INSTYTUT GENETYKI
I BIOTECHNOLOGII ZWIERZĄT
Polskiej Akademii Nauk

INSTITUTE OF GENETICS
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Preventive effect of oral supplementation with Sucrosomial® Iron in iron deficiency anemia of prematurity: studies on the animal model of preterm piglets

Profilaktyczny efekt doustnej suplementacji Sucrosomial® Iron w zapobieganiu niedokrwistości z niedoboru żelaza wcześniaków: badania na nowym eksperymentalnym prosięcym modelu wcześniaczym

Xiuying Wang, MSc

Research Supervisor

Prof. Dr hab. Rafał Radosław Starzyński

Institute of Genetics and Animal Biotechnology of the Polish Academy of Sciences

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

8-OHdG, 8-hydroxydeoxyguanosine
BMP, bone morphogenetic protein
CRP, C-reactive protein
CYBRD1, cytochrome b reductase 1
DMT1, divalent metal transporter 1
$FeSO_4$, ferrous sulfate
FLVCR, feline leukemia virus subgroup C cellular receptor
FPN, ferroportin
GDF15, growth differentiation factor 15
HFE, homeostatic iron regulator
HFT, ferritin heavy chain
HO1, heme oxygenase 1
IDA, iron deficiency anemia
IL, interleukin
LFT, ferritin light chain
MCH, mean cell hemoglobin
MCHC, mean corpuscular hemoglobin concentration
MCV, mean corpuscular volume
MRPP, Multiple Response Permutation Procedure
PCA, Principal Component Analysis
PND, postnatal day
RBC, red blood cell
RET-He, reticulocyte hemoglobin equivalent
SI, Sucrosomial® Iron
SMAD, small mother against decapentaplegic
TBI, total body iron
TFR, transferrin receptor
TIBC, total iron binding capacity
TLR, toll-like receptor
TNFα, tumor necrosis factor α
TSAT, transferrin saturation

List of publications included in this doctoral dissertation

1 Wang X, Lipiński P, Ogłuszka M, Starzyński RR. Iron status and risk of iron disorders in neonates: a narrative review of recent studies in animal models. Nutr Res Rev. 2025.
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2 Wang X, Lenartowicz M, Mazgaj R, Ogłuszka M, Szkopek D, Zaworski K, Kopeć Z, Żelazowska B, Lipiński P, Woliński J, Starzyński RR. Preterm piglets born by cesarean section as a suitable animal model for the study of iron metabolism in premature infants. Int J Mol Sci. 2024;25(20):11215.
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3 Wang X, Lipiński P, Ogłuszka M, Mazgaj R, Woliński J, Szkopek D, Zaworski K, Kopeć Z, Żelazowska B, Tarantino G, Brilli E, Starzyński RR. Oral supplementation with Sucrosomial® Iron improves the iron status of preterm piglets delivered by cesarean section. Food Funct. 2025;16(9):3525-3541.
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Summary

Iron is essential for the healthy development of newborns. A need is especially urgent for preterm infants who have low iron stores because the prenatal period during which iron accumulates is shortened. The mechanisms of iron regulation differ between adulthood and the early postnatal period, and they also vary between full-term and preterm infants. However, the regulation of iron homeostasis in preterm infants is still poorly understood. Our review (Publication 1) highlights the strengths and limitations of animal models for preterm birth research, particularly emphasizing the value of large animals in studying neonatal iron metabolism. We believe that preterm piglets may represent an excellent model for investigating iron status in human preterm infants.

In experiment 1 (Publication 2), the main goal was to develop and validate a piglet model of human prematurity to assess iron status in preterm infants. In this research, we performed a cesarean section on sow on the 109_{th} day of pregnancy, corresponding to the last trimester of human pregnancy, to obtain preterm piglets. Full-term piglets born naturally via vaginal delivery were used as controls. Blood, liver and spleen samples were collected at birth (postnatal day 0, PND0). Compared to full-term piglets, lower body weight, red blood cell indices, plasma iron level, transferrin saturation and total body iron were observed on preterm piglets, reflecting poor iron status in preterm piglets. However, preterm piglets showed high hepatic and splenic non-heme iron contents and plasma and hepatic ferritin levels. In addition, premature birth induced high mRNA expression of iron-regulatory hormone hepcidin in the liver, accompanied by changes in its regulatory factors including bone morphogenetic protein 6, erythroferrone and growth differentiation factor 15. In spite of this, no difference was observed between full-term and preterm piglets in plasma hepcidin-25 levels and in the protein abundances of the iron-exporter ferroportin in the liver and spleen. Overall, preterm piglets show a pattern of iron metabolism characteristic of functional iron deficiency and iron accumulation in the tissue. It seems that the domestic pig model of prematurity is a reliable animal model that represents iron status of late prematurity in humans.

In experiment 2 (Publication 3), we used cesarean-born piglets to investigate the effects of an innovative oral iron formulation, Sucrosomial® Iron (SI), on early iron deficiency. Full-term and preterm piglets were supplied (or not supplied) with iron from day 4 to day 10 after birth, and samples of blood, liver and spleen were collected on PND11. Similar to the results from PND0, poor growth and iron status were observed in preterm piglets on PND11. They developed iron deficiency anemia, as indicated by decreased red blood cell indices and plasma iron parameters. The iron deficiency in preterm piglets was partially improved by SI supplementation via the enhancement of red blood cell count, hemoglobin level and reticulocyte hemoglobin equivalent level in the blood, and an increase of hepatic and splenic non-heme iron contents. In addition, there were differences in the levels of hepcidin and ferroportin, two key factors related to iron metabolism regulation, as well as in the regulatory factors of hepcidin, between full-term and preterm piglets. SI supplementation can regulate the hepcidin-ferroportin axis to alleviate the negative effects of iron imbalance in preterm piglets. Moreover, SI caused neither inflammatory nor oxidative responses, and showed comparable effects to those of ferrous sulfate, a commonly used iron supplement. These results reaffirm the suitability of cesarean-delivered preterm piglets as a model for humans and demonstrate that SI is a promising iron supplement for improving postpartum iron status in preterm infants.

Experiment 3 were designed to compare intestinal iron absorption mechanisms and microbial community composition between full-term and preterm piglets, and to evaluate the effects of SI on the intestine. The animal experiments were conducted as described in experiments 1 and 2. Samples of duodenum and rectal digesta were collected on PND0 and PND11. Premature birth resulted in adverse changes in the structure of villi and crypts in the duodenum, and reduced levels of key factors involved in iron absorption on PND0 and PND11. Significant differences in alpha and beta diversity, as well as in microbial composition in the rectum, were found between full-term and preterm piglets on both PND0 and PND11. Oral SI improved duodenal morphology and enhanced the protein levels of ferritin in both full-term and preterm piglets. The microbial composition varied markedly between iron-supplemented and non-supplemented piglets. SI suppressed the abnormal

growth of *Bifidobacterium* and enhanced the abundances of *Ligilactobacillus*, *Limosilactobacillus*, *Leyella* and *Segatella* in full-term or preterm piglets. These results indicate an underdeveloped iron absorption mechanism and a different composition of intestinal microbiota in preterm piglets compared to full-term piglets. SI improves intestinal structure and microbiota composition.

In conclusion, cesarean-delivered piglets serve as a suitable model for studying iron metabolism in preterm human infants. Oral SI partially mitigated the adverse effects of iron imbalance associated with preterm birth.

Streszczenie

Żelazo jest niezbędne do zdrowego rozwoju noworodków. Potrzeba ta jest szczególnie ważna w przypadku wcześniaków, które wykazują niskie zapasy żelaza, ponieważ okres prenatalny, w którym żelazo jest magazynowane, ulega skróceniu. Mechanizmy regulacji żelaza różnią się znacznie w wieku dorosłym w porównaniu z wczesnym okresem postnatalnym, podobnie jak różnią się w przypadku niemowląt urodzonych o czasie i wcześniaków. Jednakże regulacja homeostazy żelaza u wcześniaków jest nadal słabo poznana. Praca przeglądowa stanowiąca część dysertacji (publikacja 1) podkreśla mocne i słabe strony modeli zwierzęcych w badaniach nad wcześniakami, szczególnie podkreślając wartość dużych zwierząt w badaniu metabolizmu żelaza u noworodków ludzkich. Podkreślamy w niej, że wcześniaki zwierzęce mogą stanowić doskonały model do badania statusu żelaza u wcześniaków ludzkich.

Dlatego też, w części eksperymentalnej dysertacji, eksperyment 1 (publikacja 2) głównym celem było opracowanie i walidacja modelu wcześniaków prosięcych w celu oceny statusu żelaza i walidacji pod kontem wykorzystania jako model wcześniaków ludzkich. W badaniu tym w celu uzyskania wcześniaków prosięcych u loch w 109. dniu ciąży, co odpowiada ostatniemu trymestrowi ciąży u ludzi wykonaliśmy cesarskie cięcia. Prosięta urodzone drogą naturalną wykorzystane były jako zwierzęta kontrolne. Próbki krwi, wątroby i śledziony pobrano zaraz po urodzeniu (dzień 0 po urodzeniu, PND0; postnatal day). W stosunku do prosiąt urodzonych o czasie, wcześniaki miały niższą masę ciała, niższe parametry czerwonokrwinkowe, obniżony poziom żelaza w osoczu, wysycenie transferyny i obniżony całkowity poziom żelaza w organizmie, co wskazywało na niekorzystny stan homeostazy żelaza u prosiąt wcześniaczych. Co ciekawe, zmierzono wysoką zawartość żelaza niehemowego w wątrobie i śledzionie oraz wysokie poziomy ferrytyny w osoczu i wątrobie prosiąt wcześniaczych. Ponadto, wcześniactwo związane było z wysoką ekspresją w wątrobie mRNA hepcydyny, ogólnoustrojowego hormonu regulującego poziom żelaza, czemu towarzyszyły zmiany czynników regulujących poziom hepcydyny, w tym białka morfogenetycznego kości 6, erytroferronu i czynnika różnicowania wzrostu 15. Pomimo

zmian poziomu mRNA hepcydyny w wątrobie nie zaobserwowano różnicy między prosiętami urodzonymi o czasie i wcześniakami w poziomach białka hepcydyny-25 w osoczu jak również zmian w poziomie jedyne znanego eksportera żelaza, ferroportyny zarówno w wątrobie jak i śledzionie. Podsumowując, wcześniaki prosięce wykazują wzorzec metabolizmu żelaza charakterystyczny dla funkcjonalnego niedoboru żelaza z akumulacją żelaza w tkankach. Ponadto, model wcześniactwa u świni domowej wydaje się być wiarygodnym modelem zwierzęcym odzwierciedlającym status żelaza charakterystyczny dla późnego wcześniactwa u ludzi.

W eksperymencie 2 (publikacja 3) wykorzystaliśmy prosięta urodzone przez cesarskie cięcie, aby zbadać wpływ innowacyjnego doustnego suplementu żelaza, Sucrosomial® Iron (SI), w leczeniu/zapobieganiu niedoborom żelaza u wczesniaków. Wcześniakom podawano doustnie żelazo w formie SI od PND4 do PND10, a próbki krwi, wątroby i śledziony pobrano w PND11. Podobnie jak w przypadku wyników dla prosiaków w PND0, wcześniaki wykazywały osłabiony wzrost oraz obniżone ogólnoustrojowe parametry żelaza w PND11, czego skutkiem była rozwijająca się niedokrwistość z niedoboru żelaza, o czym świadczyły zarówno zmniejszona liczba czerwonych krwinek jak i obniżone parametry żelaza w osoczu. Niedobór żelaza u wcześniaków został częściowo skorygowany przez suplementację SI co skutkowało zwiększeniem liczby czerwonych krwinek, poziomu hemoglobiny i poziomu ekwiwalentu hemoglobiny w retikulocytach oraz zwiększeniem zawartości żelaza niehemowego w wątrobie i śledzionie. Ponadto, w wątrobach i śledzionach między prosiętami kontrolnymi i wcześniakami stwierdzono różnice w poziomach hepcydyny i ferroportyny, dwóch kluczowych czynników zaangażowanych w regulację metabolizmu żelaza, a także czynników regulujących hepcydynę w wątrobie. Podsumowując eksperyment drugi stwierdzamy, że w celu złagodzenia negatywnych skutków deregulacji żelaza u wcześniaków suplementacja SI wpływa na modulację osi hepcydyna-ferroportyna. Ponadto podanie wcześniakom SI nie wywołuje reakcji zapalnych jak róznież stresu oksydacyjnego i wykazuje przy tym porównywalne efekty do podania terapeutycznych dawek siarczanu żelazawego, powszechnie stosowanego suplementu żelaza. Wyniki te potwierdzają również przydatność prosięcego modelu wcześniaczego jako modelu dla wcześniaków ludzkich i

wskazują, że SI jest obiecującym suplementem żelaza e celu poprawy poporodowego statusu żelaza u wcześniaków.

Eksperyment 3 został zaprojektowany w celu porównania mechanizmów wchłaniania żelaza w dwunastnicy oraz składu mikrobioty jelitowej prosiąt urodzonych o czasie i przedwcześnie oraz w celu oceny wpływu SI na florę jelitową i same jelita. Eksperyment 3 przeprowadzono w oparciu o eksperymenty 1 i 2. Próbki treści pokarmowej z dwunastnicy i okrężnicy pobrano w PND0 i PND11. Poród wcześniaczy spowodował niekorzystne zmiany w strukturze kosmków i krypt dwunastniczych oraz obniżył poziomy kluczowych czynników zaangażowanych we wchłanianie żelaza. Wykazano istotne różnice w różnorodności alfa oraz beta, a także w składzie mikrobiologicznym okrężnicy między prosiętami urodzonymi o czasie i przedwcześnie zarówno w PND0, jak i PND11. Co ciekawe, doustne podanie SI poprawiło morfologię dwunastnicy i zwiększyło poziom ferrytyny u prosiąt zarówno urodzonych o czasie jak i przedwcześnie. Skład mikrobiologiczny różnił się wyraźnie między prosiętami otrzymującymi suplementy żelaza i zwierzętami pozbawionymi suplementacji. Podanie SI zahamowało nieprawidłowy wzrost Bifidobacterium i zwiększyło liczebność Ligilactobacillus, Limosilactobacillus, Leyella i Segatella zarówno u prosiąt urodzonych przed terminem jak i przedwcześnie. Podsumowując tę część badań, wyniki wskazują na słabo rozwinięty mechanizm wchłaniania żelaza i odmienny skład mikrobioty jelitowej u prosiąt urodzonych przed terminem w porównaniu do prosiąt urodzonych o czasie. Ponadto, podanie SI poprawia strukturę jelit i skład mikrobioty.

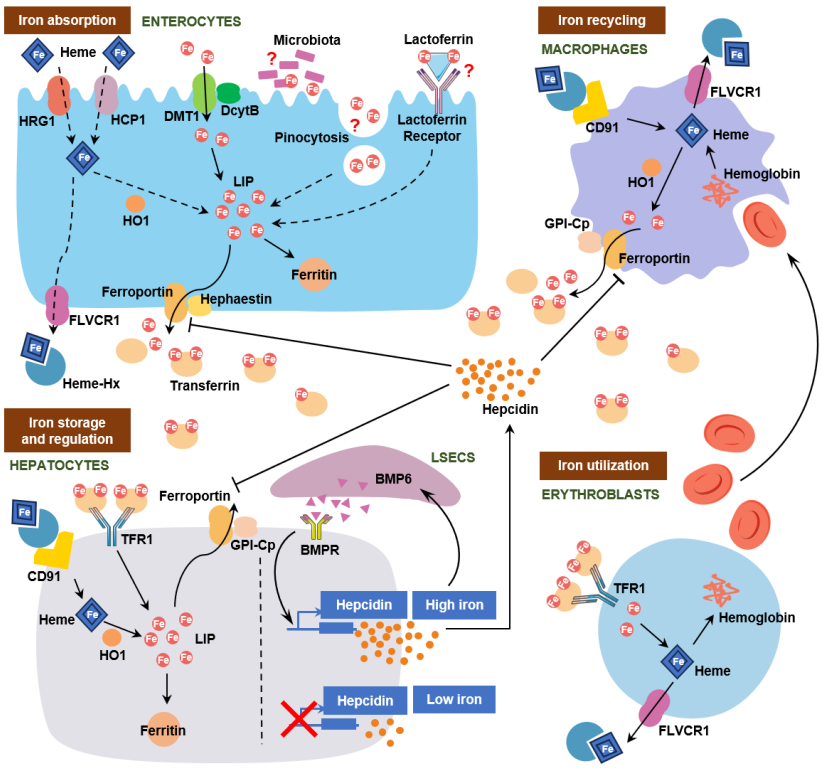
Podsumowując, prosięta urodzone przez cesarskie cięcie stanowią odpowiedni model do badania metabolizmu żelaza u wcześniaków. Doustne podanie SI wcześniakom częściowo łagodzi niekorzystne skutki zachwianej homeostazy żelaza związanej z przedwczesnym porodem.

