wild boar. In contrast to domestic pig progeny, hepatic iron stores in wild boar piglets are adequate to meet iron needs for erythropoiesis probably because during pregnancy iron transferred from the wild boar mother has to be distributed among only 4–6 fetuses [81] instead of 10–14 in domestic pig sows of high performance contemporary breeds.

5. Conclusions

In conclusion, it seems that SFP is an efficient iron supplement only in iron deficient subjects [25,26,53,54] including iron deficient pregnant mothers as it has been demonstrated in humans [26]. When pregnant females are iron replete or show slightly decreased iron status as is the case of pregnant sows, supplementation with SFP is inefficient. The mechanism of prevention of excessive iron accumulation in the body upon treatment with SFP in purpose to increase iron stores is an interesting challenge for future research. Taken together, the results of this study demonstrate the effectiveness of daily oral dose of 60 mg SFP on occurrence and prevention of anemia during swine pregnancy. In perspective the research challenge is to use oral iron supplementation to treat IDA during pregnancy in a pig model of maternal anemia. The importance of iron for feto-maternal

health and fetal development during pregnancy cannot be overestimated. Therefore the question of how iron is transported from mother to fetus is still open. There are still candidates for placental iron transport, such as ZIP8, ZIP14 or FLVCRa and b or heme iron transporters, whose altered expression may be associated with fetal or maternal iron status [82,83]. One should not forget about the possibilities of non-transferrin bound iron (NTBI) transport through the placenta. Such iron appears in the blood mostly in hemochromatic patients when saturation of transferrin exceeds 70% or 80%. There are also evidence that in pregnant woman taking iron supplements NTBI may rise [84]. Thus future research should take into account both the above-mentioned transporters as well as should explain the mechanisms of iron transport across the placenta and their effect on the regulation of iron metabolism during healthy and complicated pregnancy.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-2615/10/7/1113/s1, Table S1. The composition and chemical analysis of the basal diet for pregnant sows, Table S2: Gene specific primers sequences used in RT-PCR analyses, Table S3: Antibodies used in immunofluorescence analyses, Figure S1. Illustration of the surface of placental tissues acquired using T.E.M microscopy, Figure S2. Pregnancy outcome.

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RESEARCH ARTICLE

Marginally reduced maternal hepatic and splenic ferroportin under severe nutritional iron deficiency in pregnancy maintains systemic iron supply

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Abstract

The demand for iron is high in pregnancy to meet the increased requirements for erythropoiesis. Even pregnant females with initially iron-replete stores develop iron-deficiency anemia, due to inadequate iron absorption. In anemic females, the maternal iron supply is dedicated to maintaining iron metabolism in the fetus and placenta. Here, using a mouse model of iron deficiency in pregnancy, we show that iron recycled from senescent erythrocytes becomes a predominant source of this microelement that can be transferred to the placenta in females with depleted iron stores. Ferroportin is a key protein in the molecular machinery of cellular iron egress. We demonstrate that under iron deficiency in pregnancy, levels of ferroportin are greatly reduced in the duodenum, placenta and fetal liver, but not in maternal liver macrophages and in the spleen. Although low expression of both maternal and fetal hepcidin predicted ferroportin up-regulation in examined locations, its final expression level was very likely correlated with tissue iron status. Our results argue that iron released into the circulation of anemic females is taken up by the placenta, as evidenced by high expression of iron importers on syncytiotrophoblasts. Then, a substantial decrease in levels of ferroportin on the basolateral side of syncytiotrophoblasts, may be responsible for the reduced transfer of iron to the fetus. As attested by the lowest decrease in iron content among analyzed tissues, some part is retained in the placenta. These findings confirm the key role played by ferroportin in tuning iron turnover in iron-deficient pregnant mouse females and their fetuses.

1 | INTRODUCTION

Pregnancy is one of the phases of the mammalian life cycle most frequently characterized by the risk of iron deficiency. Consistent with the role of iron in many biological processes occurring in every cell of a growing organism, this microelement is indispensable for normal fetal development. Gestational iron deficiency anemia (IDA), a pathology resulting from the lack of iron, has been associated with pre-term delivery, low birth weight and adverse pregnancy outcomes.[1] The causes of IDA in pregnancy are the especially high physiological demand for iron (iron requirements are increased nearly 10-fold during pregnancy[2]) and insufficient iron supply due to both low maternal reserves

and inadequate dietary intake. The additional iron needs in pregnancy result mainly from the high rate of maternal erythropoiesis and red blood cell (RBC) expansion, as well as rapid embryonic/fetal growth and the formation of the placenta.[3] The use of iron supplements is strongly recommended during pregnancy, but this supposedly simple therapy is complicated by the need to consider dosing schedules that depend on the amount of supplemental iron, gestational age and the severity of anemia.[4] To address these issues, a better understanding of the handling of molecular iron in pregnancy is required. Moreover, in contrast to the well-studied regulation of iron balance in adults or even in neonates,[5] the molecular mechanisms controlling iron movement between the maternal and fetal compartments at different stages of pregnancy have received little attention until quite recently.[6] For many years it was thought that iron metabolism during pregnancy is adjusted to meet the essential iron requirements of the fetus at the expense of the mother.[7,8] This means that much of the iron present in the maternal blood plasma in complex with transferrin is transferred across the placenta to the fetal circulation and delivered to sites of utilization in the fetus. However, it was recently proposed that iron entering the placenta is retained in this tissue during iron deficiency to be then transferred to the fetus.[6] The maternal blood plasma iron pool is replenished from three main sources: hepatocytes releasing iron stored in ferritin, reticuloendothelial macrophages recycling iron derived from senescent erythrocytes, and enterocytes of the proximal duodenum absorbing iron contained in the diet. A key participant in all these processes is ferroportin (Fpn), a transmembrane protein that transfers iron to plasma apo-transferrin and the only iron exporter known in mammalian cells.[9,10] Furthermore, Fpn located on the basolateral membrane of syncytiotrophoblasts has been identified as the protein responsible for iron transport from the placenta to the fetal circulation.[11,12] The importance of Fpn in this process has been clearly demonstrated in mice by global and selective inactivation of the ferroportin (*Slc40a1*) gene.[11] A complete knockout resulted in no Fpn expression in the extraembryonic visceral endoderm (which is responsible for nutrient transfer from mother to fetus between days 5 and 10 of gestation) and early (E7.5) embryonic lethality. On the other hand, disruption of the *Slc40a1* gene in all tissues except the extraembryonic visceral endoderm and placenta did not result in embryonic death.[11] Multiple regulatory mechanisms are involved in the control of Fpn expression.[10,13] Interestingly, regulation in response to inorganic iron involves competing control mechanisms, as exemplified by the post-transcriptional induction of Fpn synthesis through the IRP/IRE system[14] and inhibition of its expression at the post-translational level, depending on the interaction with hepcidin.[15] Such bidirectional Fpn regulation highlights its crucial role in fine tuning systemic iron bioavailability.

In the present study, we exposed pregnant mouse females and their E18.5 fetuses to severe nutritional iron deficiency and analyzed Fpn expression and its localization in sites critical for materno-fetal iron movements. Under iron-deficiency conditions, the level of Fpn protein was uniformly down-regulated by about 50% compared to normal iron conditions in maternal duodenal enterocytes, the placenta and fetal liver. However, only a slight decrease occurred in maternal hepatic and splenic expression of Fpn, mainly localized in Browicz-Kupffer cells and splenic macrophages. In the absence of negative regulation, resulting from drastically lowered maternal and fetal hepcidin, the Fpn level in the examined tissues was very likely dependent on intrinsic iron content.

In the light of this finding, we postulate that iron retrieved from phagocytosed senescent erythrocytes maintains Fpn expression in macrophages, and is then efficiently recycled to the bloodstream and preferentially delivered to the placenta at the expense of the mother. A certain part of this iron is retained in the placenta as evidenced by the relatively small drop in the placental iron content, while the rest is likely transferred to the fetal circulation by the remaining Fpn in the basolateral membrane of syncytiotrophoblasts.

2 | METHODS

2.1 | Experimental animals and tissue collection

Experiments were performed using 5-month-old pregnant female mice (strain B6; 129S7) bred in the Department of Molecular Biology, Institute of Genetics and Animal Biotechnology of the Polish Academy of Sciences. All mice were housed at constant temperature (22°C) under artificial light (12-hour photoperiod). Two weeks prior to mating with males of the same strain, females were fed control or iron-deficient diets (Harlan Laboratories, Madison, WI, USA) containing 48 and 4 ppm iron, respectively. The female mice were maintained on the same diets throughout pregnancy. For timed matings, the morning plug was identified and was considered E0.5. Plugged females were sacrificed by isoflurane overdose at E18.5. In a few experiments, non-pregnant females exposed to the same dietary iron regimes were used. Blood samples were taken from the inferior vena cava of females using heparin-coated syringes. The blood was collected in heparin-coated tubes and centrifuged at 800 *g*/4°C/10 min. Plasma samples were then frozen in liquid nitrogen and stored at −80°C for later analysis. Fetuses were dissected from the uterus, sacrificed by decapitation and their livers were collected and frozen. Placentas from fetuses, chosen from each mother at random, were rapidly dissected and frozen in liquid nitrogen, before being stored at −80°C. Tissue samples (maternal and fetal livers, maternal duodenum, and placenta) for immunofluorescence analysis were fixed in 4% paraformaldehyde in PBS. Bone marrow cells for total cellular RNA extraction were flushed out from the femur of females with ice-cold Hanks balanced salt solution. All procedures were approved by the 2nd Local Ethical Commission (permission number WAW2/53/2016).

2.2 | Measurement of red blood cell indices, blood plasma iron parameters and non-heme iron content in tissues

The red blood cell (RBC) count, hemoglobin concentration (HGB), hematocrit (HCT) and mean cell volume (MCV) were determined using

an automated hematology analyser (Abacus Junior Vet 5; Diatron, Budapest, Hungary). The plasma iron concentration and total iron-binding capacity (TIBC) were evaluated using an assay based on the colorimetric measurement of an iron-chromazurol complex (absorbance at 630 nm) according to the manufacturers's protocol (Alpha Diagnostic, Poland). The percentage transferrin saturation (TSAT) was then calculated. The non-heme iron content of liver and placenta fragments (100 mg) and of spleen fragments (40 mg) was determined by acid digestion of the samples at 100°C for 10 min., followed by colorimetric measurement of an iron-ferrozine complex (absorbance at 560 nm) as described previously.[16]

2.3 | Real-time quantitative PCR (RT-qPCR)

Total cellular RNA was extracted from liver, bone marrow, kidney and placental tissue (20 mg) using Trizol reagent (Invitrogen) according to the manufacturer's protocol. Two micrograms of DNase-treated total RNA were reverse transcribed using a Transcriptor First-Strand cDNA Synthesis Kit (Roche, Switzerland). This cDNA was used as the template for real-time quantitative PCR analysis with gene-specific primer pairs (Table S1), performed in a Light Cycler U96 (Roche Diagnostics, Mannheim, Germany). The amplified products were detected using SYBR Green I (Roche Diagnostics). To confirm amplification specificity, the PCR products were subjected to melting curve analysis and agarose gel electrophoresis. Light Cycler U96 Software was used for data analysis. Transcript levels were normalized relative to the control reference genes selected using NormFinder software v0.953 (https://moma.dk/normfinder-software).