1 Introduction

1.1 Neonatal iron metabolism and regulation

Iron is one of the most vital metals for sustaining life, especially during the rapid growth and development of infancy. Most body iron is bound to hemoglobin in red blood cells (RBCs), iron storage proteins (such as ferritin and hemosiderin) in the liver, and myoglobin in muscle tissue (Dev and Babitt, 2017). The remaining part is distributed among ubiquitous heme- and non-heme-containing enzymes (such as cytochromes, catalase, and aconitase) and transferrin, the main iron transport protein in circulation (Dev and Babitt, 2017). The ability of iron to donate and accept electrons during redox reactions makes it essential for fundamental biological processes, including oxygen delivery, energy metabolism, cellular respiration, cell proliferation and differentiation (Wang et al., 2019). Importantly, it is required for myelination, synaptogenesis, and neurotransmitter synthesis during the fetal and early neonatal periods (Rao et al., 2003; Bastian et al., 2016; Cheli et al., 2018). However, iron can be toxic under aerobic conditions, as it catalyzes the propagation of reactive oxygen species via Fenton reaction, leading to oxidative stress, tissue injury and disease (Vogt et al., 2021). Since mammals lose iron through the shedding of mucosal and skin cells or bleeding but lack a regulated mechanism for iron excretion, iron balance is maintained by tightly controlling dietary absorption.

Systemic iron turnover is primarily regulated through the iron-related activities of specific cells: duodenal enterocytes absorb the heme and non-heme iron from the diet; hepatocytes serve as the main site of iron storage and synthesize the iron-regulatory hormone hepcidin; erythroblasts in the bone marrow are essential for heme and hemoglobin production; and reticuloendothelial macrophages recycle iron from aged erythrocytes (Publication 1-Fig. 1).



Publication 1-Fig. 1 Systemic iron turnover in the neonate. Duodenal enterocytes absorb two forms of dietary iron, non-heme and heme iron, through different absorption mechanisms. Heme uptake by enterocytes is primarily mediated by heme carrier protein 1 (HCP1) and possibly by heme responsive gene 1 (HRG1). Inside enterocytes, heme is catabolized by the enzyme heme oxygenase 1 (HO1) to elemental iron, biliverdin, and carbon monoxide. In turn, by means of iron reductase duodenal cytochrome b (DcytB) and divalent metal transporter 1 (DMT1), non-heme iron crosses the apical membrane of the intestinal epithelial cell to enter the cytoplasm. After that, iron enters the labile iron pool (LIP) in the cytosol, a source of iron for heme synthesis and iron-sulfur cluster biogenesis. The cytoplasmic iron is subsequently incorporated into iron-containing proteins, stored in the iron storage protein ferritin, or exported into the circulation by the only known iron exporter ferroportin, with the participation of the copper-dependent ferroxidase hephaestin (or glycosylphosphatidylinositol-linked, membrane-bound ceruloplasmin, GPI-Cp in other cell types). In the bloodstream, iron is bound by transferrin and taken up by hepatocytes, erythroblasts, and other cells through transferrin receptor 1 (TFR1)-mediated endocytosis. The potential pathway of heme iron export from enterocytes involves the transfer of intact heme (not catabolized by HO1) across the basolateral membrane by feline leukemia virus subgroup C cellular receptor 1 (FLVCR1) to the blood, where it is captured by hemopexin and delivered in the form of heme-hemopexin (Heme-Hx) via receptor CD91 to various sites in the body. Other pathways that may contribute to early iron absorption include lactoferrin receptor-mediated process, pinocytosis and gut microbiota. Iron recirculation by tissue macrophages, which phagocytose senescent erythrocytes and rapidly degrade the heme released from hemoglobin through the catabolic activity of HO-1, ensures a major part of daily iron supply to erythroid precursors. Systemic iron homeostasis is regulated by hepcidin, a hormone secreted by the hepatocytes. In cases of iron deficiency, low hepcidin facilitates the unimpeded ferroportin function of professional iron exporter cells. When the organism is saturated with iron, the hepcidin blood level increases, and this peptide has a greater potential to interact with ferroportin and consequently to reduce iron transfer into the bloodstream. In this process, bone morphogenetic protein 6 (BMP6) acts as an “iron sensor”. Its transcription in liver sinusoidal endothelial cells (LSECs) is induced by high iron levels. BMP6 is then released from LSECs and has a stimulatory paracrine effect on hepcidin expression in hepatocytes by binding to BMP receptors (BMPRs). Solid lines represent confirmed pathways, while dashed lines indicate proposed pathways that have yet to be confirmed.

Two classical intestinal iron transporters, divalent metal transporter 1 (DMT1) and ferroportin (FPN), are essential for adult iron absorption. However, an accumulating body of research has shown that the modulation of iron absorption in early life might differ from that in adulthood. A rat study by Leong et al. (2003b) demonstrated that the gene expression of intestinal *Dmt1* and *Fpn1* is age-dependent: on day 1 after birth, their expression levels were low but increased markedly by day 40. Similar trends were observed in a piglet model that the protein levels of DMT1 and FPN were barely detectable during the first two days of life and only started to increase around the fourth day after birth (Lipiński et al., 2010). These can be explained by the findings of Lopez et al. (2006), who show that DMT1 is mislocalized during late gestation, expressed at minimal levels in early life, and predominantly present in its deglycosylated form until postnatal day 20 (PND20). Thompson et al. (2007) who used rat pups with a *Dmt1* gene mutation, demonstrated that DMT1 is not essential for iron absorption from breast milk. Until the development-dependent expression of DMT1 in the intestine is induced, alternative pathways such as the lactoferrin receptor, pinocytosis, and gut microbiota may facilitate iron uptake (Lopez et al., 2006; Deschemin et al., 2016; Helman et al., 2019). Conflicting reports exist; for example, Ramakrishnan et al. (2015) found that disrupting DMT1 expression in the intestines of mouse pups caused rapid anemia at weaning, suggesting that DMT1 is crucial for neonatal iron absorption. Another iron transporter, FPN, can facilitate the transport of iron out of cells. The embryonic lethality observed in $Fpn^{null/null}$ mice indicates that FPN is essential for early development (Donovan et al., 2005). Iron absorption was severely impaired in suckling FPN knockout mice, confirming the critical role of FPN in intestinal iron absorption (Frazer et al., 2017). At this stage, enterocyte FPN shows reduced responsiveness to hepcidin, which may enable sustained high iron absorption regardless of hepcidin levels, thereby minimizing the risk of iron deficiency during development (Frazer et al., 2017). In fact, it remains largely unclear which characteristics of the immature intestine contribute to the high absorption of iron from milk and whether this process involves components of the adult iron absorption machinery.

Systemic iron homeostasis is tightly regulated by hepcidin, which is produced in the liver. This hormone regulates plasma iron levels by controlling the absorption of dietary iron

from the intestine, the release of recycled hemoglobin iron from macrophages, and the mobilization of stored iron from hepatocytes (Nemeth et al., 2004). The process may be related to the hepcidin-FPN axis, where hepcidin binds to FPN, leading to its internalization and degradation, which reduces the export of cellular iron (Nemeth et al., 2004). The synthesis of hepcidin is influenced by various processes, such as iron status, inflammation, erythropoiesis, and hypoxia (Lee and Beutler, 2009; Liu et al., 2016). Increased intracellular iron in the liver enhances the production of bone morphogenetic proteins (BMPs) by liver sinusoidal endothelial cells, which bind to BMP receptors and a BMP co-receptor hemojuvelin to activate small mother against decapentaplegic (SMAD) phosphorylation and promote hepcidin transcription (Babitt et al., 2007; Andriopoulos et al., 2009; Canali et al., 2017). Hepcidin synthesis is also regulated by serum iron concentrations. Holo-transferrin competes for homeostatic iron regulator (HFE) binding to transferrin receptor 1 (TFR1), causing HFE to become displaced and form a complex with TfR2 (Goswami and Andrews, 2006; Schmidt et al., 2008; D'Alessio et al., 2012). This TfR2-HFE complex induces hepcidin expression by interacting with the BMP6/hemojuvelin/SMAD pathway (Goswami and Andrews, 2006; Schmidt et al., 2008; D'Alessio et al., 2012). In addition, inflammatory cytokines, in particular interleukin 6 (IL6), can upregulate hepcidin by binding to the IL6 receptor to activate the Janus kinase, which in turn switches on latent transcription factor-signal transducer and activator of transcription 3 (Wrighting and Andrews, 2006; Verga Falzacappa et al., 2007). During erythropoietic stress, the increased erythropoietin level in the kidney induces secretion of the erythroblast-produced erythroid factor erythroferrone and thereby suppresses the hepatic production of hepcidin (Kim and Nemeth, 2015).

Though hepcidin is important in iron homeostasis in adults, the role of this peptide during early life is poorly understood. A prospective observational study that included 221 infants reported that the hepcidin concentration in the cord blood of full-term babies (gestational age ≥37 weeks) was more than twice as high as that of moderate preterm infants (gestational age 30–36 weeks), and that the concentration was lowest in very preterm infants (gestational age <30 weeks) (Lorenz et al., 2014). The findings of Cross et al. (2020) support these observations. Kitajima et al. (2011) also reported that levels of serum pro-hepcidin (a

60-amino acid form of the prohormone hepcidin) at birth positively correlated with gestational age and birth weight. However, Tiker et al. (2006) found no relationship between pro-hepcidin levels and gestational age, and both healthy preterm and full-term newborns have high pro-hepcidin levels. In addition, hepcidin concentrations in full-term neonates are similar to those in adults (Lorenz et al., 2013). Studies in pigs have also shown a high abundance of hepcidin transcripts in the liver of 1- and 2-day-old full-term piglets, which is similar to that of 200-day-old adult pigs (Lipiński et al., 2010). Yet, Cross et al. (2020) presented other data showing that full-term cord blood hepcidin concentration was higher than adults' serum hepcidin concentration. These conflicting findings complicate our understanding of the role of hepcidin in regulating neonatal iron metabolism. Aside from gestational age, hepcidin levels in infants have been shown to correlate with both the size of their iron stores (Lipiński et al., 2010; Lia et al., 2011; Lorenz et al., 2014; Uijterschout et al., 2016) and the mode of delivery (cesarean section or vaginal delivery) (Lorenz et al., 2014).

1.2 Premature birth and iron deficiency

According to the World Health Organization (2023a)'s definition, premature birth occurs when baby is born alive before 37 weeks of pregnancy are completed, whereas a baby born after 37 weeks of gestation is considered full-term. An estimated 15 million babies have been born too early in the last decade due to the effects of multiple pregnancies, infections, genetic factors, and unidentified causes (World Health Organization, 2023a, b). Premature birth has become the leading cause of child mortality, responsible for over 20% of all deaths in children under the age of five years (World Health Organization, 2023a, b).

Healthy full-term infants born to mothers with sufficient iron levels typically have enough iron reserves to sustain their growth and development during the first 4–6 months of life (Domellöf et al., 2014), whereas premature infants often lack these reserves (Akkermans et al., 2016; Moreno-Fernandez et al., 2019). Compared with full-term infants, premature infants face a significantly higher risk of iron deficiency. The majority of iron stored at birth is accumulated during the last trimester of pregnancy (Raffaeli et al., 2020), making total

iron stores positively related to gestational age; thus, the more premature the infant, the poorer its iron status. Research has shown that preterm small-for-gestational-age infants have less total iron stores than preterm appropriate-for-gestational-age infants, who in turn have lower stores than full-term infants (Mukhopadhyay et al., 2012). After birth, premature infants experience further depletion of their already low iron stores, driven by rapid catch-up growth involving blood volume expansion and increased hemoglobin demand (Raffaeli et al., 2020). The postnatal drop in hemoglobin in full-term neonates is well tolerated, but this decline is more rapid in preterm neonates (Jopling et al., 2009; Strauss, 2010). Other factors affecting the iron status of premature infants include maternal factors, such as severe maternal iron deficiency (Kemppinen et al., 2020; Raffaeli et al., 2020), obesity (Phillips et al., 2014; Jones et al., 2016), and diabetes (Georgieff et al., 1990; Petry et al., 1992), which can impair neonatal iron endowment. In addition, phlebotomy for laboratory testing and disorders causing bleeding or hemolysis both contribute to anemia of prematurity by leading to additional iron loss (Strauss, 2010).

Iron deficiency in early life adversely affects infant development, leading to dysfunction in multiple organs, including the heart (Alioglu et al., 2013), intestine (Berant et al., 1992) and brain (Georgieff, 2011). In severe neonatal iron deficiency, iron was preferentially allocated to the production of RBCs rather than brain accretion (Zamora et al., 2016). A clinical trial found that preterm infants with anemia and low iron stores exhibited a higher occurrence of abnormal neurologic reflexes compared with nonanemic, iron-replete infants (Armony-Sivan et al., 2004). These reflexes included glabella reflex, Babinski reflex, plantar and palmar grasp, as well as passive movements of the arms and legs. Premature infants with iron deficiency also exhibit impaired auditory neural maturation (Amin et al., 2010; Choudhury et al., 2015). It is noteworthy that early iron deficiency impacts brain and behavioral function not only during the deficiency period but also long after treatment, with mechanisms involving long-term alterations in dopamine metabolism, myelination, and hippocampal structure and function (Georgieff, 2011). Therefore, maintaining proper iron balance is essential in newborns, particularly preterm infants, as iron deficiency can have lasting adverse effects.

1.3 Iron supplementation

Several professional societies have published recommendations for iron supplementation in human infants. According to guidelines of the European Society for Paediatric Gastroenterology, Hepatology and Nutrition, there is no need for general iron supplementation of healthy, full-term, breast-fed infants during the first months of life (Domellöf et al., 2014). Given that exclusive breast-feeding beyond 6 months has been associated with an increased risk of iron deficiency and iron deficiency anemia (IDA), all infants should receive iron-containing complementary foods (Domellöf et al., 2014). The American Academy of Pediatrics also proposes that term infants up to 6 months of age should receive 1 mg/kg/day of iron if iron cannot be provided by dietary sources (Domellöf et al., 2014). Both the European Society for Paediatric Gastroenterology, Hepatology and Nutrition and the Canadian Paediatric Society's Nutrition and Gastroenterology Committee recommend an elemental iron intake of 1–2 mg/kg/day for marginally low birth weight infants (a birth weight of 2–2.5 kg) starting at 2 to 6 weeks of age and continuing to 6 months of age, as well as of 2–3 mg/kg/day for those weighing less than 2 kg (Domellöf et al., 2014; Unger et al., 2019; Bahr et al., 2021). Similarly, the American Academy of Pediatrics suggests a daily iron supplementation of 2 mg/kg/day for preterm infants aged 1 to 12 months (Bahr et al., 2021). It is especially important to avoid iron supplementation for any child with severe malnutrition until recovery, because iron can make infections worse (World Health Organization, 2013).

Oral iron administration is the simplest and most cost-effective intervention to reduce iron deficiency. Prototypical examples of orally administered iron are inorganic and organic iron salts, e.g., ferrous sulfate ($FeSO_4$), citrate, gluconate, fumarate, and lactate (Man et al., 2022). Orally administering $FeSO_4$ to iron-deficient rat pups normalized iron transporter expression levels, body iron uptake, and mucosal iron retention (Leong et al., 2003a). However, oral administration is usually associated with poor iron absorption and bioavailability, as well as potential adverse effects, including inhibition of micronutrient absorption, imbalance in intestinal microbiota, gastrointestinal distress, nausea, diarrhea,

constipation, and neonatal morbidity (Long et al., 2012; Man et al., 2022). New formulations of slow-release iron, such as chelated and microencapsulated iron compounds, have been developed to address this problem (Man et al., 2022). These compounds may increase iron absorption and decrease side effects by preventing contact between iron and the intestinal mucosa.

Sucrosomial® Iron (SI) is an innovative oral iron formulation, where ferric pyrophosphate is encased within a phospholipid bilayer and a sucrester matrix (sucrosome) (Fig. 1) (Gómez-Ramírez et al., 2023). The sucrester matrix protects the iron from acidic gastric fluid, allowing SI to reach the intestinal mucosa intact (Bastida et al., 2021). There, it can be absorbed through two pathways: para-cellular and trans-cellular (including M cells), independently of DMT1 mediation (Fig. 2) (Fabiano et al., 2018a; Bastida et al., 2021; Gómez-Ramírez et al., 2023). This unique structure ensures that SI provides high iron bioavailability and excellent gastrointestinal tolerance. Evidence from clinical studies supports the use of SI as a valid option for treating iron deficiency and IDA associated with chronic kidney disease, inflammatory bowel disease, bariatric surgery, post-operative anemia, congestive heart failure, and other conditions (Gómez-Ramírez et al., 2023). Comparative studies on newborn full-term piglets have shown that SI better relieves IDA compared to five other iron supplements: $FeSO_4$, phospholipid-coated iron nanoparticles, dextran-coated iron nanoparticles, pure iron nanoparticles, and iron dextran (Mazgaj et al., 2021). Other studies supporting the superiority of SI over other iron supplements have been reported in both *in vitro* (Tarantino et al., 2015) and *in vivo* (Asperti et al., 2018; Suva and Tirgar, 2024) experiments. Currently, there are no reports regarding the effects of SI on premature infants.

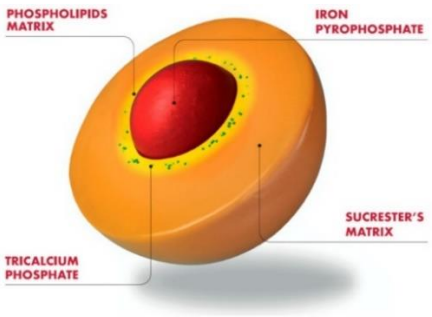


Fig. 1 Schematic structure of Sucrosomial® Iron (Gómez-Ramírez et al., 2023)

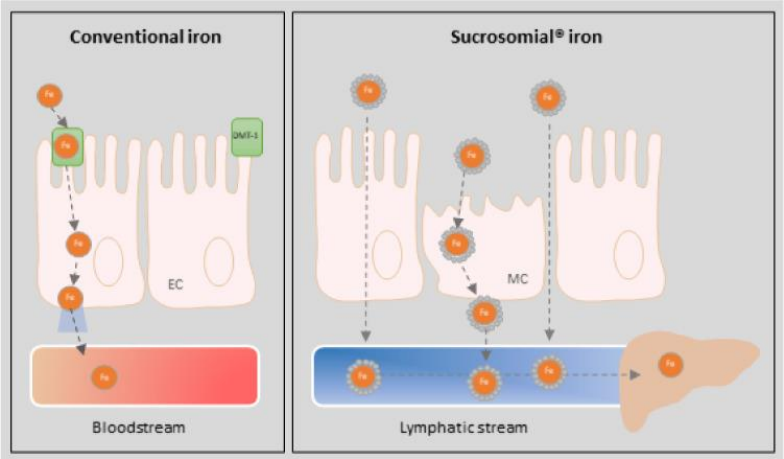


Fig. 2 Schematic representation of absorption mechanisms of conventional iron and Sucrosomial® Iron. EC, enterocytes; MC, M cells (Bastida et al., 2021)

2 Hypotheses

- Cesarean-born piglets represent a suitable model for investigating iron homeostasis in late preterm human infants.
- SI is a promising iron supplement with significant potential to alleviate the negative effects of iron imbalance caused by premature birth.

3 Objectives

- To develop a model of human late prematurity using cesarean-born piglets.
- To compare the iron status of prematurely born piglets with that of full-term piglets.
- To evaluate the efficacy of oral SI supplementation in preterm piglets, focusing on its effects on systemic iron homeostasis and gastrointestinal microbiome colonization.
- To compare the efficacy of oral SI supplementation in preterm piglets with the oral "golden standard" supplement, $FeSO_4$.

4 Materials and methods

4.1 Animals and experimental design

All experimental procedures were approved by the 2nd Local Ethics Committee in Warsaw (WAW2/125/2021). In this experiment, we used six Polish Landrace sows: three to obtain preterm piglets and three to obtain full-term piglets. Pregnancy was confirmed by ultrasound examination at 35–40 days after insemination. Each sow was housed in an individual pen and provided a balanced diet twice daily, meeting their nutritional requirements according to gestational stage. Water was available *ad libitum*. On the 109_{th} day of gestation, a cesarean section was conducted on three sows to deliver preterm piglets. Twenty-four preterm piglets of both sexes were obtained. For the control group, eighteen naturally born Polish Landrace piglets (with an average gestation period of 115 days) were delivered vaginally. Six preterm piglets and six full-term piglets were immediately weighed and sacrificed for sampling at birth (PND0) to compare their characteristics with those of human newborns.

The remaining thirty piglets were used in iron supplementation experiments. They were transferred to an environmentally controlled room in the Laboratory of Large Animal Models (The Kielanowski Institute of Animal Physiology and Nutrition of the Polish Academy of Sciences, Jabłonna, Poland), where the temperature was maintained between 35-37°C and humidity around 56%. Within 3 to 4 hours after birth, each preterm piglet was inserted with an intragastric feeding tube. The tube was used to feed the preterm piglets until they learned to use a nursing bottle, usually by day 2 or 3. Newborn piglets were assigned to one of five groups: (1) full-term piglets without iron supplementation; (2) full-term piglets supplemented with SI (PharmaNutra, Pisa, Italy; 2 mg Fe/piglet/day) from day 4 to day 10 after birth; (3) preterm piglets without iron supplementation; (4) preterm piglets supplemented with SI (2 mg Fe/piglet/day) from day 4 to day 10 after birth; (5) preterm piglets supplemented with $FeSO_4$ (Gambit, Kutno, Poland; 2 mg Fe/piglet/day) from day 4 to day 10 after birth. All full-term and preterm piglets were housed in special cages equipped with dry bedding and heating pads. They were allowed to move freely within the cages and

had visual and tactile contact with each other, but no contact with the sow. During the experiment, all piglets were given every hour with warm (37°C) human milk (in collaboration with Milk Bank, Saint Family Hospital, Warsaw, Poland). Piglets were weighed every morning before iron was administered.

4.2 Sample collection

On PND0 and PND11, blood was collected directly from the heart under isoflurane anesthesia (1.28%) into heparin tubes. Plasma was isolated by centrifugation at 1000 × g for 15 minutes and stored at −80°C until further use. After euthanasia via intravenous injection with 0.5 mL\kg b.w. of Morbital (133.3 mg/mL of sodium pentobarbital + 26.7 mg/mL of pentobarbital, Biowet, Puławy, Poland) in piglets, liver, spleen and duodenum samples were quickly dissected and flushed with PBS. One portion of each tissue sample was immediately fixed in a 4% formaldehyde solution (Sigma-Aldrich, St. Louis, MO, USA) for histological examination. The remaining portions were frozen in liquid nitrogen and stored at −80 °C until analysis. Bone marrow cells for Perls' staining were smeared onto slides and fixed with methanol. The digesta was obtained from the rectum and stored at −80 °C for microbial diversity analysis.

4.3 RBC and reticulocyte indices and plasma iron parameters

RBC and reticulocyte indices were measured by ProCyte Dx Hematology Analyzer (IDEXX Laboratories, Westbrook, ME, USA). Plasma iron concentration and total iron binding capacity (TIBC) were detected using the colorimetric method for an iron–chromazurol complex (absorbance at 630 nm), with reagents from Biomaxima (Lublin, Poland). Percent of transferrin saturation (TSAT) was calculated according to the following formula: TSAT (%) = (plasma iron (μg/dL) / TIBC (μg/dL)) × 100. Plasma ferritin concentration was determined using a porcine ELISA kit (Fine Biotech, Wuhan, China).

4.4 Total body iron (TBI) and non-heme iron contents in tissues

TBI was calculated as described previously (Miller et al., 2003): TBI = (0.2776 × hemoglobin (g/L) × body weight (kg)) + [(log10 plasma ferritin (ug/L) - 1.345) / 0.0439 × body weight (kg)]. Tissue non-heme iron content was determined by acid digestion of the samples at 100 °C for 10 minutes, followed by colorimetric measurement of an iron–ferrozine complex (absorbance at 562 nm, Beckman DU-68, Beckman Coulter, Brea, CA, USA).

4.5 Perls' staining and intestinal morphology

After a 24 h fixation in a 4% formaldehyde solution, hepatic, splenic and duodenal segments were dehydrated, embedded in paraffin, and cut into 5 μm sections with a Hyrax M25 rotary microtome (Zeiss, Oberkochen, Germany). The sections were then mounted on glass slides, deparaffinized, stained with Perls' Prussian blue for 30 min, counterstained with nuclear red (Sigma-Aldrich, St. Louis, MO, USA) for 2 min and analyzed under a light microscope (Eclipse E200, Nikon, Amsterdam, The Netherlands). The same staining procedure with Prussian Blue was applied to bone marrow smears. The 10 longest and straightest villi, along with their associated crypts from each sample, were measured at 20× magnification using Capture2.3 software. The villus height/crypt depth ratio was calculated.

4.6 Plasma levels of hepcidin and its regulatory factors

Plasma levels of hepcidin-25, erythropoietin, erythroferrone, growth differentiation factor 15 (GDF15) and IL6 were quantified using commercial ELISA kits (Hepcidin-25, DRG Instruments GmbH, Marburg, Germany; Erythropoietin, erythroferrone and GDF15, BlueGene Biotech, Shanghai, China).

4.7 C-reactive protein (CRP), malondialdehyde and 8-hydroxydeoxyguanosine (8-OHdG) levels

Commercial ELISA kits were used to quantify the levels of CRP (ELK Biotechnology,

Denver, CO, USA), malondialdehyde (ELK Biotechnology), and 8-OHdG (Elabscience, Houston, TX, USA), in accordance with the manufacturers' instructions.

4.8 Real-Time PCR

Total RNA was prepared using the SV Total RNA Isolation System (Promega, Madison, WI, USA). The first-strand cDNA was synthesized using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). Quantitative analysis of PCR was carried out on a LightCycler® 96 Instrument (Roche Diagnostics, Basel, Switzerland) using SYBR Green I (FastStart Essential DNA Green Master, Roche Diagnostics) as recommended by the manufacturer. The primer sequences for the target genes are listed in Supplementary Table 1. The stability of reference genes was assessed using NormFinder software (MOMA, Aarhus, Denmark). The expression of target genes relative to housekeeping genes (GAPDH or HPRT1) was calculated using the formula $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001).

4.9 Western blot analysis

The method for measuring levels of membrane (DMT1 and FPN) and cytosolic ((ferritin light chain (LFT) and ferritin heavy chain (HFT)) protein was the same as our previously described methods (Starzyński et al., 2013). In brief, 40 μg of membrane extracts (or cytosolic extracts) were prepared and separated by electrophoresis on 9% (or 16%) SDS-PAGE gels. Electroblotting of the resolved proteins onto the PVDF transfer membrane (Thermos Scientific, Waltham, MA, USA), blocking, and incubation with primary and secondary antibodies were performed. Supplementary Table 2 shows the details of the primary and secondary antibodies. For quantitative analysis of protein abundance, reactive bands were quantified relative to actin using a Molecular Imager with Quantity One 4.6 software (Bio-Rad, Hercules, CA, USA).

4.10 Microbial diversity analysis

Total DNA was extracted from rectal digesta using PureLink™ Microbiome DNA

Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA). PCR amplification, products purification, library construction and sequencing were conducted in accordance with the standardized protocol of BGI (Hong Kong, China). Raw data were filtered to generate high quality clean reads using iTools Fqtools fqcheck (v0.25, BGI, Hong Kong, China), cutadapt (v2.6, https://cutadapt.readthedocs.io/en/v2.6/installation.html) and readfq (v1.0, BGI, Hong Kong, China). If paired-end reads overlap with each other, then a consensus sequence will be generated by FLASH (v1.2.11, https://ccb.jhu.edu/software/FLASH/). The sequences with similarity levels exceeding 97% were clustered into operational taxonomic units using USEARCH (v7.0.1090, https://drive5.com/uparse/). Representative sequences of operational taxonomic units were matched to the Silva database (v138, https://www.arb-silva.de/) for taxonomic annotation with the RDP classifier software (v2.2, https://sourceforge.net/projects/rdp-classifier/). Alpha diversity indices were analyzed using Mothur (v1.31.2, https://mothur.org/wiki/). For the beta diversity analysis, Principal Component Analysis (PCA) was conducted with the R package "ade" (v3.5.1, https://www.r-project.org/), while the Multiple Response Permutation Procedure (MRPP) was performed using the R package "vegan". In addition, the sample clustering tree was computed and visualized with QIIME (v1.8.0, http://qiime.org/scripts/assign_taxonomy.html) and R.

4.11 Statistical analysis

Data from PND0 were statistically analyzed using independent samples t-tests (SPSS Statistics 26, IBM, Armonk, NY, USA). Data from PND11 (excluding preterm piglets supplied with $FeSO_4$) were analyzed by two-way ANOVA, with delivery mode (full-term normal vaginal delivery and premature delivery by cesarean section), dietary treatment (with or without SI supplementation), and the interactions between them as sources of variables (SPSS). If there was a significant interaction or an interaction trend, a post hoc test was conducted using Fisher's LSD, comparing: full-term piglets supplied with and without SI; full-term and preterm piglets supplied without SI; preterm piglets supplied with and without SI; and full-term and preterm piglets supplied with SI (SPSS). To compare the effects of SI

and $FeSO_4$ supplementation on preterm piglets, results among the preterm piglets supplied without iron, with SI, and with $FeSO_4$ were statistically analyzed using one-way ANOVA with Fisher's LSD (SPSS). Figures were prepared using R and GraphPad Prism 9.1.1 (GraphPad Software, San Diego, CA, USA). All data were presented as means with SEM. Differences at $P \leq 0.05$ were considered statistically significant, and trends were declared with $0.05 < P \leq 0.10$.

5 Results

5.1 Experiment 1 (Publication 2): Preterm piglet model preparation and its validation (samples collected on PND0)

5.1.1 Preterm piglets exhibited a considerable loss of body weight

Premature birth by cesarean section resulted in a reduction in body weight compared to full-term vaginal delivery ($P < 0.05$; Publication 2-Fig. 1). At birth, preterm piglets showed spontaneous respiration and stable hemodynamic conditions.

5.1.2 Premature birth altered RBC and reticulocyte indices

Because erythropoietic cells are the major users of iron in the body, iron homeostasis and erythropoiesis are tightly intertwined. Hemoglobin level, RBC count, hematocrit value and reticulocytes count were lower, while mean corpuscular volume (MCV) and mean cell hemoglobin (MCH) values were higher in preterm piglets compared with full-term piglets ($P < 0.05$; Publication 2-Fig. 2).

5.1.3 Preterm piglets showed a sharp decline in biochemical plasma iron parameters

Plasma iron levels, TIBC and TSAT are clinical indicators used to reflect body iron states and monitor iron deficiency and IDA (Elsayed et al., 2016; Wang et al., 2019). In preterm piglets, plasma iron levels, TIBC and TSAT were markedly lower than those in full-term piglets ($P < 0.05$; Publication 2-Fig. 3).

5.1.4 Premature birth decreased TBI

TBI allows for the evaluation of the full range of iron status from deficiency to excess and has been shown to predict anemia better than each of its constituents (hemoglobin concentration, plasma ferritin level and birth weight) (Miller et al., 2003). Babies born with low TBI confers a substantially greater risk of anemia from 3 to 12 month of age (Miller et al., 2003). Here, we found a marked drop in TBI in preterm piglets compared with full-term animals ($P < 0.05$; Fig. 3).

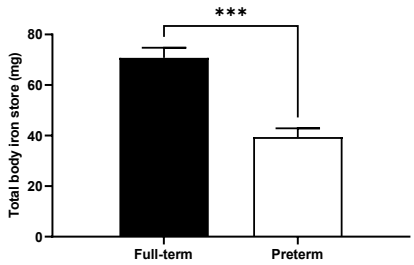


Fig. 3 Total body iron content. Data are presented as the mean ± SEM. *** asterisk denotes a statistically significant difference at $P < 0.001$ between full-term and preterm piglets.

5.1.5 Hepatic, splenic and bone marrow iron status in preterm piglets

Iron is stored in the liver, spleen and other organs of the reticuloendothelial system. Its distribution and storage are typically evaluated by quantifying tissue iron content and the levels of the iron storage protein ferritin. Preterm piglets had higher levels of hepatic and splenic non-heme iron, plasma ferritin and hepatic LFT and HFT than full-term animals ($P < 0.05$; Publication 2- Fig. 4a, Fig. 4b, Fig. 4c and Fig. 5a), but no change in the splenic ferritin protein level (Publication 2- Fig. 5b). In addition, hepatic sections with Perls' staining showed strong iron deposits in preterm piglets, while full-term animals displayed weaker staining of non-heme iron (Publication 2-Fig. 4d). However, no iron deposits were found in the spleen or bone marrow of either preterm or full-term piglets (Publication 2-Fig. 5c and Fig. S1).

5.1.6 Changes in levels of hepcidin and its regulators in preterm piglets

The peptide hepcidin, which is primarily synthesized by the liver in response to high iron levels, is regarded as the key regulator of systemic iron balance (Viatte and Vaulont, 2009). Preterm piglets showed nearly eightfold higher hepatic hepcidin mRNA expression than full-term piglets ($P < 0.05$), but no significant difference was observed in plasma hepcidin-25

concentrations between the two groups (Publication 2-Fig. 6a). In preterm piglets, the mRNA expression of BMP6, an important endogenous inducer of hepcidin synthesis (Parrow and Fleming, 2014), was upregulated ($P < 0.05$; Publication 2-Fig. 6b). In addition, plasma levels of erythroid hepcidin inhibitors, such as erythroferrone and GDF15, were lower in preterm piglets than in full-term piglets ($P < 0.05$; Publication 2-Fig. 6b). Moreover, the plasma erythropoietin concentration in preterm piglets was reduced compared to that in full-term piglets ($P < 0.05$; Publication 2-Fig. 6b). Finally, no significant differences were observed between preterm and full-term piglets in the levels of IL6 and tumor necrosis factor α (TNFα) (Publication 2-Fig. 6b and Fig. S2), cytokines responsible for inducing hepcidin during inflammation (Cross et al., 2020).

5.1.7 No difference in hepatic and splenic FPN levels between full-term and preterm piglets

The sole iron exporter in mammalian cells, FPN, transfers iron to plasma apo-transferrin and is a key target of hepcidin (Hentze et al., 2010). Both FPN mRNA and protein levels in the liver and spleen were similar between full-term and preterm piglets (Publication 2-Fig. 7).

5.2 Experiment 2 (Publication 3): SI supplementation and its therapeutic consequences on preterm piglets (samples collected on PND11)

5.2.1 Premature birth induced poor growth performance, low levels of plasma biochemical iron parameters and TBI

Decreased initial body weight, final body weight, average daily gain, plasma iron level, TSAT and TBI were observed in preterm piglets ($P < 0.05$; Publication 3-Fig. 1 and Fig. 2). Supplementation with SI did not affect these indicators.