2.4 | Western blot analysis

For the detection of ferroportin (Fpn) and L-ferritin (L-Ft) protein levels, membrane and cytosolic extracts, from maternal liver, spleen, duodenum, fetal liver, and from the placenta, were prepared as previously described[17] and separated by electrophoresis on 10% and 14% SDS-PAGE gels, respectively. Electroblotting of the resolved proteins (100 μg) onto polyvinylidene difluoride membrane (Millipore), blocking and incubation with primary antibodies were performed as previously described.[18] The following primary antibodies were used: a rabbit antiserum raised against recombinant mouse L-Ft, (kindly provided by Dr. P. Santambrogio, Department of BioTechnology, San Raffaele Scientific Institute, Milan, Italy), and a rabbit polyclonal anti-Fpn antibody (Alpha Diagnostic), affinity purified rabbit polyclonal anti-mouse Fpn antibody kindly provided by Dr. François Canonne-Hergaux (INSERM UMR 1043, Centre de Physiopathologie de Toulouse Purpan, Toulouse, France), transferrin receptor 1 monoclonal antibody (Thermo Fisher), anti-human Natural Resistance-Associated Protein 2 (DMT1/NRAMP2), (Alpha Diagnostic Intl. Inc). Membranes were then washed and incubated with peroxidase-conjugated anti-rabbit secondary antibody (Santa Cruz Biotechnology) for 1 h at room temperature (20°C). Immunoreactive bands were detected using the ECL (enhanced chemiluminescence) Plus Western blotting detection system (Amersham Life Sciences). Band intensities were quantified by densitometric analysis using Quantity One software, v.4.6.6 (Bio-Rad).

2.5 | Immunofluorescence (IF) analysis and confocal microscopy of liver, placenta and duodenum sections

After laparotomy, livers and placentas from pregnant mothers (E18.5) were immediately dissected and fixed in 4% paraformaldehyde (Sigma) in phosphate-buffered saline (PBS) at 4°C for 24 h. Following two 30-minutes washes in PBS, the tissues were successively soaked in 12.5% and 25% sucrose (Bioshop) at 4°C for 2 h and 7 days, respectively. Hepatic and placental samples were then embedded in Cryomatrix medium (Thermo Scientific), frozen in liquid nitrogen and sectioned in 20-μm slices using a cryomicrotome (Shandon). The sections were washed in PBS for 10 min and permeabilized by bathing in PBS/0.1% Triton X-100 (Sigma) for 20 mins. Non-specific antibody binding was blocked by incubating the tissue sections in PBS/3% BSA (Bioshop) at room temperature (RT) for 1.5 h. For protein detection, sections were incubated overnight at RT with primary antibodies diluted in PBS/3% BSA. As a negative control, some sections were incubated without primary antibody. The primary and fluorochrome-conjugated secondary antibodies used in IF analysis are described in Table S2. Next, the sections were washed for 5×6 min with PBS/0.1% Triton X-100 and incubated for 1.5 h at RT with secondary antibody diluted in PBS/3% BSA. Finally, sections were washed for 10 min in PBS and mounted in Vectashield medium with DAPI (Vector Labs). The presence of Fpn in Browicz-Kupffer cells (liver macrophages) was determined by double immunofluorescence staining of the investigated protein and the macrophage marker F4/80. In this case, liver sections were first incubated with anti-Fpn followed by anti-F4/80 primary antibody, and then they were incubated with a mixture of secondary antibodies conjugated with different fluorochromes: Cy3 for Fpn and Alexa488 for F4/80. Note, IF was analysed with a Zeiss LSM 710 confocal microscope (Carl Zeiss) using a $\times 40$ objective and Zeiss ZEN software.

2.6 | ImageJ analysis of immunofluorescence images

ImageJ analysis was performed to characterize IF produced using the ferroportin antibody. Samples from each group of animals were prepared for analysis at the same time. Liver and placenta sections from three different females per experimental group were examined by confocal microscopy at $40 \times$ magnification. For quantitative comparisons between experimental groups, the same acquisition parameters were applied to capture images within the same experimental set. Subsequently, ImageJ software (NIH, Bethesda) was used to measure mean fluorescence in each tubule section. The signal intensity was manually quantified to generate a Mean Gray Value: the sum of grey

values in the selected area divided by the number of pixels within that area. For each experimental group, 30 measurements of livers and placentas were made, that is, 10 measurements per female.

2.7 | Measurement of blood plasma erythroferrone (ERFE) level

An indirect ERFE ELISA kit designed for quantification of mouse erythroferrone in serum was purchased from Intrinsic Lifesciences The BioIron Company (catalog no. SKU# ERF-200). Serum samples, standards and controls mixed with sample diluent (100 μL/well) were incubated for 1 h with immobilized polyclonal antibody in the precoated plate. The plate was than washed and unbound mouse ERFE was removed. A second anti-mouse Erfe antibody conjugated with biotin was added for 1 h, washed and followed with HRP-SA conjugated which binds to biotinylated anti-mouse erythroferrone for the next 30 min. After washing the enzyme-TMB reaction formed a blue colored complex within 15 min. The reaction was terminated by the addition of a stop solution, which turned the reaction mixture yellow. Colorimetric measurement (absorbance at 450 nm) was performed using a microplate reader (ELx800 BIO-TEK INSTRUMENTS. Inc, Winoski, VT, USA). The intensity of the yellow color was proportional to the Erfe concentration in the samples. A standard curve was prepared by plotting the log concentration of standard curve vs its corresponding OD (450).

2.8 | Statistical analysis

Statistical analysis was performed using ANOVA and Kruskal-Wallis ANOVA tests (for parametric and non-parametric data distributions, respectively) combined with proper post-hoc tests (Tukey and Dunn tests, respectively) with $p \leq .05$ (*), $p \leq .01$ (**) and $p \leq .001$ (***) being considered statistically significant and highly significant, respectively.

3 | RESULTS

3.1 | Iron deficiency anemia (IDA) in pregnant females fed a low-iron diet

Exposure of pregnant rodent females to an iron-poor diet is a common procedure used to induce iron deficiency and iron-deficiency anemia in pregnancy.[6,19] In this study we found that pregnant female mice subjected to dietary iron restriction had considerably decreased iron parameters in the blood plasma, such as iron concentration and transferrin saturation, compared with those fed a control diet (Figure S1). Consequently, pregnant iron-deficient females manifested symptoms of microcytic anemia characterized by significantly diminished RBC count, hemoglobin concentration, hematocrit and MCV values (Table S3). Pregnancy in females fed the control diet, and iron deficiency in non-pregnant females led to deterioration of RBC status, although to a lesser degree than in iron-deficient mothers (Table S3).

3.2 | Decreased hepatic iron stores in iron-deficient pregnant females and their fetuses

Depletion of iron stores in the bone marrow and the liver remains the most definitive test for differentiating iron-deficiency anemia from other microcytic anemias.[20] Measurement of the hepatic non-heme iron content in anemic pregnant females revealed a five-fold decrease compared with iron-replete animals (Figure S2(A)). Unsurprisingly, the level of iron in the livers of fetuses from anemic mothers was also lowered; however, this decrease was only three-fold. Ferritin is a major iron-storage protein and its expression is highly positively correlated with cellular and tissue iron status.[21] Hepatic ferritin in mammals is mostly comprised of L-chains (ca. 80%) and therefore the L-ferritin level in the liver reflects hepatic iron abundance. The assessment of L-ferritin protein levels in maternal and fetal livers by Western blotting largely confirmed the results of direct measurement of iron content. Iron-deficient mothers and their fetuses both showed drastic reductions in the level of L-ferritin, but again, this decrease was much greater in the former than the latter (Figure S2(B)).

3.3 | Hepatic hepcidin and BMP6 mRNA levels in iron-deficient females and fetuses

Hepcidin is a small peptide hormone produced by hepatocytes that orchestrates body iron fluxes (including iron transfer across the placenta) by adjusting iron supply to body iron requirements. Hepcidin binds to Fpn to induce its degradation, thus inhibiting iron release from exporting cells.[22] Its expression is controlled by iron levels (circulating and liver iron content), erythropoietic activity and inflammatory cues.[23] In pregnancy, maternal and fetal hepcidins are regulated by both maternal and fetal iron conditions.[24] To assess the functioning of the hepcidin-ferroportin regulatory axis under iron deficiency in pregnancy, we examined hepcidin mRNA abundance in maternal and fetal livers, comparing the obtained results with hepcidin transcript levels in the livers of iron-deficient non-pregnant females (Figure 1(A)). In all examined iron-deficient subjects, hepcidin mRNA expression was down-regulated compared to the respective controls. Importantly, even in iron-replete fetuses the hepcidin transcript was barely detectable. Comparison of hepcidin expression in pregnant and non-pregnant females receiving the standard iron diet demonstrated that pregnancy triggers a nearly three-fold drop in hepcidin mRNA abundance. Hepatic hepcidin expression in hepatocytes is activated predominantly at the transcriptional level by a member of the TGFβ superfamily, bone morphogenetic protein 6 (BMP6),[25] the expression of which is induced by iron in non-parenchymal cells of the liver.[26] To establish the relationship between the expression of this activator and that of its target gene hepcidin, we examined BMP6 mRNA levels in the liver samples. In both iron-deficient non-pregnant and pregnant

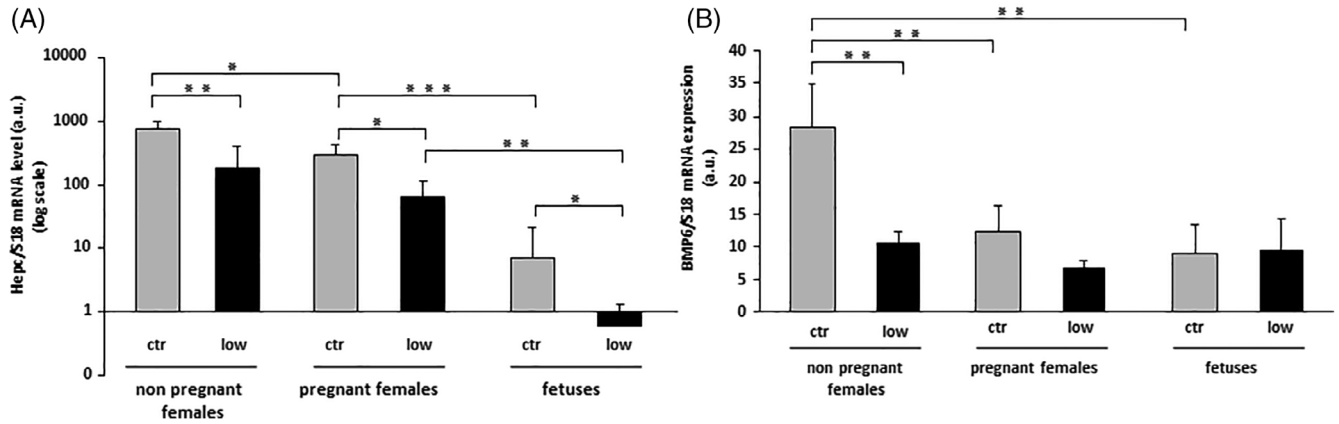

FIGURE 1 Decrease in hepatic hepcidin and BMP6 mRNA levels in iron-deficient non-pregnant females, pregnant females and their E18 fetuses. RT-qPCR analysis of hepcidin (A) and BMP6 (B) transcript levels in livers. The histograms display the relative hepcidin and BMP6 mRNA levels in arbitrary units (means ± S.D.). Liver samples were obtained from eight pregnant and non-pregnant females of the iron-replete (ctr) and iron-deficient (low) groups. For fetuses, values were calculated for three sets of pooled fetal liver samples obtained from three pregnant females of each group. The histogram displays the relative hepcidin and BMP6 mRNA levels in arbitrary units (means ± S.D.). Significant differences are indicated (*$p < .05$, **$p < .01$, ***$p < .001$)

females, hepatic BMP6 expression was substantially reduced to 24% and 55% of the levels found in the respective iron-replete controls (Figure 1(B)), and corresponded to the similar decreases seen in hepcidin mRNA expression. As in the case of hepcidin, pregnancy caused a three-fold decrease in BMP6 transcript levels in animals receiving the standard iron diet. We observed no differences in hepatic BMP6 mRNA level between fetuses according to their iron status, which was consistent with their very low levels of hepcidin mRNA.

3.4 | Increased expression of erythroid inhibitors of hepcidin in iron-deficient pregnant females

Erythropoietic regulation of hepcidin is one of the major signaling pathways controlling hepatic hepcidin expression.[27] Enhancement of the erythropoiesis induced by increased erythropoietin (Epo) produced in kidney, causes suppression of hepcidin to increase iron availability for hemoglobin synthesis in erythroblasts. Assessment of Epo mRNA expression in the kidneys of anemic mothers by Real-Time PCR revealed a substantial increase compared to control ones, but also to anemic non-pregnant females (Figure S3(A)). Among the erythroid regulators that reduce hepcidin expression during expanded erythropoiesis, erythroferrone (ERFE) has been identified as a soluble factor produced and released by erythroblasts after bleeding or erythropoietin treatment.[28] To investigate the possible contribution of ERFE to the negative regulation of hepcidin in pregnant females fed a low-iron diet, we measured its expression at the mRNA level in bone marrow as well as its concentration in blood plasma. We found that both indexes of ERFE expression were dramatically elevated compared to pregnant females receiving the standard iron diet and non-pregnant iron-deficient females (Figure S3(B) and (C)). Apart from ERFE, it has been proposed that other erythroid factors, including GDF15,[29] suppress hepcidin during erythropoiesis. Although this regulatory function of GDF15 has been questioned,[30] we found that GDF15 mRNA expression in bone marrow was significantly increased in iron-deficient pregnant females (Figure S3(D)), suggesting that it may play a role in the negative regulation of hepcidin expression.

3.5 | A slight decrease in hepatic and splenic ferroportin in iron-deficient mothers vs a prominent decrease in other tissues

Ferroportin (Fpn), the sole exporter of non-heme iron known to date, transfers cellular iron to apo-transferrin, which then transports this microelement via the blood to various tissues in the body, including the bone marrow.[10] Ferroportin is particularly abundant in hepatic and splenic macrophages, where it exports iron recycled from senescent erythrocytes, a process of critical importance for sustaining physiological erythropoiesis.[31] Assessment of Fpn levels in the livers of anemic mothers by Western blotting revealed a relatively small decrease (14%) compared to control females (Figures 2(A) and (B)). A much greater decrease in hepatic Fpn protein abundance (>50%, Figure 2(B)) was detected in iron-deficient E18.5 fetuses compared to the controls.

Immunofluorescent (IF) co-localization studies of Fpn and F4/80, a well-known macrophage marker,[32] demonstrated that Fpn was mainly located in Browicz-Kupffer cells in the livers of both control and iron-deficient pregnant females (Figure 2(C)). Comparative quantitative analysis of the Fpn IF signal using ImageJ revealed no significant difference in the expression of Fpn between livers of control and iron-deficient pregnant females (Figure 2(D)). Considering that spleen (ie, splenic macrophages) largely participates in recycling of iron from senescent RBC, we also determined Fpn protein level by Western blotting in the spleen of anemic mothers. Despite a strong decrease in splenic iron content (Figure S4(A)) we found no significant changes in

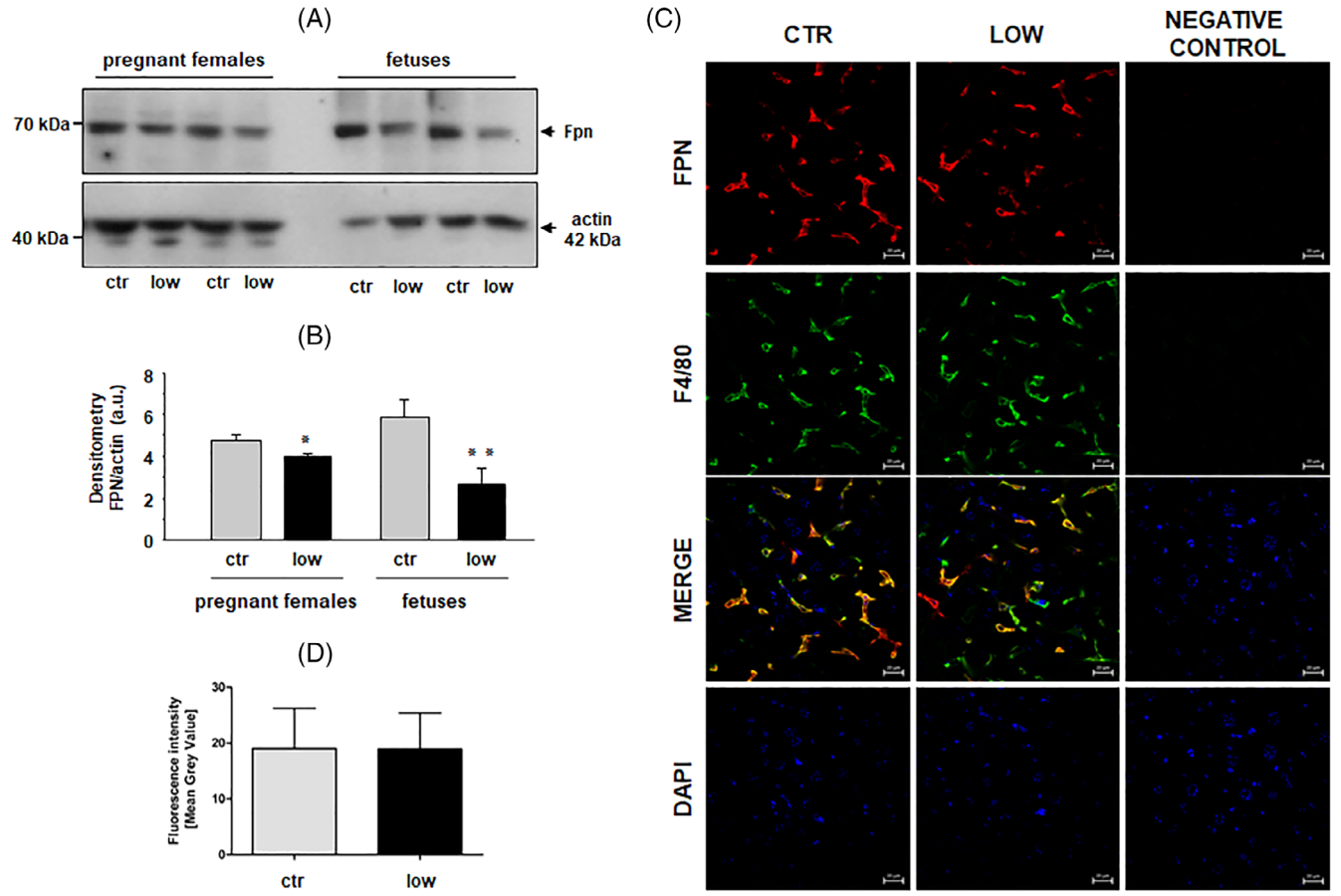

FIGURE 2 Differential decrease in hepatic ferroportin (Fpn) expression in pregnant females fed a low-iron diet and in their E18 fetuses. (A) Western blot analysis of Fpn protein levels in liver membrane fractions prepared from pregnant females (individuals) of the iron-replete (ctr) and iron-deficient (low) groups, and their fetuses (pooled samples). A representative immunoblot is shown (B) Immunoreactive Fpn bands from separate blots performed on hepatic membrane extracts obtained from four females and three sets of pooled fetal liver samples obtained from three pregnant females of each group were quantified by densitometry using a Molecular Imager, and Fpn protein levels (means ± S.D.) are plotted in arbitrary units (a.u.). Significant differences are indicated (*$p < .05$, **$p < .01$). (C) Immunofluorescent staining of Fpn (red channel; top panel) and macrophage marker F4/80 (green channel; second panel from the top) in livers obtained from pregnant females of each group analyzed by confocal microscopy. Co-localization of Fpn and F4/80 is shown in the merged image (second panel from the bottom) and the location of nuclei is disclosed by counterstaining with DAPI (bottom panel). To confirm the specificity of Fpn and F4/80 detection, liver sections of pregnant females were incubated with only the respective secondary antibodies. No staining was detected in these negative controls (third column). (D) Quantitative analysis of the Fpn fluorescent signal in female livers. Immunofluorescence in 30 liver sections from pregnant females of each group was quantified manually by ImageJ analysis and Fpn signal intensities as Mean Gray Values (means ± S.D.) are plotted in arbitrary units (a.u.). Scale bars correspond to 20 μm [Color figure can be viewed at wileyonlinelibrary.com]

Fpn level compared neither to pregnant females fed control diet nor to females from other experimental groups (Figure S4(B) and (C)). Taken together, the results of both Western blotting and IF suggested that the drop in hepatic and splenic Fpn level in anemic mothers is at best marginal. To check the role of duodenal Fpn in iron delivery to the circulation in pregnant females receiving the low-iron diet, we assessed its localization on absorptive enterocytes in the duodenum and then quantified the intensity of the IF signal. In the duodenum of females from both experimental groups, Fpn was located along the basal and lateral membranes of absorptive enterocytes (Figure 3 (A) and (B)). However, the intensity of the Fpn IF signal was much lower (46%) in the duodenum of iron-deficient pregnant females compared to those fed the standard iron diet (Figure 3(C)). Western blot analysis of Fpn protein level in the duodenum of iron-deficient, anemic mothers revealed even greater decrease (52%) compared to mothers fed control iron diet (Figure 3(D) and (E)).