5.2.2 Oral SI altered the levels of RBC and reticulocyte indicators in preterm piglets

An interaction between delivery mode and dietary treatment was found for RBC count, hemoglobin level, reticulocyte count and percentage, and reticulocyte hemoglobin equivalent (RET-He) level ($P < 0.05$; Publication 3-Fig. 3). In the control groups without iron supplementation, preterm piglets had lower RBC count and RET-He level, and higher

reticulocyte count and percentage compared to full-term piglets ($P < 0.05$). The administration of oral SI enhanced RBC count, hemoglobin level and RET-He level, but reduced reticulocyte count and percentage in preterm piglets ($P < 0.05$), whereas no similar effect was observed in full-term piglets. In addition, preterm piglets fed with SI had higher RBC count and hemoglobin level than full-term piglets fed with SI ($P < 0.05$). No interaction was observed between delivery mode and dietary treatment with respect to MCV, MCH and mean corpuscular hemoglobin concentration (MCHC). Compared to full-term vaginal delivery, premature delivery by cesarean section resulted in piglets with higher MCV and MCH, and lower MCHC ($P < 0.05$).

5.2.3 Oral SI enhanced hepatic and splenic iron deposits in preterm piglets

There was a significant interaction between delivery mode and dietary treatment for hepatic non-heme iron content ($P < 0.05$; Publication 3-Fig. 4A), and a trend was observed for splenic non-heme iron content ($P < 0.10$; Publication 3-Fig. 5A). In the groups without iron supplementation, preterm piglets showed an increase in non-heme iron content in the liver compared to full-term piglets ($P < 0.05$). In the preterm groups, an increase in non-heme iron content was observed in the liver and spleen of piglets supplemented with SI compared to those without iron supplementation ($P < 0.05$). In addition, SI raised higher hepatic and splenic non-heme iron contents in preterm piglets than full-term piglets ($P < 0.05$). Delivery mode and dietary treatment did not show any interaction in relation to the ferritin level (Publication 3-Fig. 4B, Fig. 4C and Fig. 5B). Plasma ferritin, LFT and HFT protein levels in the liver and spleen of preterm piglets were higher than full-term piglets ($P < 0.05$). SI tended to increase hepatic LFT and HFT protein levels ($P < 0.10$). Perls' staining displayed localized iron deposits in the liver and spleen of preterm piglets (Publication 3-Fig. 4D and Fig. 5C).

5.2.4 Oral SI regulated hepcidin-FPN axis in preterm piglets

There was a trend for interaction between delivery mode and dietary treatment for hepatic hepcidin mRNA expression and plasma hepcidin-25 concentration ($P < 0.10$; Publication 3-Fig. 7A). In cesarean-born piglets, oral SI enhanced hepatic hepcidin mRNA expression and plasma hepcidin-25 concentration in comparison to the absence of iron supplementation (*P*

< 0.05). In the SI-supplied groups, hepcidin levels in the liver and blood were higher in preterm piglets than in full-term piglets ($P < 0.05$).

For hepcidin regulators, in the plasma, an interaction was identified regarding erythroferrone concentration ($P < 0.05$), and an interaction trend was observed for erythropoietin concentration ($P < 0.10$; Publication 3-Fig. 7B). In non-iron-supplemented groups, higher erythroferrone ($P < 0.05$) and erythropoietin ($P < 0.10$) concentrations were observed on preterm piglets compared to full-term piglets. Among the preterm piglets, oral SI reduced erythroferrone and erythropoietin concentrations ($P < 0.05$). There was no interaction affecting levels of plasma GDF15 and IL6 (Publication 3-Fig. 7B). Preterm piglets had lower plasma GDF15 and IL6 concentrations ($P < 0.05$).

No interaction between delivery mode and dietary treatment was found regarding the FPN protein levels (Publication 3-Fig. 6). Preterm piglets showed a significant increase in FPN protein levels in the liver and spleen ($P < 0.05$). Oral SI tended to decrease FPN protein level in the spleen ($P < 0.10$).

5.2.5 Oral SI did not induce inflammatory and oxidative responses

Toll-like receptors (TLRs), as key sensors of the innate immune system, regulate the activation of various immune and inflammatory mediators, including proinflammatory cytokines (Wang et al., 2021). Excessive production of these cytokines can exacerbate the inflammatory response, potentially leading to tissue damage (Wang et al., 2021). In addition, CRP is a mediator of the innate immune system and functions as an acute-phase protein, secreted in response to cytokine signaling at sites of tissue injury or infection (Olson et al., 2023). No interaction between delivery mode and dietary treatment was found for the plasma CRP level (Fig. 4), and the mRNA expression of *TLR4* and proinflammatory cytokines in the liver and spleen (Publication 3-Fig. 8A). Preterm piglets had low mRNA expression of hepatic *TLR4*, *IL1β*, *IL6* and *TNFα*, as well as splenic *TNFα* ($P < 0.05$). They also tended to have low splenic *IL1β* mRNA expression and plasma CRP level ($P < 0.10$). A significant downregulation of splenic *TNFα* mRNA expression was observed following oral SI administration ($P < 0.05$).

As a product of lipid peroxidation, malondialdehyde serves as a widely recognized

marker of oxidative stress and tissue injury (Badria et al., 2015). Besides, 8-OHdG is considered a biomarker for DNA oxidative damage (Nakano et al., 2003; Tuomainen et al., 2007). There was a trend for interaction between delivery mode and dietary treatment for plasma 8-OHdG content (Fig. 4) and splenic malondialdehyde content (Publication 3-Fig. 8B) ($P < 0.10$). Compared to full-term piglets without SI supplementation, preterm piglets without supplementation and full-term piglets with supplementation showed reduced 8-OHdG content ($P < 0.05$). Splenic malondialdehyde content was higher in preterm piglets than in full-term piglets, regardless of SI supplementation ($P < 0.05$). There was no interaction effect on plasma malondialdehyde levels, while preterm piglets exhibited higher levels than full-term piglets ($P < 0.05$).

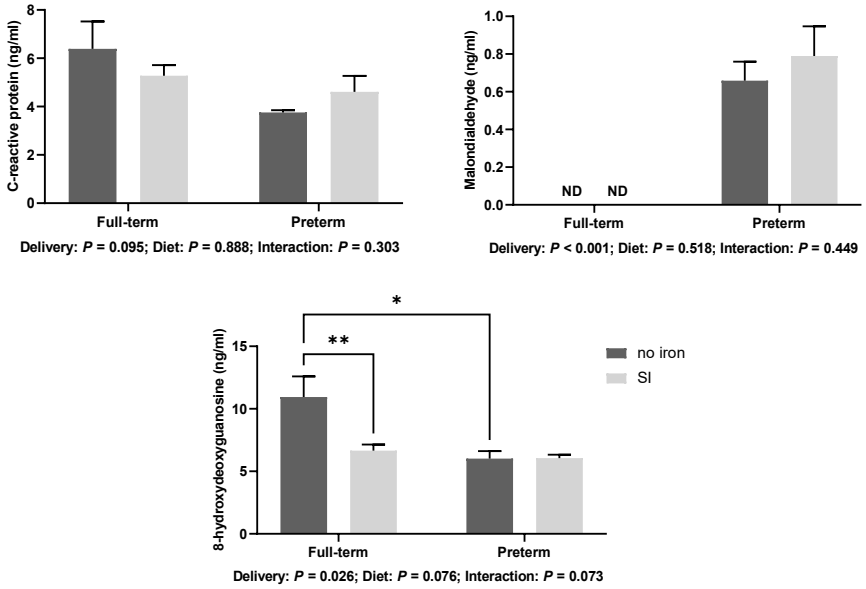


Fig. 4 Plasma C-reactive protein, malondialdehyde and 8-hydroxydeoxyguanosine contents. Data are presented as the mean ± SEM. * and ** asterisks denote statistically significant differences at $P < 0.05$ and $P < 0.01$, respectively. ND, not detectable.

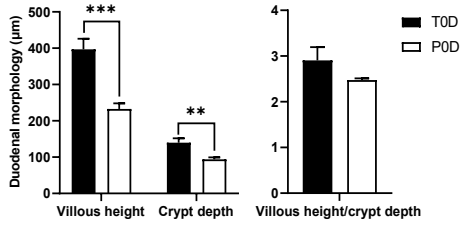
5.2.6 The supplemental effectiveness of SI is comparable to that of FeSO4 in preterm piglets

In preterm piglets, $FeSO_4$ supplementation elevated plasma iron and RET-He levels compared to those without iron supplementation ($P \leq 0.05$; Publication 3-Table 1). Yet, aside from average daily gain and plasma iron level, no significant differences in other iron indicators were detected between preterm piglets administered $FeSO_4$ and SI.

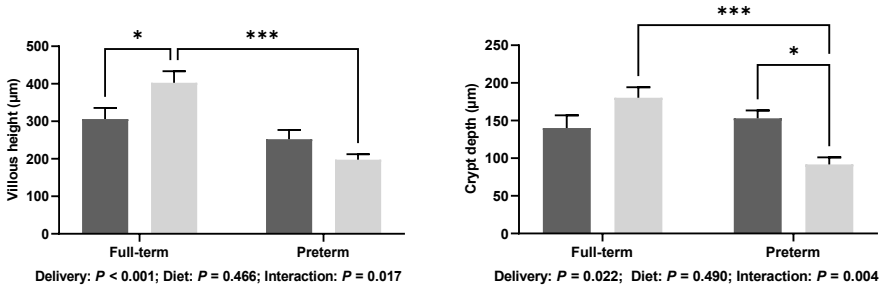
5.3 Experiment 3: The role of premature birth and SI supplementation on gut microbiota composition (samples collected on PND0 and PND11)

5.3.1 Oral SI improved duodenal morphology but had no effect on iron deposits

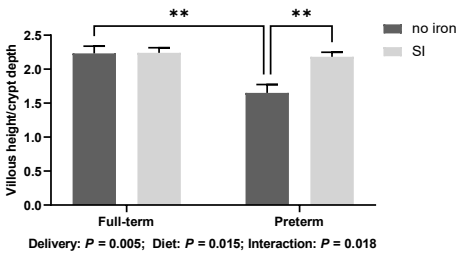
The integrity of the intestinal structure is important for nutrient digestion and absorption. Morphological alterations in the digestive tract, such as villus atrophy and crypt hyperplasia, indicate malabsorption and hinder growth (Wang et al., 2021). In addition, the villous height/crypt depth ratio is a commonly used index to assess the effects of diet on gut structure and to quantify intestinal responses to disease (Montagne et al., 2003). On PND0, compared to full-term vaginal delivery, premature delivery by cesarean section led to piglets with lower villous height and crypt depth in the duodenum ($P < 0.05$; Fig. 5A). On PND11, villous height, crypt depth and villous height/crypt depth ratio in the duodenum were influenced by an interaction between delivery mode and dietary treatment ($P < 0.05$; Fig. 5B). Without iron supplementation, preterm piglets showed a lower villous height/crypt depth ratio than full-term piglets ($P < 0.05$). Oral SI increased villous height in full-term piglets and villous height/crypt depth ratio in preterm piglets, whereas decreased crypt depth in preterm piglets ($P < 0.05$). Duodenal iron deposits were not detected by Perls' staining in either full-term or preterm piglets, regardless of SI supplementation (Fig. 5C).



(A)



(B)



(C)

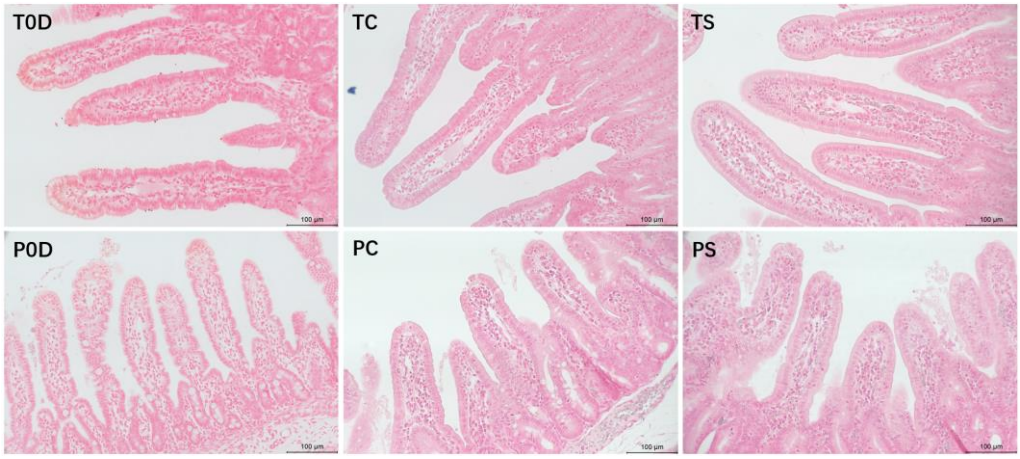


Fig. 5 Duodenal morphology of piglets at birth (A) and on postnatal day 11 (B) and histological examination of iron loading (C). (C) Non-heme iron deposits were detected by staining with Prussian Blue (Original magnification: 200×. Scale bars = 100μm). Data are presented as the mean ± SEM. *, **, and *** indicate statistically significant differences at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively. T0D, full-term piglets at birth; TC, 11-day-old full-term piglets without iron supplementation; TS, 11-day-old full-term piglets with Sucrosomial® Iron supplementation; P0D, preterm piglets at birth; PC, 11-day-old preterm piglets without iron supplementation; PS, 11-day-old preterm piglets with Sucrosomial® Iron supplementation.

5.3.2 Oral SI affected levels of key factors involved in duodenal iron absorption

The duodenum is the main site of iron absorption. Some key factors, including DMT1, cytochrome b reductase 1 (CYBRD1), heme oxygenase 1 (HO1), LFT, FPN and feline leukemia virus subgroup C cellular receptor (FLVCR), participate in the heme and non-heme iron absorption (McKie et al., 2001; Kikuchi et al., 2005; Hentze et al., 2004; Yeh et al., 2009; Hvidberg et al., 2005; Staroń et al., 2017). On PND0, lower mRNA expression of *DMT1* ($P < 0.05$), *CYBRD1* ($P < 0.10$), *HO1* ($P < 0.10$), *FPN* ($P < 0.05$), *FLVCR* ($P < 0.05$), and protein levels of DMT1 ($P < 0.05$) and FPN ($P < 0.05$) were found in the duodenum of preterm piglets compared to full-term piglets (Fig. 6A). On PND11, a trend for a delivery mode × dietary treatment interaction was observed for duodenal DMT1 protein level ($P < 0.10$; Fig. 6B). Without iron supplementation, decreased DMT1 protein level was observed

in preterm piglets in comparison to full-term piglets ($P < 0.05$). Full-term piglets fed with SI had a lower DMT1 protein level than those fed without SI ($P < 0.05$). No interaction was found for the protein levels of FPN and LFT in the duodenum. Cesarean section led to a reduction in FPN protein level compared to full-term vaginal delivery ($P < 0.05$). Oral supplementation of SI increased the LFT protein level ($P < 0.05$).

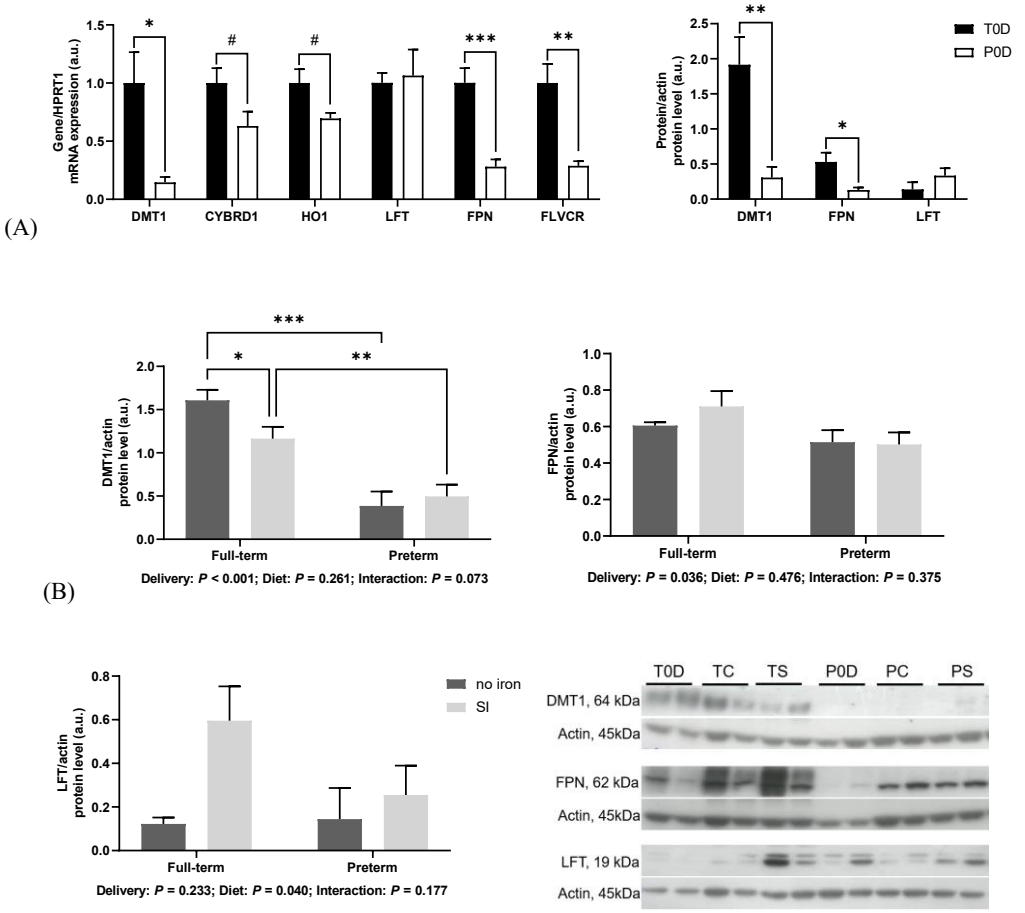


Fig. 6 Levels of key factors involved in iron absorption in the duodenum of piglets at birth (A) and on postnatal day 11 (B). (B) Representative western blot images and relative densitometric bar graphs. Actin was used as protein loading control. Data are presented as the mean ± SEM. *, **, and *** indicate statistically significant differences at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively; # indicates a trend toward significance at $0.05 < P \leq 0.10$. T0D, full-term piglets at birth; TC, 11-day-old full-term piglets without iron supplementation; TS, 11-day-old full-term piglets with Sucrosomial® Iron supplementation; P0D, preterm piglets at birth; PC, 11-day-old preterm piglets without iron supplementation; PS, 11-day-old preterm piglets with Sucrosomial® Iron supplementation. a.u., arbitrary units; DMT1, divalent metal transporter 1; CYBRD1, cytochrome b reductase 1; HO1, heme oxygenase 1; LFT, Ferritin light chain; FPN, ferroportin; FLVCR, feline leukemia virus subgroup C cellular receptor.

5.3.3 Oral SI altered bacterial composition in the rectum

In total, 1,954,605 high-quality sequence reads were generated. The Good's coverage of each

group was over 99%, indicating near-complete sampling of the community (Table 1 and Table 2). Alpha diversity measures the within-sample microbial diversity. On PND0, higher community richness (Sobs, Chao and Ace) and diversity (Shannon and Simpson) estimators were observed in preterm piglets compared to full-term piglets ($P < 0.05$; Table 1). On PND11, there was a trend for interaction between delivery mode and dietary treatment for Ace index ($P < 0.10$; Table 2). In the SI-supplied groups, Ace index was higher in full-term piglets than in preterm piglets ($P < 0.05$). Except for Ace index, no interaction was observed for other indices. Premature birth decreased Sobs, Chao and Shannon indices, and increased Shannon index ($P < 0.05$).

Table 1 Alpha diversity indices in rectal digesta of piglets at birth

Item	Full-term	Preterm	*P*-value
Sobs	351±21	471±7	<0.001
Chao	488±21	563±9	0.009
Ace	487±25	579±10	0.007
Shannon	2.25±0.20	3.60±0.03	<0.001
Simpson	0.273±0.067	0.082±0.004	0.018
Goods coverage	0.99777±0.00008	0.99837±0.00040	<0.001

Data are presented as means ± SEM. The observed species (Sobs) represent the number of operational taxonomic units identified.

Table 2 Alpha diversity indices in rectal digesta of piglets on postnatal day 11

Item	Full-term		Preterm		*P*-value						
	No iron (TC)	SI (TS)	No iron (PC)	SI (PS)	Delivery	Diet	Interaction	TC*vs.* TS	TC*vs.* PC	PC*vs.* PS	TS*vs.* PS
Sobs	367±5	367±13	292±60	261±5	<0.001	0.467	0.467				
Chao	501±10	506±17	417±54	379±10	<0.001	0.449	0.336				
Ace	525±19	553±19	541±9	477±36	0.227	0.463	0.077	0.357	0.672	0.117	0.033
Shannon	2.80±0.27	3.01±0.16	1.42±0.52	1.20±0.35	<0.001	0.984	0.492				
Simpson	0.188±0.046	0.144±0.023	0.507±0.175	0.530±0.154	0.002	0.912	0.721				
Goods coverage	0.99772±0.00005	0.99773±0.00007	0.99826±0.00003	0.99821±0.00009	<0.001	0.841	0.646				

Data are presented as means ± SEM. The observed species (Sobs) represent the number of operational taxonomic units identified.

For the beta diversity analysis, the MRPP, complemented by ordination methods such as PCA, was used to determine whether the differences between groups were significant. The results of PCA revealed clear segregation and dissimilarities of microbiota composition among all groups on PND0 and PND11, except for the two full-term piglet groups on PND11 (Fig.7A). This was further supported by the result of bray_curtis cluster tree (Fig. 7B). The results of MRPP also showed clear differences in microbiota composition not only between two groups on PND0 but also among four groups on PND11 ($P < 0.05$, Table 3).

Table 3 Multiple Response Permutation Procedure analysis of gut microbiota

Group	Distance	A	Observe_delta	Expect_delta	*P*-value
T0D-P0D	bray	0.2740	0.3593	0.4948	0.003
TC-PC-TS-PS	bray	0.3517	0.4154	0.6408	0.001
TC-PC	bray	0.3340	0.4266	0.6405	0.014
TC-TS	bray	0.0001	0.4298	0.4298	0.440
PC-PS	bray	0.3355	0.3908	0.5881	0.024
TS-PS	bray	0.3430	0.4054	0.6170	0.006

A value > 0 means the difference between groups is greater than the difference within groups; A greater expect_delta value represents a greater difference between groups; $P < 0.05$ indicates a significant difference. T0D, full-term piglets at birth; TC, 11-day-old full-term piglets without iron supplementation; TS, 11-day-old full-term piglets with Sucrosomial® Iron supplementation; P0D, preterm piglets at birth; PC, 11-day-old preterm piglets without iron supplementation; PS, 11-day-old preterm piglets with Sucrosomial® Iron supplementation.

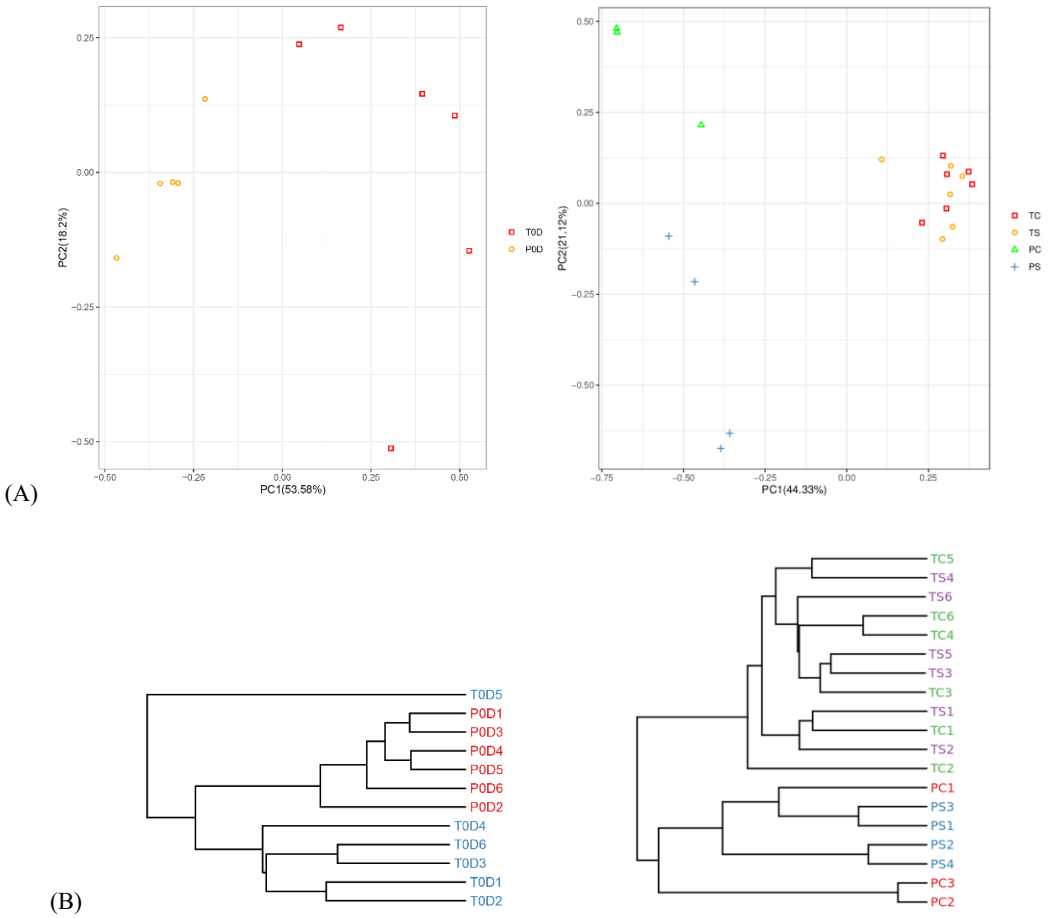


Fig. 7. Beta diversity indices in rectal digesta of piglets. (A) Principal Component Analysis at the level of operational taxonomic unit. Points in different colors and shapes represent different groups. The scales on the horizontal and vertical axes indicate relative distances and have no practical meaning. PC1 and PC2 represent potential factors contributing to the migration of microbial components. (B) Bray_curtis cluster tree. Shorter branch lengths or closer distances between samples indicate higher similarity. T0D, full-term piglets at birth; TC, 11-day-old full-term piglets without iron supplementation; TS, 11-day-old full-term piglets with Sucrosomial® Iron supplementation; P0D, preterm piglets at birth; PC, 11-day-old preterm piglets without iron supplementation; PS, 11-day-old preterm piglets with Sucrosomial® Iron supplementation.

The relative abundance of bacterial communities at the phylum and genus levels was analyzed (Fig. 8, Supplementary Table 3 and Supplementary Table 4). On PND0, preterm piglets showed higher relative abundances of Actinomycetota ($P < 0.05$), Bacillota ($P < 0.10$) and Bacteroidota ($P < 0.05$), and lower Pseudomonadota abundance ($P < 0.10$) at the phylum level than full-term piglets. At the genus level, premature delivery led to PND0 piglets with high abundances of *Bifidobacterium*, *Blautia*, *Holdemanella*, *Lactobacillus*, *Ligilactobacillus*, *Mediterraneibacter*, *Megasphaera*, *Phascolarctobacterium*, *Veillonella*, *Leyella*, *Segatella, Citrobacter* and *Klebsiella*, and low abundances of *Clostridium sensu stricto* and *Escherichia* ($P < 0.05$). On PND11, delivery mode and dietary treatment interacted to influence the abundances of phyla Actinomycetota and Pseudomonadota, and genera *Bifidobacterium*, *Ligilactobacillus*, *Limosilactobacillus* and *Escherichia* ($P < 0.05$), and an interaction trend was observed for genera *Bacteroides*, *Leyella*, *Segatella* and *Klebsiella* ($P < 0.10$). When comparing groups without iron supplementation, the preterm piglets had higher Actinomycetota and *Bifidobacterium* abundances than the full-term piglets ($P < 0.05$). Oral SI let to a decrease in the abundances of Actinomycetota and *Bifidobacterium* in preterm piglets, while resulted in an increased in the abundances of *Ligilactobacillus*, *Leyella*, *Segatella* and *Klebsiella* in full-term piglets and the abundances of Pseudomonadota, *Limosilactobacillus* and *Escherichia* in preterm piglets ($P < 0.05$). Oral administration of SI also tended to reduce *Bacteroides* abundance in preterm piglets ($P < 0.10$). In the SI-supplied groups, the abundances of Pseudomonadota, *Limosilactobacillus* and *Escherichia* were higher, whereas those of *Ligilactobacillus*, *Leyella*, *Segatella* and *Klebsiella* were lower in preterm piglets than in full-term piglets ($P < 0.05$). No interaction was observed for other bacteria. Compared to preterm groups, Bacillota, Bacteroidota, *Blautia*, *Holdemanella*, *Lactobacillus*, *Mediterraneibacter*, *Megasphaera*, *Phascolarctobacterium* and *Veillonella* were found to be more abundant in full-term piglets ($P < 0.05$), with a trend toward increased *Hallella* and *Citrobacter* abundances ($P < 0.10$). In contrast, *Enterococcus* abundance was lower in full-term piglets ($P < 0.05$). SI treatment tended to decrease the abundance of *Fusobacterium* ($P < 0.10$).

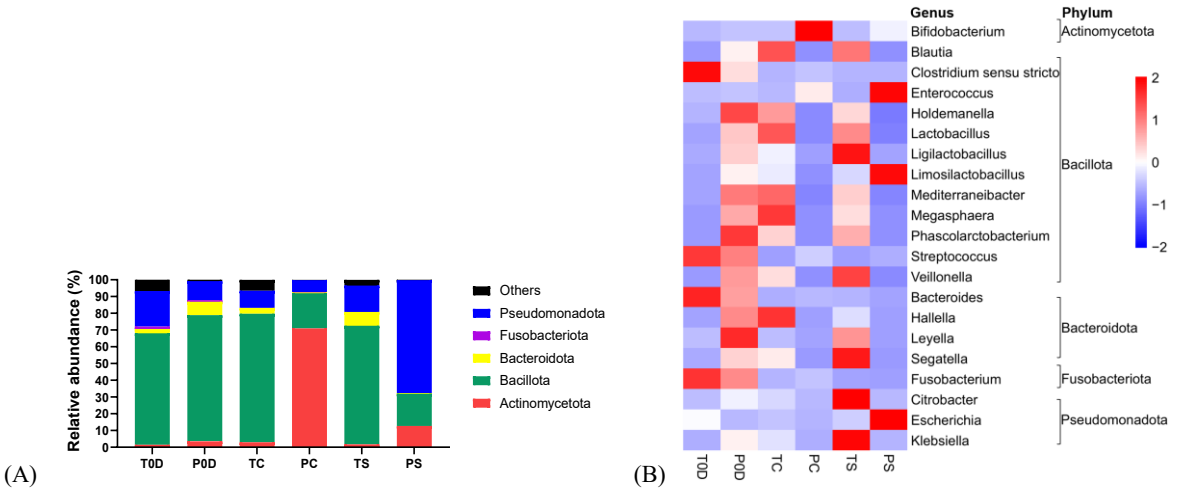


Fig. 8. Relative abundance of rectal bacteria in piglets at the phylum (A) and genus (B) levels. Phyla and genera with a relative abundance greater than 1% were selected for comparison. T0D, full-term piglets at birth; TC, 11-day-old full-term piglets without iron supplementation; TS, 11-day-old full-term piglets with Sucrosomial® Iron supplementation; P0D, preterm piglets at birth; PC, 11-day-old preterm piglets without iron supplementation; PS, 11-day-old preterm piglets with Sucrosomial® Iron supplementation.

5.3.4 Comparison of the effects of SI and $FeSO_4$ supplementation on the intestine in preterm piglets

Compared to preterm piglets without iron supplementation, $FeSO_4$ increased duodenal LFT protein level ($P < 0.05$) and rectal *Blautia* ($P < 0.10$) and *Escherichia* ($P < 0.05$) abundances, and decreased rectal *Megasphaera* abundance ($P < 0.10$) in preterm piglets (Supplementary Table 5). Higher duodenal crypt depth ($P < 0.05$) and LFT protein level ($P < 0.05$), and rectal *Blautia* ($P < 0.05$), *Mediterraneibacter* ($P < 0.10$) and *Bacteroides* ($P < 0.05$) abundances, as well as lower duodenal villous height/crypt depth ratio ($P < 0.05$) and rectal *Limosilactobacillus* abundance ($P < 0.05$), were found in preterm piglets fed with $FeSO_4$ than those fed with SI.

6 Discussion

An increasing number of studies indicate that the regulation of iron metabolism differs between infancy and adulthood (Lönnerdal et al., 2015). Compared with full-term infants, preterm infants are more likely to develop iron imbalances caused by an inadequate iron storage due to shortened pregnancy. So far, the regulation of iron homeostasis in both full-

term and preterm infants is not yet fully understood. Further research, particularly using animal models, is needed, as experimental studies of neonatal iron deficiency are challenging to conduct in humans. In our review (Publication 1), we discussed the application of different animal models in studying neonatal iron imbalance. While rodents are more convenient for experimental manipulation, particularly genetic manipulation, large animals are more similar to humans in terms of organ structure and nutritional requirements. However, the use of large animals, especially for simulating premature infants, remains relatively rare.

For large animals, sheep have been proposed as a relevant and valuable species for modeling preterm birth. Not only sheep's gestation period is closer to that of humans, but also their relatively large size allows for specific operation of either fetus or pregnant ewe. But differences in stomach architecture and function between ruminants and monogastric species, limit its utility for studying human iron metabolism. In this context, piglets appear convenient animals for prematurity research. One of the main advantages of using preterm piglets is the occurrence of impaired respiratory, nutritional, immune, and metabolic responses, which are similar to those observed in human premature newborns (Sangild et al., 2006, 2013; Lennon et al., 2011; Oosterloo et al., 2014; Burrin et al., 2020). We believe that piglets are a suitable model to study iron metabolism in perinatal and neonatal periods. First, IDA is the most common deficiency disorder in the early postnatal period of pigs because of their low hepatic iron content, and often progresses to critical illness (Svoboda and Drabek, 2005). Second, the pig is increasingly being used in biomedical research to study human genetic and nutritional diseases that are not accurately represented by rodent models (Schook et al., 2005). Third, the porcine genome has been sequenced, and molecular tools are now available to study iron-related genes in the porcine model (Ayuso et al., 2020). Taking the above into account, one aim of our study was to develop a new model of premature IDA in pigs that reflects the characteristic features of human premature anemia.