The placenta serves as the interface between mother and fetus, and it mediates nutrient transport to the latter, including the transport of iron. The uni-directional transfer of iron taken up by the placenta to the fetal circulation is effected by Fpn located on the basolateral membrane of syncytiotrophoblasts.[12] In the placentas of iron-deficient females, the Fpn level determined by Western blotting (Figure 4(A)) was decreased by about 42% compared to iron-replete females (Figure 4(B)). Strong down-regulation of Fpn was also clearly shown by IF analysis of placental sections from females receiving the iron-deficient diet (Figure 4(C)). Microscopic analysis of IF staining

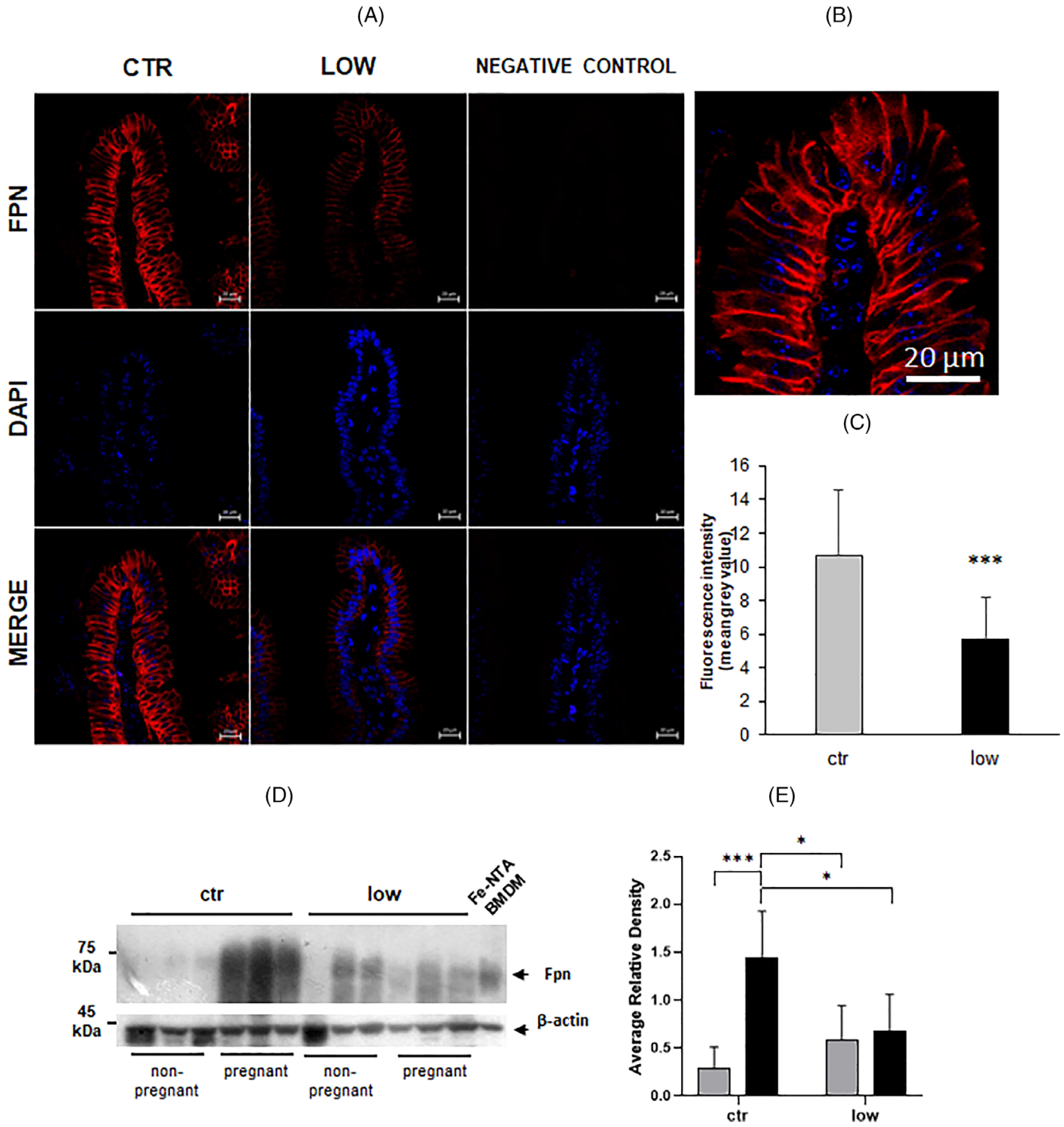

FIGURE 3 Substantial decrease in duodenal ferroportin (Fpn) protein level in pregnant females fed a low-iron diet. (A) Immunofluorescent staining of Fpn in the duodenum of pregnant females of the iron-replete (ctr) and iron-deficient (low) groups analyzed by confocal microscopy. To confirm the specificity of Fpn detection, the duodenum sections were incubated with only the secondary antibody. No Fpn staining was detected in these negative controls. Counterstaining of nuclei was performed with DAPI. Scale bars correspond to 20 µm. (B) High magnification image of duodenal enterocytes showing clear basolateral localization of Fpn. (C) Quantitative analysis of the Fpn fluorescent signal in duodenum. Immunofluorescence in 30 duodenum sections from pregnant females of each group was quantified manually by ImageJ analysis and Fpn signal intensities as Mean Gray Values (means ± S.D.) are plotted in arbitrary units (a.u.). Significant difference is indicated (***$p < .001$). D, Western blot analysis of Fpn protein levels in duodenum membrane fractions prepared from pregnant and non-pregnant females of the iron-replete (ctr) and iron-deficient (low) groups. Fe-NTA BMDM, ferric nitrilotriacetate (100 µM) treated mouse bone marrow-derived macrophages E, Immunoreactive Fpn bands from the blot shown in D were quantified by densitometry using a Molecular Imager, and Fpn protein levels (means ± S.D.) are plotted in arbitrary units (a.u.). Significant differences are indicated (*$p < .05$, ***$p < .001$). gray bars – non-pregnant females; black bars – pregnant females [Color figure can be viewed at wileyonlinelibrary.com]

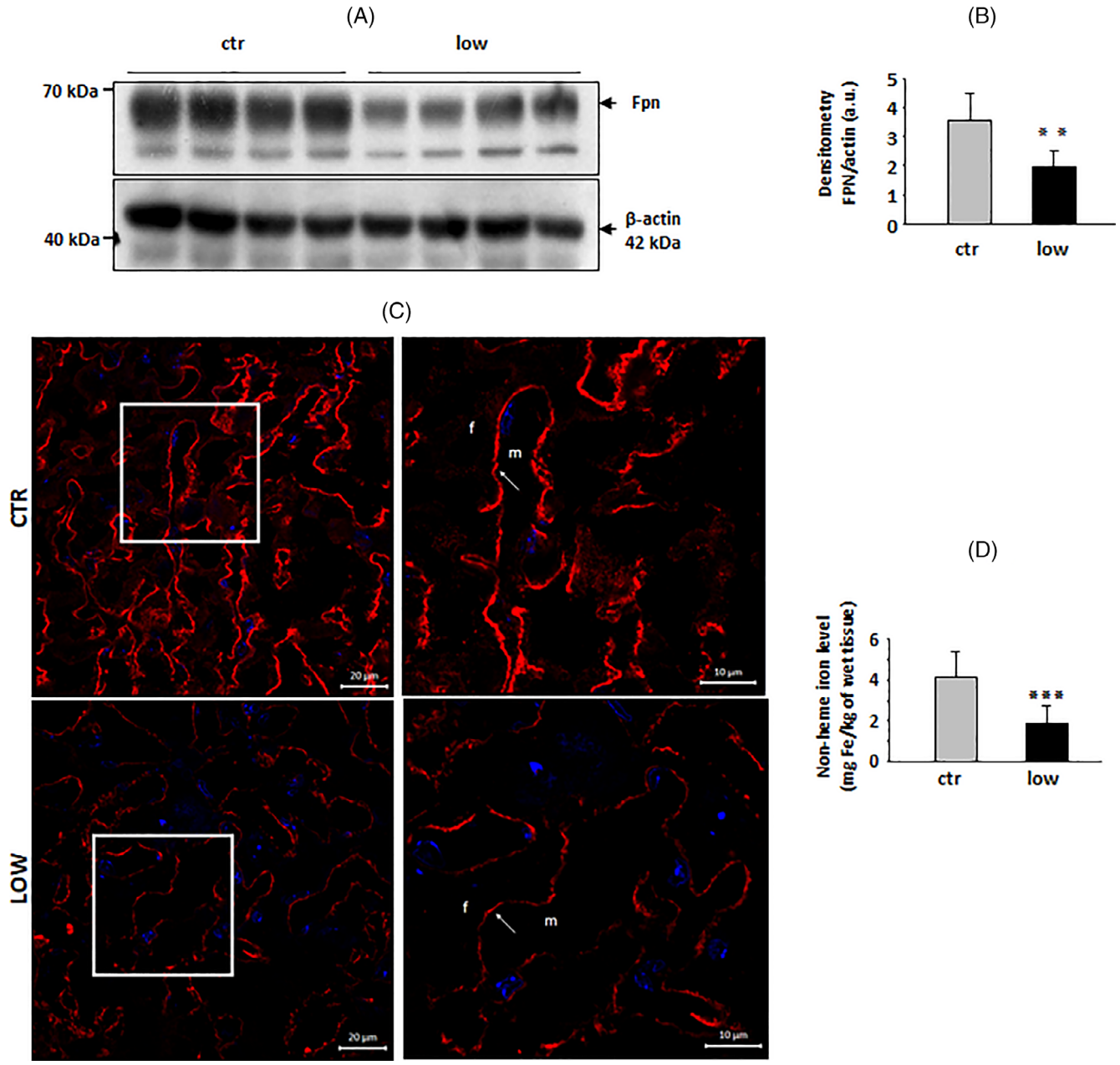

FIGURE 4 Decreased expression of ferroportin (Fpn) in the placenta of females fed a low-iron diet. (A) Western blot analysis of Fpn protein levels in placenta membrane fractions prepared from pregnant females (individuals) of the iron-replete (ctr) and iron-deficient (low) groups. A representative immunoblot is shown. (B) Immunoreactive Fpn bands from separate blots performed on placental membrane extracts obtained from four females of each group were quantified by densitometry using a Molecular Imager, and Fpn protein levels (means ± S.D.) are plotted in arbitrary units (a.u.). Significant difference is indicated (**$p < .01$). C Immunofluorescent staining of Fpn in the placenta of pregnant females of each group analyzed by confocal microscopy. Cell nuclei were counterstained with DAPI (blue). Scale bars correspond to 20 µm. The right-hand panels show high magnification images of stained placenta (areas boxed in the left-hand panels), confirming the basolateral localization of Fpn in syncytiotrophoblasts. Scale bars correspond to 10 µm. m, maternal compartment. f, fetal compartment; D, Non-heme iron content in placenta samples obtained from six pregnant females of each group (means ± S.D.). Significant difference is indicated (***$p < .001$) [Color figure can be viewed at wileyonlinelibrary.com]

indicated typical localization of Fpn in the basal (fetal facing) membrane of placental syncytiotrophoblasts (Figure 4(C)). Interestingly, the fall in iron content in the placentas of iron-deficient females was the lowest (only two-fold) among the three tissues examined in our study, that is, maternal and fetal liver, and placenta (Figure 4(D)).