In experiment 1 (Publication 2), we evaluated the validity of our model by comparing the iron status of full-term and preterm newborn piglets, and by assessing their similarity to human neonates. In our study, preterm piglets exhibited poor growth and a significant drop in TBI compared to full-term piglets. Similarly, human studies have shown that decreases in

body weight (Arad et al., 1988; Alur et al., 2000) and TBI (Singla et al., 1985; Miller et al., 2003) vary with decreasing gestational age, suggesting that preterm infants have smaller iron stores at birth as compared to those born at term. In addition, the results of biochemical and hematological tests strongly indicated iron deficiency in preterm piglets, as evidenced by low RBC count, hemoglobin level, plasma iron level and TSAT. The analysis of blood indices in human preterm infants aligns closely with our findings in preterm piglets (Singla et al., 1979; Halliday et al., 1984; Olivares et al., 1992; Alur et al., 2000; Rolim et al., 2019). Paradoxically, while low iron levels typically lead to an increase in TIBC due to upregulated transferrin synthesis in the liver, preterm infants often exhibit low TIBC (Sweet et al., 2001; Yamada et al., 2014). This may be due to immature liver function in preterm infants, which reduces transferrin production. Obviously, our piglet model accurately reflects the condition of human preterm infants with poor iron status who are at risk of iron deficiency.

Interestingly, despite the low TBI, the increase in hepatic and splenic non-heme iron contents, along with high tissue and plasma ferritin levels, were observed in preterm piglets, which is somewhat puzzling. Consistent with our findings, Georgieff et al. (1995) reported that premature infants have higher levels of non-heme iron in the liver compared to full-term infants. One possible explanation for this phenomenon is the intensive mobilization of iron from organ stores at the end of pregnancy to meet the increased demand for RBC production, likely driven by elevated erythropoietin levels in full-term piglets, as demonstrated in our study. Indeed, during mammalian prenatal development, definitive erythropoiesis primarily occurs in the fetal liver (Palis, 2014). The liver serves as the main extramedullary erythropoietic tissue in the human fetus at midterm and continues to produce blood cells during the first week of life (Hom et al., 2015; Punia and Elghetany, 2018). The spleen also contributes to hematopoiesis in mice up to 3 months of age, but does not appear to play a role in this process in humans, and its role in pigs remains unknown (Hom et al., 2015). Our data, showing a nearly two-fold difference in body weight between full-term and preterm piglets, strongly indicate that during the last 6 days of pregnancy, iron is intensively mobilized from the liver to meet the demand for this micronutrient in other tissues. In summary, we speculate that the decreased non-heme iron levels in the liver of full-term

piglets result from extensive iron utilization in erythropoiesis, accompanied by a shift of this iron fraction to the heme compartment in erythroid cells, as well as the intensive growth of fetuses during the final stage of pregnancy.

The next step of our research was to examine the level of hepcidin, a key regulator of systemic iron metabolism, in preterm piglets to investigate the characteristics of iron metabolism in premature infants. Hepcidin can control plasma iron level by managing iron absorption in the intestine, release from macrophages, and storage mobilization in the liver (Nemeth et al., 2004). We observed an increase in hepatic hepcidin mRNA expression in preterm piglets compared to full-term piglets. Given that hepcidin expression is transcriptionally regulated by increased liver iron (Wang and Babitt, 2019), this finding aligns with the elevated hepatic iron content observed in preterm piglets. Elevated hepatic iron stimulates BMP6–SMAD signaling and hepcidin transcription (Babitt et al., 2007; Andriopoulos et al., 2009; Canali et al., 2017), which may explain the strong BMP6 induction in preterm piglets. Apart from iron levels, hepcidin synthesis can be inhibited by erythroid factors secreted, including erythroferrone (Kautz and Nemeth, 2014) and GDF15 (Tanno et al., 2007). During erythropoiesis, the secretion of erythroferrone is induced by erythropoietin levels (Kim and Nemeth, 2015). These erythroid factors were found to be decreased in preterm piglets, suggesting that the high hepcidin levels in these animals may also result from the reduced activities of erythroid hepcidin suppressors. It is also worth noting that anemia of prematurity is characterized by reduced erythropoietin production (Kling, 2005; Aher and Ohlsson, 2006). These data suggest low efficiency of erythropoiesis in preterm piglets. In some cases, hepcidin levels in preterm infants may be elevated after cesarean section due to inflammation (Cross et al., 2020). However, this was not observed in our study, as the levels of inflammatory cytokines were similar in full-term and preterm piglets.

Despite the highly upregulated hepcidin mRNA expression in the liver of preterm piglets, blood hepcidin-25 levels in these animals did not change significantly and remained low. Recent studies have supported that fetal hepcidin levels in cord blood from preterm births are comparable to those from full-term births (Liu et al., 2022). Hepcidin is initially

synthesized as an 84-amino acid precursor, which undergoes cleavage to produce the bioactive 25-amino acid peptide secreted into the circulation (Hunter et al., 2002; Valore and Ganz, 2008). We hypothesize that during the perinatal period, the regulation of hepcidin processing and release may be immature, which could explain why circulating hepcidin levels do not correspond to its hepatic mRNA expression. Therefore, it is not surprising that FPN levels—a key target of hepcidin and an iron exporter—did not differ significantly between full-term and preterm piglets in either the liver or spleen.

Overall, it appears that preterm piglets exhibit an iron metabolism pattern typical of functional iron deficiency and tissue iron accumulation. Our study is the first to establish an animal model of prematurity using the Polish Landrace breed, and this validated pig model closely mirrors systemic iron metabolism in preterm human infants. This model enables comprehensive biochemical and molecular analysis of tissue samples, which would be difficult or impossible to obtain from humans. In addition, due to their consistent genetic background, pigs serve as an excellent research model for both basic and applied studies. Therefore, preterm piglets represent a promising and competitive model for studying iron metabolism in premature infants.

In experiment 2 (Publication 3), we used the preterm piglet model to explore the effects of a novel iron supplement, SI, whose role in preterm infants is currently unknown. The condition of preterm piglets at PND11 was similar to that at PND0, showing poor growth performance, low TBI, plasma iron levels, TSAT, and overall iron deficiency. This once again shows that preterm piglets are at a greater risk of iron deficiency than full-term piglets due to insufficient iron stores. Regarding SI supplementation, an interesting finding emerged: it had little effect on full-term infants with relatively good iron status, but it was effective in alleviating iron deficiency in premature infants.

In this study, SI enhanced RBC count, hemoglobin level and Ret-He value, and induced iron accumulation in preterm piglets, but not in full-term piglets. Similar results were reported by a recent study (Asperti et al., 2018): SI-treated mice with IDA recovered from anemia; however, SI had no effect on healthy, non-anemic mice. Our previous research showed that SI supplementation neither altered systemic iron homeostasis nor affected the

hematological status of healthy, iron-replete pregnant sows (Mazgaj et al., 2020). Other studies, including animal (Asperti et al., 2021; Zakrzewski et al., 2022) and clinical studies (Pisani et al., 2015; Elli et al., 2018; Bastida et al., 2021) on subjects with iron deficiency conditions, also confirm that SI increases iron content in tissues and benefits the improvement of iron deficiency. What's more, our results are consistent with the changes in the hepcidin-FPN axis. Regardless of iron supplementation, relatively low levels of hepcidin and FPN in term-born piglets (compared to preterm piglets) were sufficient to maintain the body's iron levels at term. However, in preterm piglets, levels of hepcidin and FPN are higher and influenced by iron supplementation, indicating that the regulatory mechanisms of iron differ between full-term and preterm piglets. We also examined the regulators of hepcidin, such as erythroferrone and GDF15, and found that they were consistent with the changes in hepcidin in both full-term and preterm piglets. Therefore, SI may be an effective iron supplement only in iron-deficient subjects, potentially involving the regulation of the hepcidin-FPN axis.

Finally, we compared SI with the commonly used iron supplement $FeSO_4$ and found that they were equally effective, with SI not causing inflammatory response and oxidative damage. This experiment confirmed the poor iron status in preterm infants and highlighted the importance of iron supplementation for iron-deficient preterm infants. It also confirmed the differences in iron regulation mechanisms between preterm and full-term infants. Oral SI may be a valuable iron supplement option for preterm infants, especially for those who cannot tolerate iron salts or who do not respond to them.

The aforementioned results of our studies have shown that premature infants are at risk of iron deficiency and that supplemental SI has a beneficial effect in these cases. In experiment 3, we aimed to investigate the potential mechanisms underlying this effect, focusing on intestinal absorption mechanism and microbial regulation in early life. First, we investigated the impact of preterm birth and iron supplementation on the morphology of the duodenum, the primary intestine responsible for iron absorption. In our study, preterm birth resulted in piglets with low duodenal villous height and crypt depth on PND0, and low villous height/crypt depth ratio on PND11. Structural changes in the digestive tract are

indicative of malabsorption and impaired body growth (Wang et al., 2021). Recent studies have shown results consistent with ours: lower gut weight, villus height, crypt depth, goblet cell density and disaccharidase activity in preterm piglets at birth compared to full-term piglets, indicating gut immaturity in preterm neonates (Ren et al., 2018; Pan et al., 2020). SI can partially improve the intestinal morphology of neonates, and its effect in preterm piglets is better than $FeSO_4$ supplementation.

In line with the immature duodenal morphology, our findings showed that the iron absorption mechanism in the duodenum of premature newborns was underdeveloped, as evidenced by low levels of iron absorption-related factors DMT1, CYBRD1, HO1, FPN and FLVCR. The intestinal development of preterm piglets is less mature than that of full-term piglets, which may contribute to inadequate iron absorption and could be a contributing factor to the higher prevalence of iron deficiency observed in preterm piglets.

Reduced DMT1, significantly increased LFT, and slightly elevated FPN were observed in the duodenum of full-term piglets supplied with SI. These changes might reduce iron uptake by the duodenum while enhancing its storage and excretion, thereby ensuring an adequate supply to the duodenum and preventing toxicity. *In vitro* studies, Caco-2 cells treated with SI exhibited approximately a 30-fold increase in ferritin formation compared to those treated with ferric pyrophosphate salt (Fabiano et al., 2018a). Unlike full-term piglets, preterm piglets exhibited lower levels of DMT1 and FPN, with no significant changes observed following SI supplementation. However, SI supplementation clearly increased the blood RBC count and hemoglobin level, as well as the hepatic and splenic non-heme iron contents of preterm piglets, as demonstrated in experiment 2. In both *in vitro* and *ex vivo* models, SI effectively retained ferric iron in simulated gastric fluid, prevented its reduction by intestinal enzymes, and enhanced iron absorption across intestinal epithelium, without relying on DMT1-mediated transport (Fabiano et al., 2018a). SI is absorbed across the intestinal epithelium as a vesicle-like form through both paracellular and transcellular routes (Fabiano et al., 2018b; Gómez-Ramírez et al., 2018; Bastida et al., 2021). Most SI is directed to the lymphatic system rather than the portal bloodstream, eventually entering the arterial circulation before reaching the liver (Gómez-Ramírez et al., 2018). This may explain why

SI does not alter duodenal DMT1 and FPN levels, while hepatic non-heme content remain elevated in preterm piglets. In summary, for premature infants, SI may bypass the classic DMT1 iron absorption pathway, circumventing the immaturity or damage to this pathway caused by prematurity, thereby improving iron status.

Iron is not only important for the host, but also for the growth of intestinal microbiota. According to the results of alpha and beta diversity analyses, the rectal microbiota of full-term and preterm piglets differed significantly on PND0 and PND11. Preterm piglets exhibited greater microbial diversity on PND0 compared to full-term piglets. However, by PND11, the failure of some bacteria to successfully colonize the intestine resulted in reduced microbial diversity in preterm piglets. This pattern is consistent with findings from studies on human preterm infants, which report similar observations 3–4 days after birth (Hiltunen et al., 2022). Recent studies have shown that preterm infants harbor an initial intestinal microbiota distinct from that of full-term infants, characterized by lower stability, which may delay the transition to an adult-like microbiome (Milani et al., 2017).

In addition, we investigated differences in microbial composition between full-term and preterm piglets. In full-term piglets, phyla Bacillota and Pseudomonadota dominated the gut microbiota on PND0 and PND11. In preterm piglets, these two phyla were dominant on PND0, but on PND11, phyla Actinomycetota and Bacillota prevailed. This shift in preterm piglets was characterized by significant changes in two probiotic genera, with a 36-fold increase in *Bifidobacterium* and a 7-fold decrease in *Lactobacillus*. Although *Bifidobacterium* is generally considered beneficial, its growth in preterm piglets is abnormally elevated compared to full-term piglets (assuming the term groups represent a normal microbiota). Increased *Bifidobacterium* abundance was observed in the ascending colon of iron-deficient pigs at PND61 (Knight et al., 2019). Our previous research confirmed iron deficiency in PND11 preterm piglets (as shown in experiment 2), suggesting that the increase in *Bifidobacterium* may be linked to iron deficiency, though further validation is needed. In early preterm infants, high-abundance bacteria like *Lactobacillus* are typical in the gut microbiota during the first 2 days (Ardissone et al., 2014). Obviously, in our study, most *Lactobacillus* strains failed to colonize on PND11 in preterm piglets. These changes

may be influenced by the mode of delivery. Vaginal birth exposes infants to maternal microbes, including fecal strains that colonize the gut, whereas cesarean section prevents this exposure, disrupting vertical transmission (Korpela et al., 2018). Also, maternal strains are more stable than nonmaternal strains (Korpela et al., 2018). Overall, these results indicate a significant developmental disruption in the microbiota of preterm piglets.

SI relieved abnormal *Bifidobacterium* proliferation and promoted the growth of *Ligilactobacillus*, *Limosilactobacillus*, *Leyella* and *Segatella* in full-term or preterm piglets. As mentioned above, bacteria *Bifidobacterium* may be associated with iron deficiency, while SI alleviates iron deficiency (as shown in experiment 2) and significantly affects *Bifidobacterium* abundance. At the same time, $FeSO_4$ supplementation also resulted in a 2-fold decrease in this bacterium, while SI caused a more than 5-fold decrease. *Ligilactobacillus* has significant antioxidant and antibacterial properties, and its abundance is closely related to intestinal health (Yang et al., 2024). Similarly, *Limosilactobacillus* has been proven to be beneficial in reducing gut dysbiosis, strengthening the epithelial barrier and modulating the immune system (Zhang et al., 2022; Wu et al., 2024). *Limosilactobacillus* strain, *L. reuteri*, could reduce the risk of necrotizing enterocolitis and late-onset sepsis in preterm infants (Ang et al., 2023). The genera *Leyella* and *Segatella* are classified within the family Prevotellaceae. Prevotellaceae represents an important bacterial group involved in polysaccharide degradation and the formation of short-chain fatty acids (Heinritz et al., 2016). Notably, SI supplementation also caused an increase in *Escherichia* abundance in preterm piglets, and a similar effect was observed with $FeSO_4$ supplementation in this study. Similar phenomena have been shown in studies on different forms of iron supplementation, e.g., $FeSO_4$, ferrous fumarate, and sodium iron EDTA (Cross et al., 2015; Jaeggi et al., 2015), indicating that iron supplementation appears to inevitably increase the growth of *Escherichia*. *Escherichia* is one of the first bacteria to colonize newborns at birth. It includes both pathogenic strains (such as Enteropathogenic *E. coli*; Pokharel et al., 2023) and non-pathogenic strains (such as probiotic *E. coli* Nissle 1917; Zyrek et al., 2007). Based on existing data, we cannot determine whether SI causes pathogenic or nonpathogenic *Escherichia* in this study.

This experiment shows different mechanism of iron absorption in full-term and preterm piglets. It also confirms the beneficial effects of SI on the intestine, such as improving morphology and promoting the growth of certain beneficial bacteria, which are more pronounced than $FeSO_4$ supplementation. However, iron supplementation inevitably promotes the growth of *Escherichia*; for example, if the strain is Enteropathogenic *E. coli*, it can cause acute and prolonged diarrhea in infants (Pokharel et al., 2023). In addition, unlike experiment 2, where SI affects only indices in the blood, liver and spleen of iron-deficient preterm piglets, experiment 3 suggests that SI affects intestinal indices both in full-term and preterm piglets. This may indicate that SI supplementation has different effects on various organs and tissues involved in iron metabolism. Therefore, when considering iron supplementation, especially for vulnerable preterm infants, it is crucial to assess its effects on these organs comprehensively.

7 Conclusions

- Piglets delivered by cesarean section are an appropriate model for preterm human babies, suitable for both basic and applied research of iron metabolism. Preterm piglets exhibit tissue iron accumulation alongside functional iron deficiency, and their regulatory mechanisms of iron metabolism differ from those of full-term piglets.
- Oral SI alleviates the negative effects of iron imbalance caused by preterm birth, potentially by improving blood indices and iron storage in the liver and spleen, and by regulating the hepcidin-FPN axis through effects on iron status and erythropoiesis.
- In preterm piglets, the gastrointestinal system is underdeveloped and displays abnormal microbial colonization, while SI may partially counteract these phenomena.