3.6 | Increased expression of iron importers in the placenta of iron-deficient females

While Fpn mediates unidirectional transport of iron accumulated in syncytiotrophoblasts to the fetal circulation, transferrin receptor 1 (TfR1) and divalent metal transporter 1 (DMT1) are responsible for

iron loading to these cells from the maternal circulation.[12] In the placenta of iron-deficient females the expression of these two iron importers was strongly up-regulated at the mRNA (Figures S5(A) and S6(A)) and protein (Figures S5(C),(D) and S6(C),(D)) levels, consistent with the paradigm of preferential iron transport across the placenta to satisfy fetal iron requirements[7,8] or placenta requirements as recently assumed.[6] Immunofluorescent detection of TfR1 and DMT1 in placental sections (Figures S5(B) and S6(B), respectively) showed clearly enhanced fluorescent signals for these proteins localized on the apical side of syncytiotrophoblasts facing the maternal circulation. Interestingly, while TfR1 displayed exclusively linear localization on the apical membrane (Figure S5(B)), DMT1 was also detected in intracellular vesicles in the form of discrete dots (Figure S6(B)). This localization pattern suggests that, according to a well-characterized mechanism, DMT1 participates in the transport of iron liberated from transferrin within the endosome to the cytoplasm.[33]

4 | DISCUSSION

Pregnancy is a physiological condition frequently associated with iron deficiency anemia.[1] During pregnancy, iron needs are greatly increased because of the expansion of maternal red blood cell (RBC) mass, and growth of the placenta and the fetus. Efficient mobilization of iron from maternal hepatic stores is required to satisfy the iron requirements of both the mother and fetus. Relatively little is known about the regulation of iron fluxes between the iron-deficient mother, placenta and fetus. In particular, there is a dearth of knowledge concerning gestational control of the expression of Fpn, the sole cellular iron exporter, operating not only in maternal and fetal compartments, but also at the maternal-fetal interface, that is, the placenta.[10,12] Therefore, we performed a comprehensive evaluation of Fpn expression and distribution in the maternal duodenum, liver, spleen and in the placenta: tissues with a high potential to release iron. We also determined the expression of Fpn in the fetal liver. This analysis was performed on subjects under conditions of dietary iron restriction, which in pregnant females led to drastic decreases in both plasma iron concentration and transferrin saturation, and consequently a decline in RBC indices. Severe iron deficiency in pregnant mothers was also confirmed by substantial decreases in hepatic and splenic iron content and hepatic L-ferritin protein level. Unsurprisingly, maternal iron deficiency caused a strong reduction in hepcidin, a liver peptide that negatively regulates dietary iron absorption and iron release from macrophages and hepatocytes by binding and degrading Fpn.[15] Suppression of hepcidin has been observed in normal and iron-deficient pregnancies,[24] and is perceived as a means of regulating iron mobilization from stores to meet requirements for the expansion of maternal red cell mass, and placental and fetal growth. A possible mechanism underlying this modulation may involve down-regulation of bone morphogenetic protein 6 (BMP6), a main activator of hepcidin,[25] expressed predominantly in hepatic endothelial sinusoidal cells and modulated by iron fluctuations.[26] Interestingly, in comparison with iron-replete non-pregnant females, hepatic BMP6 mRNA levels declined to the same extent in both non-pregnant iron-deficient females and in pregnant females fed the standard iron diet. The restriction of dietary iron in pregnancy did not induce any further reduction in maternal hepatic BMP6 expression. These data indicate that even with an adequate dietary iron supply during pregnancy, BMP6-dependent signaling pathways regulating hepcidin are strongly attenuated to increase overall iron availability. It has been reported that apart from iron scarcity, hepcidin expression can also be suppressed by secreted erythroid factors, such as erythroferrone (ERFE)[28] and GDF15[29] thereby increasing iron availability for hemoglobin synthesis. Kautz and co-workers described how ERFE synthesis is promoted by both erythropoietin (EPO) injection and expanded erythropoiesis caused by bleeding.[28] An increase in erythropoietic activity in the later stages of pregnancy appears to depend upon elevated plasma EPO levels and increases ERFE expression by erythroblasts.[34] Indeed, in iron-deficient pregnant females, we observed a concerted regulations: increased EPO mRNA expression in the kidney and both dramatic rise in ERFE transcript level in bone marrow accompanied by highly elevated blood plasma concentrations of ERFE, which can lead to hepcidin suppression. Similarly, in the bone marrow cells of these animals we detected increased expression of GDF15, another inhibitor of hepcidin expression, originally reported to be secreted at high levels during erythroblast maturation.[29] Thus, our results suggest that the inhibition of maternal hepcidin in pregnancy is mediated by both iron deficiency and erythroid factors. The post-translational regulation of Fpn by hepcidin means that in the absence of this peptide hormone or where levels are low, the concentration of Fpn on the cell surface is high.[15] In the case of absorptive enterocytes the lack of this negative regulation results in enhanced absorption of dietary iron, a phenomenon that is central to the pathophysiology of hereditary hemochromatosis.[35] However, it is important to bear in mind that apart from hepcidin-mediated control, Fpn expression also depends on intracellular downstream regulatory circuits mediated by heme and iron.[36,37] These intrinsic adjustments determine a basal level of Fpn on the cell membrane, which is then susceptible to regulation by Hepc. In iron-deficient pregnant mothers, Fpn expression was strongly down-regulated in the duodenum and only slightly reduced or unchanged in the liver and spleen, respectively. We hypothesize that despite hepcidin deficiency, Fpn levels in the basolateral membrane of enterocytes are highly depressed as a result of post-transcriptional IRP-dependent regulation in response to low intra-enterocytic iron levels caused by an iron-deficient diet.[38] Where intra-enterocytic iron concentrations are low, the iron absorption genes including Fpn are transcriptionally induced by stabilized hypoxia-inducible factor 2α (HIF2α).[39] On the other hand, under these conditions, de novo synthesis of HIF2α is subject to IRP-mediated translational inhibition due to the presence of an IRE in the 5'UTR of the HIF2α mRNA.[40] With prolonged iron deficiency this IRP-dependent regulation can prevail and contributes to the decrease in Fpn. It should be underlined that Fpn transcript containing IRE sequence (and thus amenable to regulation by IRPs) accounts only for 25% of total Fpn mRNA in duodenum.[41] With regard to maternal hepatic Fpn, our IF colocalization and Western blot analyses clearly demonstrated that it is mainly expressed in

liver macrophages (Browicz-Kupffer cells), and this expression is only slightly decreased by an iron-deficient diet. This may be due to the specificity of iron metabolism in Browicz-Kupffer cells, which play an essential role in iron recycling through the phagocytosis of senescent RBCs, and thus continuously acquire iron in large amounts.[37,42] Although overall iron content in the liver was strongly decreased, it seems that this more accurately reflects the iron status in hepatocytes. In macrophages, Fpn is sequentially up-regulated by heme-dependent transcriptional induction followed by post-transcriptional IRP/IRE system regulation mediated by iron continuously released by heme catabolism, as described previously.[37] Importantly, under absolute iron-deficiency conditions, hypochromic and microcytic erythrocytes have a shortened lifespan coupled with accelerated reticuloendothelial cell sequestration once released into the circulation.[43] We therefore postulate that during severe maternal iron deficiency, iron recycling into the plasma through Fpn by erythrophagocytosing hepatic and splenic macrophages is the only efficient pathway of iron delivery for the mother and her fetuses.

Animal model studies indicate that a hierarchy of iron delivery during pregnancy is established in order to maintain fetal iron levels at the expense of the mother.[7,8] Despite this priority, a sufficient supply of iron to the fetus is difficult to maintain and iron deficiency can, lead to neonatal morbidity with an increased risk of prematurity, low birth weight and deleterious effects on brain development.[44,45] Recently, it was also demonstrated that under conditions of iron deficiency in pregnancy, this microelement is preferentially retained in the placenta at the expense of the fetus in order to maintain iron-dependent placental processes that are indispensable for the overall function of this organ.[6] Our results indicate the strong potential of the placenta to take up iron from the maternal circulation as visualized by the high level expression of TfR1 and DMT1, proteins involved in iron uptake, localized to the apical membrane of the placental syncytiotrophoblasts.[12] Similar results have been reported for the placenta of iron-deficient female rats.[46] In contrast, the level of Fpn expressed at the basal membrane[12] was only half that observed in control, iron-replete pregnant mice. This expression pattern of iron transport proteins in pregnant females under severe iron deficiency only causes a moderate reduction in placental iron content, which is much less drastic than the decrease observed in both maternal and fetal livers and maternal spleens. This observation largely confirms recent results showing mildly decreased placental iron level at E18.5 in iron-deficient pregnant females compared to iron replete ones.[6] Molecular mechanisms controlling iron transporter protein expression in the placenta seem to involve the concerted action of the post-transcriptional intracellular IRP/IRE system and post-translational regulation by fetal hepcidin. The importance of IRPs (especially IRP1[47]) in handling placental iron has been clearly demonstrated in human[47-49] and animal[6,50] studies. Using an almost identical model of dietary iron deficiency in pregnant mouse females, Sanghke and co-workers observed a 50% reduction in placental Fpn expression that was very likely mediated by IRP1.[6] It is tempting to speculate that under conditions of iron deficiency, this regulatory mechanism provides a compromise solution for maintaining the balance between iron delivery to developing fetuses and protection of the placenta from excessive iron depletion. In the light of our results, it is unlikely that hepcidin, which is barely detectable in the fetal liver, can modulate the Fpn protein level at the basolateral membrane of syncytiotrophoblasts and thus Fpn-dependent export from the placenta to the fetal circulation under normal physiological conditions. Therefore, the findings of the present study and those of others[6,51] demonstrate that the contribution of fetal hepcidin to the regulation of placental Fpn in both normal and iron-deficient conditions is of minor importance. Furthermore, the similar low-level expression of BMP6 in the fetal liver under normal and iron-deficient conditions, (despite a three-fold difference in hepatic iron content), suggests that fetal BMP6 may be unresponsive to iron signaling and thus a BMP6-dependent pathway of hepcidin regulation may be impaired.

In summary, our study emphasizes the complex role of Fpn, which is differentially expressed in maternal and fetal tissues, and in the placenta, in the distribution of iron between the mother, the fetus and the placenta under conditions of iron deficiency. First, the unaltered ferroportin-dependent iron transport from liver and spleen macrophages seems to be the only efficient pathway of iron delivery to the maternal circulation. Second, the great decrease in placental ferroportin is responsible for the reduced iron export into the fetal circulation and for the maintenance of iron levels in the placenta. Finally, the reduction in fetal hepatic Fpn may secure greater iron availability for fetal erythropoiesis, which takes place in this organ.[51-53]

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CONFLICT OF INTEREST

The authors declare no conflict of interests.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article and the data that support the findings of this study are available from the corresponding author upon reasonable request.

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Review

Iron Supplementation of Pregnant Sows to Prevent Iron Deficiency Anemia in Piglets: A Procedure of Questionable Effectiveness

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Abstract: In pigs, iron deficiency anemia (IDA) is a common disorder that occurs during the early postnatal period, leading to the stunted growth and increased mortality of piglets. The main cause of IDA is low iron stores in the liver of newborn piglets; these stores constitute the main source of iron needed to satisfy the erythropoietic requirements of the piglets in their first weeks of life. Insufficient iron stores in piglets are usually due to the inadequate placental iron transfer from the sow to the fetuses. Therefore, iron supplementation in pregnant sows has been implemented to enhance placental iron transfer and increase iron accumulation in the liver of the fetuses. Over the years, several oral and parenteral approaches have been attempted to supplement sows with various iron preparations, and consequently, to improve piglets' red blood cell indices. However, there is debate with regard to the effectiveness of iron supplementation in pregnant sows for preventing IDA in newborn piglets. Importantly, this procedure should be carried out with caution to avoid iron over-supplementation, which can lead to iron toxicity. This article aims to critically review and evaluate the use of iron supplementation in pregnant sows as a procedure for preventing IDA in piglets.