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9 Appendices

9.1 Supplementary data

Supplementary Table 1 Specific primer sequences for pigs used for real-time PCR

Gene	Forward (5' - 3')	Reverse (5' - 3')	Amplicon Size, bp	Gene Bank
Bone morphogenetic protein 6	TTGTGAACCTGGTGGAGTAC	GATTCGGAATTCTGCAGCCG	122	NM_001168001.1
Cytochrome b reductase 1	ACCCTCATTTGGGTCCTCCAC	TATACGATGATGGCGATGCC	128	NM_001128452.1
Divalent metal transporter 1	GCAGGTGGTTGACGTCTGTA	CACGCCCCCTTTGTAGATGT	100	NM_001128440.1
Feline leukemia virus subgroup C cellular receptor	AGGTGTTCATCCTCGGCTTG	TGTTATTCTGTGTGTTGGGCA	239	NM_001142846.1
Ferritin light chain	AAAACCCAGGACGCTATGGA	GAGCTTCACCTCCTCATCCA	150	NM_001244131.1
Ferroportin	GCAGCAAAAGAACGAGTGGG	AAGGATCCACAGCATCCTCC	84	XM_003483701.4
GAPDH	CCCCTTCATTGACCTCCACT	CCCATTTGATTTTGGCGGGA	158	AF017079.1
Heme oxygenase 1	ATGGCGTCCTTGTACCACAT	AGACAGGTCACCCATGTAGC	276	NM_001004027.1
Hepcidin	ATCCCAGACAAGACAGCTCA	TCTTGCAGCACATCCCACAGA	164	NM_214117.1
HPRT1	GGCCATCACATCGTAGCCCT	TCGCCCGTTGACTGGTCATT	164	NM_001032376.2
Interleukin 1β	GCTAACTACGGTGACAACAATAATG	CTTCTCCACTGCCACGATGA	186	NM_214055.1
Interleukin 6	AAGGTGATGCCACCTCAGAC	TCTGCCAGTACCTCCTTGCT	151	JQ839263.1
Toll-like receptor 4	TCAGTTCTCACCTTCCTCCTG	GTTCATTCCTCACCCAGTCTTC	166	GQ503242.1
Tumor necrosis factor α	AAGACACCATGAGCACTGAGA	CGACCAGGAGGAAGGAGAAG	132	JF831365.1

Supplementary Table 2 Antibodies used for western blot analysis

Target protein	Primary antibody	Dilution	Secondary antibody	Dilution
Divalent metal transporter 1	Rabbit polyclonal, Alpha Diagnostic, #NRAMP22-A	1:1000	Goat anti-rabbit polyclonal, Sigma-Aldrich, #A6154	1:10000
Ferroportin	Rabbit polyclonal, Novus Biologicals, #NBP1-21502	1:1000	Goat anti-rabbit polyclonal, Sigma-Aldrich, #A6154	1:10000
Actin	Mouse polyclonal, Thermo Fisher Scientific, #MA5-11869	1:1000	Goat anti-mouse polyclonal, Sigma-Aldrich, #A5278	1:10000
Ferritin light chain	Rabbit polyclonal, ABCAM, #ab69090	1:1000	Goat anti-rabbit polyclonal, Sigma-Aldrich, #A6154	1:10000
Ferritin heavy chain	Rabbit polyclonal, ABCAM, #ab65080	1:1000	Goat anti-rabbit polyclonal, Sigma-Aldrich, #A6154	1:10000

Supplementary Table 3 Bacterial composition in rectal digesta of piglets at birth

Item	Full-term	Preterm	*P*-value
Phylum			
Actinomycetota	1.463±0.167	3.547±0.281	<0.001
Bacillota	66.555±4.059	75.295±0.726	0.060
Bacteroidota	2.624±0.550	8.044±1.274	0.003
Fusobacteriota	1.435±0.334	1.005±0.173	0.280
Pseudomonadota	21.175±4.360	11.320±0.865	0.051
Genus			
Actinomycetota			
Bifidobacterium	0.182±0.057	1.955±0.286	0.001
Bacillota			
Blautia	0.184±0.074	1.908±0.270	<0.001
Clostridium sensu stricto	33.325±6.928	11.059±1.794	0.022
Enterococcus	0.157±0.024	0.190±0.033	0.430
Holdemanella	0.242±0.099	1.297±0.069	<0.001
Lactobacillus	7.000±3.277	24.306±1.244	0.002
Ligilactobacillus	0.069±0.034	1.447±0.230	0.002
Limosilactobacillus	4.602±1.344	7.260±0.423	0.108
Mediterraneibacter	0.186±0.122	1.523±0.392	0.017
Megasphaera	0.241±0.112	4.675±0.871	0.004
Phascolarctobacterium	0.103±0.017	1.763±0.399	0.009
Streptococcus	5.106±0.887	3.935±0.590	0.297
Veillonella	0.210±0.052	2.800±0.319	<0.001
Bacteroidota			

Item	Full-term	Preterm	*P*-value
Bacteroides	1.390±0.532	0.840±0.211	0.371
Hallella	0.014±0.008	0.813±0.374	0.086
Leyella	0.142±0.045	1.391±0.416	0.014
Segatella	0.195±0.118	1.474±0.341	0.005
Fusobacteriota			
Fusobacterium	1.422±0.333	1.003±0.173	0.291
Pseudomonadota			
Citrobacter	0.037±0.013	0.207±0.045	0.012
Escherichia	19.975±4.436	7.220±0.277	0.017
Klebsiella	0.031±0.005	0.717±0.160	0.008

Data are presented as means ± SEM.

Supplementary Table 4 Bacterial composition in rectal digesta of piglets on postnatal day 11

Item	Full-term		Preterm		*P*-value						
	No iron (TC)	SI (TS)	No iron (PC)	SI (PS)	Delivery	Diet	Interaction	TC*vs.* TS	TC*vs.* PC	PC*vs.* PS	TS*vs.* PS
Phylum											
Actinomycetota	2.945±0.692	1.693±0.352	70.942±16.808	12.743±7.331	<0.001	<0.001	<0.001	0.865	<0.001	<0.001	0.192
Bacillota	76.780±4.165	70.844±4.059	21.035±13.241	19.245±5.907	<0.001	0.540	0.741				
Bacteroidota	3.455±1.201	8.221±2.609	0.481±0.166	0.387±0.015	0.014	0.248	0.231				
Fusobacteriota	0.031±0.011	0.024±0.013	0.161±0.160	0.002±0.001	0.293	0.114	0.147				
Pseudomonadota	10.260±2.966	15.759±2.354	7.170±3.114	67.437±12.691	0.001	<0.001	<0.001	0.465	0.736	<0.001	<0.001
Genus											
Actinomycetota											
Bifidobacterium	2.248±0.838	0.947±0.203	70.346±17.202	12.456±7.326	<0.001	<0.001	<0.001	0.862	<0.001	<0.001	0.183
Bacillota											
Blautia	4.281±0.806	3.729±1.513	0.055±0.024	0.012±0.002	0.004	0.801	0.829				
Clostridium sensu stricto	0.234±0.124	0.160±0.075	1.644±1.566	0.061±0.013	0.196	0.107	0.140				
Enterococcus	0.114±0.027	0.008±0.002	0.943±0.410	3.219±1.861	0.024	0.198	0.161				
Holdemanella	0.947±0.322	0.692±0.151	0.082±0.080	0.002±0.001	0.006	0.498	0.724				
Lactobacillus	38.116±5.891	32.291±4.318	3.508±2.742	2.194±0.685	<0.001	0.488	0.660				
Ligilactobacillus	0.788±0.157	3.282±0.828	0.010±0.002	0.047±0.028	0.003	0.042	0.048	0.002	0.371	0.969	<0.001
Limosilactobacillus	6.410±0.961	5.919±0.979	4.065±0.586	13.311±2.954	0.131	0.014	0.008	0.800	0.331	0.002	0.003
Mediterraneibacter	1.654±0.662	1.021±0.161	0.005±0.001	0.002±0.001	0.011	0.500	0.505				
Megasphaera	7.476±2.283	3.384±0.922	0.022±0.012	0.010±0.002	0.005	0.237	0.240				
Phascolarctobacterium	0.909±0.365	1.102±0.167	0.055±0.038	0.055±0.006	0.003	0.728	0.728				

Item	Full-term		Preterm		*P*-value						
	No iron (TC)	SI (TS)	No iron (PC)	SI (PS)	Delivery	Diet	Interaction	TC*vs.* TS	TC*vs.* PC	PC*vs.* PS	TS*vs.* PS
Streptococcus	0.318±0.143	0.283±0.101	1.035±0.771	0.511±0.320	0.131	0.359	0.421				
Veillonella	1.888±0.658	3.901±1.502	0.049±0.046	0.007±0.004	0.021	0.389	0.370				
Bacteroidota											
Bacteroides	0.065±0.020	0.072±0.034	0.115±0.064	0.005±0.001	0.813	0.141	0.098	0.863	0.318	0.055	0.157
Hallella	1.143±0.568	0.246±0.105	0.001±0.001	0.002±0.001	0.096	0.270	0.269				
Leyella	0.129±0.050	0.913±0.302	0.013±0.012	0.002±0.001	0.026	0.082	0.074	0.007	0.711	0.972	0.005
Segatella	1.232±0.532	3.351±0.548	0.010±0.008	0.002±0.001	<0.001	0.059	0.058	0.004	0.130	0.993	<0.001
Fusobacteriota											
Fusobacterium	0.092±0.061	0.024±0.012	0.160±0.159	0.002±0.001	0.722	0.098	0.490				
Pseudomonadota											
Citrobacter	0.111±0.031	1.072±0.459	0.013±0.006	0.011±0.004	0.082	0.144	0.143				
Escherichia	9.192±2.940	10.852±1.966	6.662±2.880	66.876±12.601	<0.001	<0.001	<0.001	0.821	0.778	<0.001	<0.001
Klebsiella	0.389±0.111	2.453±0.807	0.017±0.003	0.060±0.028	0.024	0.075	0.087	0.007	0.654	0.962	0.006

Data are presented as means ± SEM.

Supplementary Table 5 Effects of iron supplementation in prematurely born piglets

Item	PC	PS	PF	*P*-value		
				PC*vs.*PS	PC*vs.*PF	PS*vs.*PF
Duodenal morphology						
Villous height (μm)	252±25	198±15	240±19	0.070	0.675	0.147
Crypt depth (μm)	153±10	92±9	129±11	0.003	0.169	0.031
Villous height/crypt depth	1.65±0.12	2.18±0.07	1.86±0.06	0.002	0.152	0.024
Levels of key factors involved in duodenal iron absorption						
Divalent metal transporter 1/actin (a.u.)	0.387±0.166	0.497±0.136	0.459±0.176	0.639	0.757	0.872
Ferritin light chain/actin (a.u.)	0.144±0.142	0.255±0.134	0.920±0.169	0.612	0.005	0.012
Ferroportin/actin (a.u.)	0.514±0.066	0.502±0.066	0.496±0.051	0.895	0.841	0.944
Alpha diversity indices						
Sobs	292±60	261±5	239±4	0.466	0.226	0.571
Chao	417±54	379±10	412±21	0.385	0.907	0.413
Ace	541±9	477±36	547±62	0.372	0.930	0.296
Shannon	1.42±0.52	1.20±0.35	1.49±0.27	0.690	0.903	0.576
Simpson	0.507±0.175	0.530±0.154	0.407±0.084	0.912	0.631	0.526
Goods coverage	0.99826±0.00003	0.99821±0.00009	0.99815±0.00009	0.710	0.410	0.616
Rectal bacterial composition						
Phylum						
Actinomycetota	70.942±16.808	12.742±7.331	34.498±17.533	0.024	0.120	0.295
Bacillota	21.035±13.241	19.244±5.907	18.352±7.762	0.891	0.837	0.941
Bacteroidota	0.481±0.166	0.387±0.015	0.911±0.317	0.774	0.211	0.111
Fusobacteriota	0.161±0.160	0.002±0.001	0.005±0.002	0.170	0.178	0.973

Item	PC	PS	PF	*P*-value		
				PC*vs.*PS	PC*vs.*PF	PS*vs.*PF
Pseudomonadota	7.170±3.114	67.437±12.691	41.072±15.153	0.012	0.106	0.164
Genus						
Actinomycetota						
Bifidobacterium	70.346±17.202	12.456±7.326	34.255±17.530	0.025	0.126	0.297
Bacillota						
Blautia	0.055±0.024	0.012±0.002	0.193±0.076	0.571	0.096	0.028
Clostridium sensu stricto	1.644±1.566	0.061±0.013	0.053±0.011	0.165	0.163	0.994
Enterococcus	0.943±0.410	3.219±1.861	0.841±0.461	0.245	0.957	0.194
Holdemanella	0.082±0.080	0.002±0.001	0.003±0.002	0.166	0.174	0.973
Lactobacillus	3.508±2.742	2.194±0.685	0.391±0.166	0.515	0.145	0.343
Ligilactobacillus	0.010±0.002	0.047±0.028	0.009±0.006	0.208	0.954	0.161
Limosilactobacillus	4.065±0.586	13.311±2.954	0.687±0.205	0.011	0.262	0.001
Mediterraneibacter	0.005±0.001	0.002±0.001	0.005±0.001	0.055	0.673	0.087
Megasphaera	0.022±0.012	0.010±0.002	0.006±0.002	0.181	0.099	0.677
Phascolarctobacterium	0.055±0.038	0.055±0.006	0.021±0.002	0.998	0.235	0.202
Streptococcus	1.035±0.771	0.511±0.320	0.197±0.101	0.407	0.199	0.587
Veillonella	0.049±0.046	0.007±0.004	0.004±0.001	0.212	0.181	0.909
Bacteroidota						
Bacteroides	0.115±0.064	0.005±0.001	0.095±0.025	0.053	0.686	0.080

Item	PC	PS	PF	*P*-value		
				PC*vs.*PS	PC*vs.*PF	PS*vs.*PF
Hallella	0.001±0.001	0.002±0.001	0	0.353	0.651	0.154
Leyella	0.013±0.012	0.002±0.001	0.003±0.001	0.178	0.228	0.857
Segatella	0.010±0.008	0.002±0.001	0.002±0.001	0.202	0.207	0.986
Fusobacteriota						
Fusobacterium	0.160±0.159	0.002±0.001	0.005±0.002	0.171	0.180	0.970
Pseudomonadota						
Citrobacter	0.013±0.006	0.011±0.004	0.006±0.001	0.711	0.209	0.319
Escherichia	6.662±2.880	66.876±12.601	51.219±10.836	0.005	0.022	0.312
Klebsiella	0.017±0.003	0.060±0.028	0.045±0.019	0.217	0.403	0.634

Data are presented as means ± SEM. a.u., arbitrary units; PC, 11-day-old preterm piglets without iron supplementation; PS, 11-day-old preterm piglets with Sucrosomial® Iron supplementation; PF, 11-day-old preterm piglets with ferrous sulfate supplementation.

9.2 Percentage contribution of authors to each publication

I hereby give the percentage of each author's contribution to the publication:

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Lp.	**Imię i nazwisko** ***(name and surname)***	**Opis udziału autora** ***(Description of the author's participation)***	**% udział w opracowaniu publikacji** ***(participation in the publication in %)***	**Jednostka** ***(Institution)***
1.	Xiuying Wang	Conceptualization, Visualization, Validation, Writing-Original Draft	60	Laboratory of Iron Molecular Biology, Department of Molecular Biology, Institute of Genetics and Animal Biotechnology, Polish Academy of Sciences, 05-552 Jastrzębiec, Poland
2.	Paweł Lipiński	Supervision, Writing-review & editing	15	Laboratory of Iron Molecular Biology, Department of Molecular Biology, Institute of Genetics and Animal Biotechnology, Polish Academy of Sciences, 05-552 Jastrzębiec, Poland
3.	Magdalena Ogłuszka	Writing-review & editing	10	Department of Genomics and Biodiversity, Institute of Genetics and Animal Biotechnology, Polish Academy of Sciences, 05-552 Jastrzębiec, Poland
4.	Rafał R. Starzyński	Conceptualization, Funding acquisition, Supervision, Writing-review & editing	15	Laboratory of Iron Molecular Biology, Department of Molecular Biology, Institute of Genetics and Animal Biotechnology, Polish Academy of Sciences, 05-552 Jastrzębiec, Poland

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Lp.	**Imię i nazwisko** ***(name and surname)***	**Opis udziału autora** ***(Description of the author's participation)***	**% udział w opracowaniu publikacji** ***(participation in the publication in %)***	**Jednostka** ***(Institution)***
1.	Xiuying Wang	Methodology, validation, formal analysis, investigation, data curation, writing—original draft preparation, writing—review and editing, visualization	51	Laboratory of Iron Molecular Biology, Department of Molecular Biology, Institute of Genetics and Animal Biotechnology, Polish Academy of Sciences, 05-552 Jastrzębiec, Poland
2.	Małgorzata Lenartowicz	Investigation, writing—review and editing	1	Laboratory of Genetics and Evolutionism, Institute of Zoology and Biomedical Research, Jagiellonian University, 30-387 Kraków, Poland
3.	Rafał Mazgaj	Methodology, validation, investigation	5	Laboratory of Iron Molecular Biology, Department of Molecular Biology, Institute of Genetics and Animal Biotechnology, Polish Academy of Sciences, 05-552 Jastrzębiec, Poland
4.	Magdalena Ogłuszka	Investigation	3	Department of Genomics and Biodiversity, Institute of Genetics and Animal Biotechnology, Polish Academy of Sciences, 05-552 Jastrzębiec, Poland
5.	Dominika Szkopek	Investigation	1	Laboratory of Large Animal Models, The Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences, 05-110 Jabłonna, Poland
6.	Kamil Zaworski	Investigation	1	Department of Animal Physiology, The Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences, 05-110 Jabłonna, Poland