Keywords: sows; iron metabolism; piglets; anemia; iron supplementation; pig

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1. Introduction

In domestic pigs (*Sus scrofa domestica*), neonatal iron deficiency anemia (IDA) occurs in almost all contemporary breeds [1,2]. Iron deficiency is a long-standing problem in the pig farming industry, with the first case of this disorder reported in the late 19th century [3]. Then, in 1924, a causal link was made between iron deficiency and anemia [4]. In newborn piglets, IDA has since been an ongoing problem in pig rearing, and many studies, including our own, have been dedicated to various aspects of this pathology [1–3,5,6]. In suckling piglets, IDA is typically a hypochromic, microcytic anemia, characterized by decreased red blood cell (RBC) indices. Without iron supplementation, piglets rapidly develop IDA, and thus, by definition, the values of RBC parameters measured in these animals are below acceptable ranges. There is a general consensus that the hemoglobin concentration cut-off level for anemia in suckling piglets is 8 g/dL [7]. To prevent IDA in young piglets, supplemental iron from an exogenous source must be administered during their first days of life. Parenteral iron supplementation of piglets is usually carried out by a single intramuscular injection of 200 mg of iron dextran complex within 2–3 days after birth. This method of supplementation is routinely used in pig farming and is considered the gold standard in IDA prevention in piglets [8]. However, a single high dose of iron administered parenterally may disturb the fragile iron homeostasis of newborn piglets. Providing large amounts of iron to piglets substantially increases the expression of hepcidin—a key

systemic regulator of iron metabolism [9], and consequently, via function of the hepcidin–ferroportin regulatory axis [10], may inhibit the release of iron to the blood from enterocytes, macrophages, and hepatocytes. Parenteral iron administration can also lead to more dramatic outcomes, such as sudden cardiovascular collapse and respiratory failure [11].

The need for early postnatal supplementation is due to the low fetal iron reserves in newborn piglets (they are born with 50–70 mg of iron in the liver) [1]. The hepatic iron content in piglets at birth covers their iron requirement for only the first 3–4 days of postnatal life. Indeed, in piglets not supplemented with iron, the hepatic iron content is five times lower on day 4 after birth compared to day 1 after birth, and on day 7, it is barely detectable [12]. Moreover, the iron content in sow's milk has been reported to range from 1.4 to 2.6 μg/mL [13], which is not sufficient for the development of piglets. Notably, the daily requirement of iron for fast-growing piglets in the first weeks of life is approximately 7–18 mg of iron [1]. The low hepatic iron content of piglets at birth is possibly an outcome of increased litter sizes and high birth weights, which are two objectives of the selective breeding program of contemporary pigs. Consequently, the iron transferred from the pregnant sow must be distributed among a greater number of fetuses with greater birth weight. Mahan and Newton [14] found that highly prolific sows have lower body iron content than sows with decreased prolificacy. Moreover, highly prolific sows may provide less iron to the developing piglets [15]. In contrast, our recent study showed that RBC and iron status (including hepatic iron content) in newborn piglets are not primarily determined by the litter size [16]. Nevertheless, the main objective of iron supplementation in pregnant sows is to increase their iron levels, and in turn, to provide their fetuses with the extra iron required to meet the erythropoietic needs of newborn piglets. This review focuses on the history and results of supplementing sows with iron during pregnancy to enhance the iron transfer to the fetuses and increase their iron stores.

2. The Placenta: The Unappreciated Organ of Iron Metabolism

Knowledge about iron transport across the placenta is crucial for planning iron supplementation in pregnant sows. In 2016, after Cao and Fleming [17] recalled the importance of the placenta in iron metabolism, much research has been devoted to transplacental iron transport and its regulation [18,19]. The placenta is a highly vascularized organ responsible for the exchange of nutrients and gases between the mother and developing fetus. The porcine placenta is classified as diffuse epitheliochorial [20]. This designation distinguishes it from the placentae of other livestock species in that there are no placentomes (making it diffuse), and both the fetal and maternal epithelial cell layers are maintained throughout gestation (making it epitheliochorial). Sows are pregnant for approximately 115 days, with a normal pregnancy duration ranging from 111 to 120 days depending on the breed. In pigs, fetal iron stores are generated in the liver during the 10 weeks before birth [21]. This period is critical for building up the fetal iron stores that are used in the first weeks of postnatal life (before piglets can absorb sufficient amounts of iron from the feed [22]) to support their rapid growth and erythropoiesis. Several mechanisms improve iron availability in the maternal circulation during pregnancy, including both increased intestinal iron absorption and the release from maternal iron stores [21]. As pregnancy progresses, maternal absorption of non-heme iron increases, and heme iron absorption likely follows a similar trend [21,22]. Maternal iron stores are mobilized from the liver and spleen during pregnancy, as shown in studies of pregnant pigs [23] and rodents [24,25]. Hepcidin is a small peptide hormone produced mainly by hepatocytes that orchestrates body iron fluxes (including iron transfer across the placenta) by adjusting iron supply body iron requirements. Hepcidin binds to ferroportin (Fpn) to induce its degradation, thus inhibiting iron release from exporting cells [23]. Its expression is controlled by iron levels, erythropoietic activity, and inflammatory cues [24,25]. In pregnancy, maternal and fetal hepcidin is regulated by both maternal and fetal iron conditions [26]. Iron supplementation of sows during pregnancy is intended to increase maternal iron status, and consequently, to enhance the efficiency of iron transfer to the developing fetuses. That is why understanding

the mechanisms of placental iron transport is crucial to properly conduct this procedure. The main pathways of iron transport across the placenta are depicted in Figure 1.

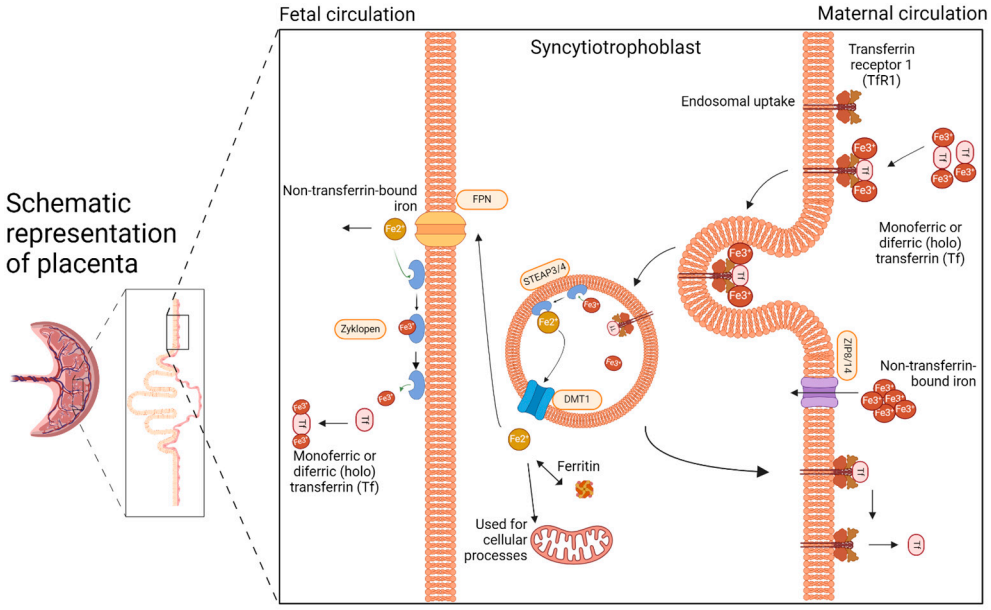

Figure 1. Pathways of iron transfer across the placenta. Created with BioRender.com.

Iron is delivered to the placenta by the maternal circulation, where it is mainly bound to transferrin (Tf), forming a monomeric or diferric (holo) Tf-Fe^{3+} complex. The primary route of iron uptake by the placenta involves the uptake of Tf-bound iron from the maternal circulation through transferrin receptor 1 (TfR1) on the apical membrane of the placental syncytiotrophoblast facing the maternal circulation [17,27]. The Tf-Fe^{3+} complex binds to TfR1 and then is internalized into the cell by clathrin-mediated endocytosis. In the acidic environment of the endosome, ferric ions dissociate from Tf and are then reduced to the ferrous (Fe^{2+}) state by ferrireductases, possibly by STEAP3 (six-transmembrane epithelial of prostate) or STEAP4 [28]. Following the release of iron, the apo-Tf-TfR1 complex is recycled back to the membrane. The neutral pH of the extracellular space facilitates the dissociation of apo-Tf from TfR1 and its release back to the maternal circulation. TfR1 is then again available for the uptake of the Tf iron complex. Within the syncytiotrophoblast, ferrous iron is transported out of the endosome into the cytoplasm by divalent metal transporter 1 (DMT1) [29,30]. Studies in knock-out DMT1 mice clearly indicate that DMT1 is dispensable for iron transport across the placenta [31]. Other potential iron transporters expressed in the placenta include the Zrt/Irt-like proteins ZIP8 and ZIP14, members of the solute carrier family 39A (SLC39A) [32]. ZIP14 has been shown to transport non-transferrin-bound iron [33]. Similarly to DMT1, ZIP14 seems to be dispensable in the placental iron transport process, as null ZIP14 mice have normal iron stores [34]. In contrast, ZIP8 deficiency was found to cause anemia and lethality in mouse embryos [35], which demonstrates that ZIP8 is essential for prenatal development. Once in the cytoplasm, iron can be stored as ferritin (Ft), used for cellular processes, or exported to the fetal circulation via Fpn, the only known mammalian iron exporter. In this process, Fpn cooperates with zyklopen, a copper-dependent ferroxidase [36], which oxidases ferrous to ferric iron that can be bound by fetal Tf. Zyklopen is not essential for iron transport to the fetus in mice [37].

3. Supplementation of Pregnant Sows with Iron

3.1. Oral vs. Parenteral Iron Supplementation

In the literature, two main ways of administering iron to sows have been described: oral supplementation, in which iron is added to feed, or parenteral supplementation, in which iron compounds are administered via intramuscular injection.

3.1.1. Oral Iron Supplementation

Oral iron supplementation was the first procedure used to treat iron deficiency in piglets by increasing iron in pregnant sows. In one of the first studies to report IDA in piglets, McGowan and Crichton [4] attempted to prevent anemia in piglets by oral supplementation of sows during pregnancy. Subsequent studies by Hart [38] and Hooks [39] also addressed this issue. Oral iron supplementation of sows is a widely employed approach in pig farming. However, it is essential to consider both the positive and negative aspects associated with this procedure.

In terms of the positive aspects of oral supplementation, ease of administration is an obvious one, as iron can be provided to sows simply by adding iron formulation to their feed [40]. Moreover, this type of iron supplementation does not cause distress to the sows in a critical condition, such as pregnancy [41]. However, iron absorption from the gastrointestinal tract can vary depending on the iron formulation used, the sow's physiology and gut health, the concurrent dietary intake, or both components. This variability in absorption may result in inconsistent iron availability and effectiveness.

High oral iron doses or inappropriate supplementation protocols can also disrupt the gut microbiota in pregnant sows, potentially leading to dysbiosis and associated gastrointestinal disorders [42]. Maintaining gut health is crucial for optimal nutrient utilization and the well-being of both the mother and developing piglets. Excessive oral iron supplementation can lead to iron overload in pregnant sows, causing oxidative stress and tissue damage [43]. Iron excess can also predispose sows to infections and negatively impact various organs, including the liver and heart [12]. Proper dosing and monitoring of supplemental iron are necessary to prevent iron overload and its associated risks. Determining the optimal timing and duration of oral iron supplementation during pregnancy is essential. Administering iron during critical stages, such as early to mid-gestation, should support maternal iron stores and promote optimal piglet development. However, prolonged supplementation beyond the necessary period should be avoided to minimize the risk of iron overload in the sow.

3.1.2. Parenteral Iron Supplementation

Parenteral intramuscular iron supplementation is a commonly employed method to alleviate iron deficiency in piglets; only a few studies have implemented this method of iron supplementation in pregnant sows [44–47]. It is essential to consider both the benefits and risks associated with this supplementation approach.

Parenteral intramuscular iron injection effectively and rapidly improves iron levels in pregnant sows, addressing iron deficiency and preventing anemia [44,45]. Improving iron levels leads to enhanced oxygen transport, energy metabolism, and overall health during pregnancy. Adequate iron applied through parenteral intramuscular supplementation positively impacts reproductive performance in pregnant sows. Improved iron levels promote better fertility, larger litter sizes, and higher birth weights, leading to healthier piglet outcomes; however, the effect of this form of supplementation on improving the level of liver iron reserves in newborn piglets is negligible [44]. Thus, parenteral supplementation of iron is limited only to preventing anemia in pregnant sows. In terms of the risks, parenteral intramuscular iron injections may cause local site reactions, such as pain, swelling, or abscess formation. Additionally, it can cause systemic iron overload [12].

The most common method of iron supplementation in pregnant sows, as described in this review, is the addition of iron formulation to the sow's fodder during pregnancy. This choice is mostly dictated by the ease of the approach and the fact that veterinary staff are not needed, unlike with parenteral iron supplementation.

3.2. Doses and Formulations of Supplemental Iron

Doses of supplemental iron described in research articles vary from low to very high amounts (milligrams to grams, respectively) given orally or intramuscularly. Table 1 presents an overview of iron formulations, doses, and corresponding effects. It is worth

mentioning that the dose of the supplementation also determines the form of iron administration. Excessive amounts of iron cannot be administered parenterally because of iron toxicity.

The simplest formulation broadly used in the pig farming industry is orally supplemented inorganic iron in the form of ferrous oxide [4] or ferrous sulfate [48,49]. Ferrous oxide, a compound that contains iron in the +2 oxidation state (Fe^{2+}), is used to provide elemental iron to the body. Ferrous sulfate, an iron salt that also contains Fe^{2+}, is often given as either ferrous sulfate monohydrate (containing ~32% elemental iron) or ferrous sulfate heptahydrate (~20% elemental iron). Ferrous sulfate is more widely used than ferrous oxide because it has better bioavailability and is relatively inexpensive. The dose of iron in its inorganic form varies from 100 ppm per day to 700 ppm per day [48,49] and is given daily with feed. This inorganic iron formulation is currently the most used form of oral supplementation in pig farming [40].

Another form of dietary supplemental iron used in pregnant sows is the chelated form of iron, mostly as a complex with amino acids [50]. Chelation is a chemical process where metal ions, such as iron, are bound to organic molecules like amino acids to create stable complexes. The resulting chelated iron is better absorbed than non-chelated iron. Amino acid chelated iron has been formulated to increase the bioavailability of iron. Some commonly used amino acids for iron chelation include glycine, lysine, and methionine. These amino acids are known to form stable complexes with iron, improving its solubility and stability in the gastrointestinal tract and facilitating its absorption [51]. Chelated iron is thought to be transferred across the intestinal barrier by routes different from those by which inorganic iron is transferred: the transfer uses amino acid transporters, thus bypassing the canonical route of iron transporters. This form is expected to allow iron to enter the circulation using an alternative pathway, which avoids tightly regulated canonical pathways of iron absorption employing iron transporters controlled by hepcidin, iron regulatory proteins, or hypoxia inducible factor 2.

The daily dose of amino acid chelated iron varies from 300 mg to 651 mg [50,52]. Formulations used in this type of supplementation are ferrous fumarate, iron lactate, iron glycine chelate, iron methionine chelate [50], and ferrous N-carbamylglycinate [53]. These forms of iron supplementation have been shown to have a positive influence on the hematological status of newborn piglets [15,50,52].

Lactoferrin [53–55], a milk protein with a high affinity for iron, is a promising carrier for delivering iron across the intestine to the circulation. Its ability to bind and transport iron in a non-toxic manner reduces the risk of iron-induced oxidative stress, which is often associated with traditional iron supplementation. By acting as a shuttle for iron, lactoferrin can facilitate its controlled and targeted release, potentially improving bioavailability and reducing the risk of iron overload [56]. Daily doses of lactoferrin-bound iron vary from 200 mg/kg of feed to 1 g/kg of feed. However, supplementing sows with lactoferrin showed no improvement in terms of the piglets' hematological status [56]. Moreover, the lactoferrin–iron complex as an iron supplementation formulation is more expensive than simple inorganic formulations.

With new technologies, it has been possible to develop new, more sophisticated oral iron formulations. Novel sucrosomial ferric pyrophosphate (SFP) can improve iron absorption while reducing gastrointestinal side effects. It consists of tiny particles of ferric pyrophosphate encapsulated in a sucrosomial matrix. The sucrosomial matrix helps to protect the iron from interacting with the acidic environment in the stomach, and thus reduces gastrointestinal irritation [57]. The main postulated benefit of sucrosomial ferric pyrophosphate is enhanced iron absorption. The sucrosomial matrix allows the iron to be absorbed more efficiently in the small intestine, bypassing canonical routes of iron absorption. It is postulated that this form of encapsulated iron is taken up by intestinal cells as a whole complex, without the mediation of specific iron transporters [58]. In our previous research, we gave 60 mg of Fe per sow per day [23] and found that SFP did not significantly affect the systemic iron homeostasis of sows during pregnancy or the hepatic

iron stores of the newborn piglets. We hypothesized that additional iron administered orally to healthy pregnant sows is insufficiently transferred across the placenta [59].

Another form of oral iron supplementation is the organic form of iron in the heme complex [58]. Heme, a ferrous iron protoporphyrin IX complex, is engaged as a prosthetic group in a number of heme proteins (including cytochromes, hemoglobin, and myoglobin) that is involved in important cellular and systemic physiological processes. Heme iron is highly bioavailable, as up to 30% can be absorbed, whereas the absorption of non-heme iron is more variable (1–10%) [60]. The absorption of heme iron occurs through specific heme transporters present in the small intestine, the main one being heme carrier protein 1 (HCP1). HCP1 imports heme iron into the enterocytes, where heme molecules are degraded by heme oxygenase 1 to yield ferrous iron, which can consequently be exported from enterocytes into the bloodstream via Fpn [61]. Later, it was shown that HCP1 has high affinity for folates, and its loss of function causes folate malabsorption [62]. Another candidate for intestinal heme absorption is the heme-responsive gene, expressed in the small intestine, where it can function as a heme importer [60]. Heme iron as a supplement looks promising, but apart from the high cost of such an iron preparation, experimental studies have yielded inconclusive results as to its effectiveness [6,63]. In our studies, we administered heme iron to pregnant sows and found no effect on the fetuses or newborn piglets (unpublished data).

The intramuscular iron supplementation of pregnant sows uses only one iron formulation, iron dextran (FeDex), which is widely used in the pig farming industry. Iron dextran is a complex of iron and the carbohydrate dextran. The structure of FeDex consists of iron compound (usually in the form of ferric hydroxide) molecules bound to dextran, a starch-derived polysaccharide [64,65]. When FeDex is administered intramuscularly, it is released slowly into the circulation; the iron is then taken up from plasma by the macrophages (called Kupffer cells) of the reticuloendothelial system of the liver, spleen, and bone marrow, where iron is released from the iron–carbohydrate complex to join a cellular low-molecular-weight iron pool. Iron is then either stored in ferritin, the major tissue iron-storage protein, or released from the cell bound to Tf, to circulate in plasma and be distributed to tissues, particularly the bone marrow [66]. In reports focusing on iron supplementation in pregnant sows, the doses used in the experimental designs ranged from 22 mg to 300 mg of Fe [44,45,47], usually given in a single injection, but Ducsey et al. [47] describe a supplementation regimen in which single dose of FeDex is divided into five smaller doses and injected on days 40, 45, 50, 55, and 60 of pregnancy. This administration aimed to avoid the negative effects of FeDex intramuscular injections, though the authors concluded that treating the sow would not likely eliminate the need for FeDex injection in piglets to prevent anemia. Current studies and newborn piglet rearing practices clearly show that FeDex given to newborn piglets by intramuscular injection can prevent them from developing IDA, but there is no clear evidence that the same treatment in sows during pregnancy can increase prenatal iron stores in fetuses through placental transfer [8,12].

All of the forms of iron supplementation mentioned above are given in addition to the standard diet for pregnant sows. The first official nutrient recommendations for swine were published in 1944 by The National Research Council, long after the first reported cases of IDA in pigs [67]. Since then, there have been 11 updates, each of which eliminated requirements and recommendations for various nutrients that were no longer relevant or appropriate and added new ones. In the 11th Edition of the Nutrient Requirements of Swine, the recommended daily amount of iron for gestating sows is 168 mg per day [40]. However, it is important to mention that in most studies, iron preparations were given in addition to the standard iron supplement in sow feed, which consisted mainly of corn. The earliest studies reported only the composition of the feed, but not the basic iron content. In later studies, the initial iron content in the diet of pregnant sows began to be reported, and ferrous sulfate was the iron preparation used due to its cost-effectiveness. In peer-reviewed studies, the dietary iron content ranged from 20 mg/kg [68] of feed to 190 mg/kg [68] of

feed. The iron content of the feed was usually calculated [68], or a standard commercial feed was used and the iron contents were provided by the supplier [54].

3.3. The Iron Levels of Pregnant Sows and Their Impact on Piglets

If left untreated, naturally developing IDA in piglets causes enormous economic losses due to slower growth rates and, more importantly, mortality [1]. All of the abovementioned iron formulations were intended to improve the iron status of pregnant sows, but more importantly, they were designed to enhance the transport of this microelement to developing fetuses and create an iron reserve in the fetal liver large enough to be sufficient for the first few weeks of the piglets' life [57]. It was assumed that improving the iron status of pregnant sows would benefit that of the offspring, thereby eliminating the need for postnatally supplementing piglets with iron during their first days of life.

In early research, Pilgrim and Quershi [59] observed that piglets born to sows supplemented with iron had higher hemoglobin levels and better growth rates than piglets born to non-supplemented sows. They found that the iron status of the supplemented sows was significantly improved compared to females from the control group. Pregnancy has a significant impact on the fitness of the sows, as reported by Pond et al. [61]. Hemoglobin levels in pregnant sows at the end of pregnancy are significantly lower if females are not supplemented with iron. O'Connor et al. [69] also noted that during pregnancy, sows that were supplemented with iron in their feed did not experience a drop in hematological parameters at the end of pregnancy. These observations suggest the need to supplement pregnant sows not only to prevent the development of IDA during pregnancy but also to possibly increase iron transport to the developing fetuses.

In some of the reviewed studies, iron supplementation was provided throughout pregnancy, while in others, supplementation was provided only during the last trimester of pregnancy, when hepatic loading with iron occurs. In both approaches, supplemented sows showed an improved hematological status during pregnancy and after parturition. Supplemented sows also had better liver iron stores than unsupplemented control sows [70,71]. However, both the hematological and iron status of the piglets born to supplemented sows were similar to those of piglets born to untreated controls [70,71]. In contrast, Li et al. [72] observed an improvement in piglet RBC parameters compared to those of the unsupplemented control group, but at the same time, suspected that this improvement could be related to the consumption of maternal feces after birth, which is a typical behavior of piglets. Indeed, it has been found that the fecal iron content of iron-supplemented sows is very high [57]. Most studies reported a slightly improved iron status of piglets born to sows supplemented with iron, but iron supplementation did not affect the further development of the piglets [46]. The fact that iron supplementation in pregnant sows does not translate clearly to a higher iron status in their progeny strongly suggests that the placental transfer of iron to fetuses is insufficient, even in the presence of excess iron. However, the iron concentration in the placenta of iron-supplemented sows is higher than that in untreated animals. This finding may be explained by the increased expression of placental iron transporters [70], especially that of TfR1, which is responsible for iron uptake and also indicates increased iron uptake by placental tissue. At the same time, increased expression of Fpn, the only known iron exporter, is to be expected, yet no changes in the expression of this protein were observed. This finding may indicate iron retention in the placental tissue [57,70], which may be due to the increased expression of fetal hepcidin, the main inhibitor of Fpn. The fetal liver can produce hepcidin during embryonic development and thus may regulate placental iron transport and fetal iron homeostasis [73]. Such fetal hepcidin upregulation can indicate high fetal iron levels and is hypothesized to be a response to fetal iron overload [19,57]. However, one report noted that hepatic iron stores in piglets from supplemented sows were indeed elevated compared to controls [72]. Piglets born to iron-supplemented sows may receive adequate iron during fetal development, yet still show signs of developing IDA shortly after delivery. This phenomenon suggests that while

iron supplementation during pregnancy is beneficial for both sows and piglets, it may still be inadequate to ensure sufficient iron stores in the early stages of the piglets' postnatal life.

It should be noted that in all of the studies mentioned in this review, the scientific research was conducted on healthy sows that did not suffer from symptoms of anemia. Whether iron supplementation would benefit sows and piglets suffering from IDA requires further investigation in the field.

3.4. The Impact of Iron Supplementation in Pregnant Sows on the Iron Content of Milk

After birth, the main dietary iron source for piglets is sow's milk; however, this milk is not rich in iron (1.3–1.5 mg/L) [74], and its concentration is not sufficient to ensure proper levels of this microelement in developing piglets. Attempts have been made to establish if iron supplementation in sows during pregnancy or during lactation could influence the iron content in milk. Experimental data have shown that supplementation of sows can slightly improve milk iron content [52] or conversely, that supplementation has no effect [57]. Xing et al. [55] attempted to increase the iron level of milk by adding iron-saturated lactoferrin to it, in amounts ranging from 1.56 to 3.76 mg/L, but even this addition was not sufficient to prevent the development of IDA and decreased growth rates in piglets.

Table 1. Studies documenting attempts to supplement pregnant sows to improve the iron status of mothers and their offspring. n.d., not defined; HGB, hemoglobin level; RBC, red blood cell level.

Author (Year)	Formulation	Dose of Fe	Length of Supplementation	Breed of Pigs	Effect on Piglets
McGowan (1924) [4]	Fe_2O_3	40 g per day	Last weeks of pregnancy	n.d.	HGB—↑ RBC—↑ Liver Fe—n.d. Serum Fe—n.d.
Hart et al. (1930) [38]	$Fe_2(SO_4)_3$ Fe_2O_3	10–250 mg per day	During pregnancy	Poland China, Duroc Jersey, and Chester White	HGB—↑ RBC—↑ Liver Fe—↑ Serum Fe—n.d.
Pilgrim and Qureshi (1952) [59]	iron dextran	100 mg per day	During pregnancy	n.d.	HGB—↑ RBC—n.d. Liver Fe—n.d. Serum Fe—n.d.
Rydberg et al. (1959) [45]	iron dextran solution	1 g in a single dose	14 days before parturition	n.d.	HGB—↑ RBC—↑ Liver Fe—n.d. Serum Fe—n.d.
Pond et al. (1961) [44]	iron dextran	1 g or 5 g in a single dose	From day 100 of pregnancy	Berkshire and Yorkshire	HGB—no change RBC—↑ Liver Fe—n.d. Serum Fe—n.d.
Spruill et al. (1971) [48]	$FeSO_4$	200 mg per day	94 days before prepartum	Hampshire and Yorkshire	HGB—↑ RBC—n.d. Liver Fe—n.d. Serum Fe—no change
Lillie and Frobish (1978) [49]	$FeSO_4$	30 or 60 mg per day	During pregnancy	Duroc	HGB—no change RBC—n.d. Liver Fe—n.d. Serum Fe—↑

Table 1. *Cont.*

Author (Year)	Formulation	Dose of Fe	Length of Supplementation	Breed of Pigs	Effect on Piglets
Ducsay et al. (1984) [47]	iron dextran	5.5 g (1.1 g per injection)	From day 40 to 60 of pregnancy	n.d.	HGB—↑ RBC—↑ Liver Fe—↓ Serum Fe—n.d.
O'Connor et al. (1989) [69]	$FeSO_4x7H_2O$	200 mg per day	During pregnancy	n.d.	HGB—↑ RBC—n.d. Liver Fe—n.d. Serum Fe—n.d.
Egeli et al. (1998) [75]	amino acid chelated iron or glutamic chelated iron	300 mg per day 650 mg per day	Last 3 weeks of pregnancy	Norwegian Landrace	HGB—↑ RBC—↑ Liver Fe—n.d. Serum Fe—no change
Peters and Mahan (2008) [15]	chelated to soy proteinor $FeSO_4$	20 mg per day	During pregnancy	Yorkshire Landrace	HGB—↑ RBC—n.d. Liver Fe—n.d. Serum Fe—n.d.
Wang (2014) [52]	organic iron complex or $FeSO_4$	650 mg per day	From day 84 of gestation	n.d.	HGB—↑ RBC—↑ Liver Fe—n.d. Serum Fe—↑
Zhao et al. (2015) [76]	chelated iron (bacterial iron)	n.d.	From day 84 of gestation	Yorkshire Landrace	HGB—no change RBC—n.d. Liver Fe—n.d. Serum Fe—no change
Jahan et al. (2017) [77]	lactoferrin	1 g per day	During pregnancy	Large White, Landrace, Duroc	HGB—n.d. RBC—n.d. Liver Fe—n.d. Serum Fe—n.d.
Buffler et al. (2017) [71]	Fe(II) SO_4 $7H_2O$	688 mg per day, on average	During pregnancy	German Landrace	HGB—↑ RBC—n.d. Liver Fe—↑ Serum Fe—no change
Li et al. (2018) [72]	Fe-Gly	150 mg 240 mg 330 mg 420 mg per day, on average	From day 86 of gestation	Landrace x Large White sows	HGB—↑ RBC—↑ Liver Fe—↑ Serum Fe—↑
	$FeSO_4xH_2O$				HGB—↑ RBC—↑ Liver Fe—n.d. Serum Fe —↑
Wan et al. (2018) [70]	ferrous N-carbamylglycinate chelate or $FeSO_4$	447 mg per day 457 mg per day	From day 86 of gestation	Landrace x Large Yorkshire	HGB—n.d. RBC—n.d. Liver Fe—↑ Serum Fe—n.d.
Barros et al. (2019) [78]	Yes Minerals Iron®	1.3 g per day	From day 84 of gestation	Topigs Norsvin®	HGB—n.d. RBC—n.d. Liver Fe—n.d. Serum Fe—n.d.
Mazgaj et al. (2020) [57]	sucrosomial ferric pyrophosphate	60 mg per day	From day 80 of gestation	990 line	HGB—no change RBC—no change Liver Fe—no change Serum Fe—no change

Table 1. *Cont.*

Author (Year)	Formulation	Dose of Fe	Length of Supplementation	Breed of Pigs	Effect on Piglets
Zhang et al. (2022) [54]	lactoferrin and Fe-Gly	400 mg 700 mg 1 g per day	From day 80 of gestation	no info	HGB—n.d. RBC—n.d. Liver Fe—no change Serum Fe—⬆
Xing et al. (2023) [55]	lactoferrin or heme iron or Fe-Gly	700 mg per day 280 mg per day 1.5 g per day	From day 33 of gestation	Landrace x Yorkshire	HGB—n.d. RBC—n.d. Liver Fe—⬆ Serum Fe—⬆

4. Discussion

Iron is an essential microelement required for various physiological functions in the body, including oxygen transport, energy metabolism, and DNA synthesis. Iron deficiency is a common problem in piglets and can lead to anemia and decreased growth rates [1]. Iron supplementation in pregnant sows has been suggested as a convenient procedure for improving piglets' iron status. Iron supplementation of pregnant sows is desirable from a farming perspective, as this strategy for preventing IDA in piglets is considered to be more cost-efficient and less labor-intensive than parenterally supplementing piglets with iron dextran, and it avoids the stressful effects of that strategy. Yet, how to ensure adequate iron stores for piglets during pregnancy remains an unanswered question. Over the years, as outlined in this review, researchers and pig farmers have been trying different methods to increase the iron in sows and thus increase iron loading to the developing fetuses. Since the early 1980s and through the 1990s, farmers and researchers have struggled with the problem of IDA in high-performing pig breeds, which are developed and crossbred to produce high numbers of piglets per litter, each growing rapidly after birth [16]. One of the first methods addressing this problem was the use of injectable iron supplements into piglets shortly after birth [8]. However, this method was found to be labor-intensive, time-consuming, and stressful for piglets. That being said, it should be underlined that the intramuscular injection of FeDex is a cheap and effective procedure for preventing IDA in piglets.

Lately, efforts have centered around enhancing the iron reserves in pregnant sows by administering oral iron supplements, in the hope of facilitating improved iron transport to the fetuses and thus preventing IDA in newborn piglets altogether. Although the effectiveness of this method in improving iron levels in piglets is still under investigation, available data indicate that its effect is likely very limited and that it does not prevent the development of anemia in piglets to a satisfactory level. A very interesting aspect of the research is reexamining the placenta as a unique player in the iron transfer from the pregnant female to fetuses. This issue of placental iron transport is being raised in an increasing number of studies, yet most have been in mice and may not include differences in the architecture of the mouse and pig placentae [19,79].

5. Conclusions

IDA is a common nutritional disorder in suckling piglets, which can negatively impact their health, growth, and development. Iron supplementation of pregnant sows has been and is still considered to be an important strategy for preventing neonatal IDA in pigs. The rationale for supplementing pregnant sows is to increase their systemic iron status and consequently enhance iron transfer across the placenta, thus fortifying the iron stores in the fetuses; these stores then serve as an abundant source of endogenous iron during early postnatal life. Over the years, pregnant sows have been subjected to various methods, doses, formulations, and durations of iron supplementation, with only limited success.

Further studies related to transplacental iron transport are needed to elucidate the limited ability of iron-supplemented sows to deliver this microelement to their fetuses.

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