7.	Zuzanna Kopeć	Investigation	1	Laboratory of Iron Molecular Biology, Department of Molecular Biology, Institute of Genetics and Animal Biotechnology, Polish Academy of Sciences, 05-552 Jastrzębiec, Poland
8.	Beata Żelazowska	Investigation	1	Laboratory of Iron Molecular Biology, Department of Molecular Biology, Institute of Genetics and Animal Biotechnology, Polish Academy of Sciences, 05-552 Jastrzębiec, Poland
9.	Paweł Lipiński	Investigation, writing—original draft preparation, writing—review and editing	5	Laboratory of Iron Molecular Biology, Department of Molecular Biology, Institute of Genetics and Animal Biotechnology, Polish Academy of Sciences, 05-552 Jastrzębiec, Poland
10.	Jarosław Woliński	Methodology, investigation, resources	1	Laboratory of Large Animal Models, The Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences, 05-110 Jabłonna, Poland; Department of Animal Physiology, The Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences, 05-110 Jabłonna, Poland
11.	Rafał R. Starzyński	Conceptualization, methodology, investigation, resources, writing—original draft preparation, writing—review and editing, supervision, project administration, funding acquisition	30	Laboratory of Iron Molecular Biology, Department of Molecular Biology, Institute of Genetics and Animal Biotechnology, Polish Academy of Sciences, 05-552 Jastrzębiec, Poland

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Lp.	**Imię i nazwisko** ***(name and surname)***	**Opis udziału autora** ***(Description of the author's participation)***	**% udział w opracowaniu publikacji** ***(participation in the publication in %)***	**Jednostka** ***(Institution)***
1.	Xiuying Wang	Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - original draft, Writing - review and editing	57	Laboratory of Iron Molecular Biology, Department of Molecular Biology, Institute of Genetics and Animal Biotechnology, Polish Academy of Sciences, 05-552 Jastrzębiec, Poland
2.	Paweł Lipiński	Investigation, Writing - review and editing	10	Laboratory of Iron Molecular Biology, Department of Molecular Biology, Institute of Genetics and Animal Biotechnology, Polish Academy of Sciences, 05-552 Jastrzębiec, Poland
3.	Magdalena Ogłuszka	Investigation, Methodology, Validation	5	Department of Genomics and Biodiversity, Institute of Genetics and Animal Biotechnology, Polish Academy of Sciences, 05-552 Jastrzębiec, Poland
4.	Rafał Mazgaj	Investigation, Methodology, Validation	1	Laboratory of Iron Molecular Biology, Department of Molecular Biology, Institute of Genetics and Animal Biotechnology, Polish Academy of Sciences, 05-552 Jastrzębiec, Poland
5.	Jarosław Woliński	Investigation, Methodology, Resources	1	Laboratory of Large Animal Models, The Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences, 05-110 Jabłonna, Poland
6.	Dominika Szkopek	Investigation	1	Department of Animal Physiology, The Kielanowski Institute of Animal Physiology and Nutrition, Polish

				Academy of Sciences, 05-110 Jabłonna, Poland
7.	Kamil Zaworski	Investigation	1	Laboratory of Large Animal Models, The Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences, 05-110 Jabłonna, Poland
8.	Zuzanna Kopeć	Investigation	1	Department of Animal Physiology, The Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences, 05-110 Jabłonna, Poland
9.	Beata Żelazowska	Investigation	1	Laboratory of Iron Molecular Biology, Department of Molecular Biology, Institute of Genetics and Animal Biotechnology, Polish Academy of Sciences, 05-552 Jastrzębiec, Poland
10.	Germano Tarantino	Resources	1	Scientific Department, Pharmanutra S.p.A., 56122 Pisa, Italy
11.	Elisa Brilli	Resources	1	Scientific Department, Pharmanutra S.p.A., 56122 Pisa, Italy
12.	Rafał R. Starzyński	Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing - review and editing	20	Laboratory of Iron Molecular Biology, Department of Molecular Biology, Institute of Genetics and Animal Biotechnology, Polish Academy of Sciences, 05-552 Jastrzębiec, Poland

9.3 Publications

Nutrition Research Reviews

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Iron status and risk of iron disorders in neonates: A narrative review of recent studies in animal models

Xiuying Wang[1], Paweł Lipiński[1], Magdalena Ogłuszka[2] and Rafał Radosław Starzyński[1]

[1]Laboratory of Iron Molecular Biology, Department of Molecular Biology, Institute of Genetics and Animal Biotechnology of the Polish Academy of Sciences, Jastrzębiec, Poland and [2]Department of Genomics and Biodiversity, Institute of Genetics and Animal Biotechnology of the Polish Academy of Sciences, Jastrzębiec, Poland

Review Article

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Corresponding author:
Rafał Radosław Starzyński;
Email: r.starzynski@igbzpan.pl

Abstract

The iron regulation mechanisms are not exactly the same between adulthood and the early postnatal period. Also, neonatal iron status is different in full-term versus preterm infants because the prenatal/gestational period, when hepatic iron accumulates, is shortened. Newborns, especially premature infants, are at high risk of iron deficiency due to inadequate iron stores, which constitute the primary source of iron to satisfy the neonate's increasing iron requirements. In addition, frequent blood transfusions and congenital haemochromatosis may induce iron overload in the affected neonate. To understand the cause of neonatal iron deficiency/overload and to promote the development of effective therapeutic interventions in humans, different animal models have been generated by genetic engineering, low-/high-iron diets, phlebotomy/transfusion and surgical manipulation. These models use various laboratory and domestic animals to study iron imbalance. They serve as surrogate models for experiments that are ethically or practically unfeasible to conduct on human neonates. Although an animal model for studying neonatal iron disorders may not fully replicate the complexities of human diseases, it is designed to model specific aspects of these conditions. Combined data from multiple models can help to offset the limitations inherent in each individual model. In this review, we outline approaches to induce neonatal iron disorders, current animal models of full-term and preterm neonates, and recommendations for diagnosis.

Introduction

Iron is indispensable during all developmental stages of mammals. The iron contained in the hepatic stores of the newborn meets most of the needs of the rapidly growing organism, particularly the need for increased blood volume and number of erythrocytes. The foetus obtains iron from the mother through the placenta, and approximately 80% of foetal hepatic iron stores accumulate during the last trimester of pregnancy[1], which can explain the iron-deficient status of the preterm neonate. At birth, most healthy, full-term newborns with adequate iron stores have normal or high haemoglobin content and then remain iron-replete until 6 months of age[2]. By contrast, premature newborns clearly show a systemic iron imbalance resulting from a shortage of iron stores caused by preterm delivery. Preterm infants have lower levels of haemoglobin, serum ferritin (SF) and total stored iron content[3–5]. It is noteworthy that the recommendations are to introduce iron-rich complementary foods at 4–6 months of age (depending on country-specific guidance) to reduce the risk of iron deficiency[6].

In severe neonatal iron deficiency, the available iron is transferred mainly to the bone marrow for erythropoiesis, at the expense of iron transport to the brain[7]. Therefore, iron deficiency during the early neonatal period may lead to long-term negative effects associated with impaired nervous system function, such as decreased attention and memory[8,9], visual and auditory system deficits[10,11], and social–emotional behaviour alterations[12,13]. If an iron deficiency in the neonate is not sufficiently corrected by exogenous iron intake, it develops into anaemia. Paradoxically, even in iron-deficient neonates, iatrogenic factors, such as chronic transfusion therapy used to treat iron deficiency anaemia (IDA), increase the risk of iron overload[14,15]. Similarly, under certain genetic conditions, such as neonatal haemochromatosis, too much iron is stored in the body[16]. It must be remembered that there is no natural pathway to excrete iron from the organism. Iron overload results in serious toxicities and oxidative damage, adversely affecting organ function[17].

Studies on human newborns provide a lot of information on iron metabolism. Nevertheless, human research of basic metabolism is expensive and difficult to perform accurately. Adult animal models provide valuable information about iron metabolism and regulation; however, considering the differences between adults and newborns, appropriate full-term and preterm

animal models that focus on iron metabolism in early development are essential and still needed. In addition, because of the high risk to babies and ethical considerations, experimental research on iron deficiency or overload, for example, induced by dietary iron supplementation, genetic engineering or surgical operation, is difficult to execute in humans. Newborn human babies are also unable to undergo frequent laboratory testing. Therefore, the use of animal models is necessary to test hypotheses developed from clinical observational studies, and to test therapeutic interventions for iron imbalance. In particular, due to the paucity of data on the mechanism by which rare anaemias and iron disorders occur, animal models of this clinical problem are invaluable. Yet, it is difficult to fully reflect neonatal iron disorders in humans using only one specific animal model. Here, we discuss different animal models of neonatal iron imbalance and characterise their advantages and disadvantages in relation to neonatal iron disturbances in humans.

Neonatal iron metabolism and regulation

Systemic iron turnover in the body is primarily modulated by regulating the iron-related function of duodenal enterocytes, hepatocytes, erythroblasts and reticuloendothelial macrophages (located in the liver, spleen and bone marrow). In brief, duodenal enterocytes absorb iron from the diet; hepatocytes release iron stored in ferritin and synthetise the iron homeostatic hormone hepcidin; bone marrow erythroblasts play a crucial role in producing haem and haemoglobin; and reticuloendothelial macrophages recycle iron derived from senescent erythrocytes (summarised in Figure 1)[18–34].

In the neonatal period, dietary iron absorption through enterocyte cells is a major way to meet the needs of the rapidly growing organism. However, some studies have shown that levels of classical iron transporters divalent metal transporter 1 (DMT1) and ferroportin (Fpn) are very low during early postnatal life. Leong *et al.*[35] investigated the expression of *Dmt1* and *Fpn1* genes in the duodenum in iron-deficient rat pups at early postnatal day (PND) 10 and in late infancy (PND 20). They showed a lack of *Dmt1* and *Fpn1* expression at PND 10, but a dramatic increase at PND 20[35]. Another study performed on normal juvenile rats confirmed the age-dependent expression of *Dmt1* and *Fpn1*, but their dramatic increase was observed only by PND 40[36]. Leong *et al.*[35,36] concluded that the molecular mechanisms for iron absorption during early infancy differ from those of late infancy, when they are similar to adult animals. Similar trends were observed in a piglet model, which showed that *DMT1* and *FPN1* were barely detectable on PNDs 1 and 2 and that their up-regulation started only around PND 4[37]. Belgrade (*b*) rat pups with a DMT1 mutation were found to have lower blood iron levels but higher tissue iron levels, which indicates that DMT1 function is not essential for iron assimilation from milk during early development[38]. Meanwhile, some results indicate that Fpn is essential for iron absorption during the suckling period, as iron absorption is very low in Fpn-knockout mice[39]. It is worth noting that, during this period, Fpn present on the basolateral membrane of enterocytes is hyporesponsive to hepcidin[39]. This phenomenon may allow the high level of iron absorption to be maintained regardless of hepcidin expression level, reducing the likelihood of iron deficiency during development. Because the developmental maturation of the DMT1–Fpn pathway occurs a few days after birth, it has been proposed that there is an alternative receptor-mediated mechanism for intestinal iron transport in the newborn. For example, lactoferrin (also known as lactotransferrin), pinocytosis and gut microbiota may contribute to iron absorption[29,30], but the exact function of these factors in iron homeostasis is still under debate and needs to be elucidated.

Iron homeostasis is precisely regulated at the systemic level by the 25-amino-acid peptide hepcidin synthesised mainly in hepatocytes. This hormone regulates iron absorption by enterocytes, iron recycling by macrophages, and iron release from hepatocytes[32]. It can bind to Fpn and cause Fpn's rapid internalisation and degradation[32]. Although hepcidin is important in iron homeostasis in adults, there are gaps in our understanding of the role of this peptide during early life. Some studies have found that hepcidin level at birth positively correlated with gestational age and birth weight[5,40]. However, others argue that no relationship exists between hepcidin levels and gestational age, and that newborns have hepcidin levels that are similar to or higher than those in adults[37,41–43]. These opposing results hinder our understanding of the role of hepcidin in the regulation of neonatal iron metabolism. In addition, it has been established that hepcidin concentration is correlated with the size of iron stores. Human research has shown that hepcidin concentrations are lower in infants with iron deficiency than in those without iron deficiency, both in cord blood (from infants born after 24–42 weeks of gestation)[40] and in serum (from infants born after 32–37 weeks of gestation)[44]. Studies in pig found that hepatic hepcidin mRNA levels were sharply reduced in piglets with iron deficiency[37,45]. Moreover, hepcidin cord blood concentrations in infants delivered by elective caesarean section were lower than those in infants after spontaneous vaginal delivery or secondary caesarean section[40]. It is noteworthy that both serum hepcidin concentrations in late preterm infants[44] and hepatic hepcidin gene expression in full-term piglets[37] were decreased during early life, which parallels a decrease in postnatal iron content.

Considering that there are still many unknown issues in neonatal metabolism and regulation, animal experiments can further explore these issues in depth, as these experiments cannot be performed on humans. Next, we list the factors that cause iron deficiency and overload, which will be used to construct models of animal iron imbalance.

Factors influencing neonatal iron status

Factors causing iron deficiency

A number of factors cause iron imbalance and may explain why newborns are so vulnerable to iron deficiency and anaemia.

(1) Low iron stores at birth. Iron stores in neonates are generated primarily during the last period of gestation[1,46]. Jopling *et al.*[4] reported that neonatal haematocrit and haemoglobin concentrations increased approximately linearly from 22 to 40 weeks of gestation. It has been found that SF levels are lower in both the cord blood and blood of 4 weeks postpartum in preterm infants than those in term infants[47], which demonstrated that premature babies had lower iron stores. Neonatal iron stores are also related to maternal health. Placental insufficiency induced by, for example, multiple pregnancies, diabetes and hypertension with intra-uterine growth restriction, can block iron transport to the foetus[46].

(2) Immaturity of iron absorption mechanisms. As mentioned earlier, the lack or reduced level of DMT1 and Fpn1 expression early in life is one potential reason for iron deficiency[35,36].

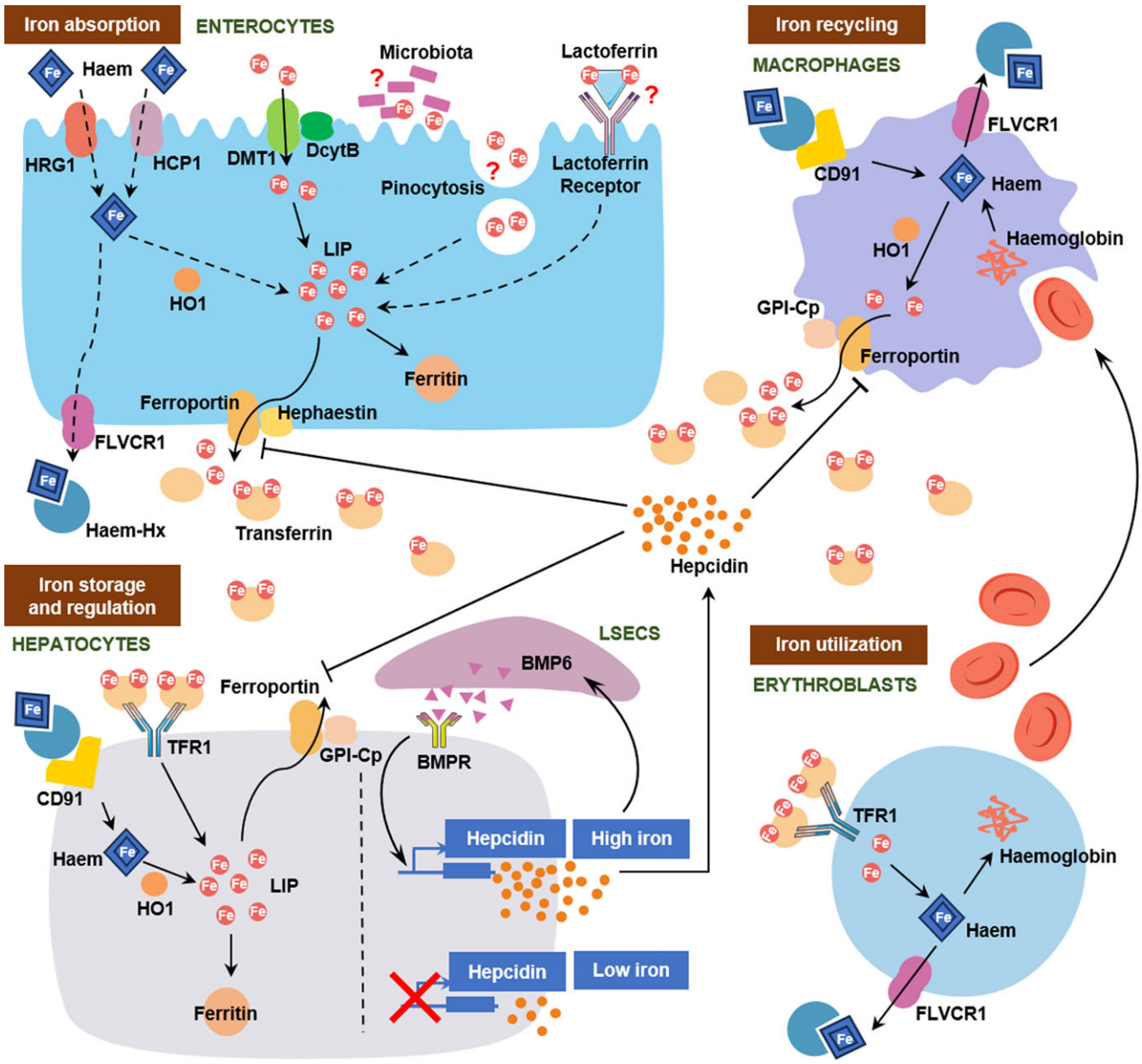

Fig. 1. Systemic iron turnover in the neonate. Duodenal enterocytes absorb two forms of dietary iron, non-haem and haem iron, through different absorption mechanisms. Haem uptake by enterocytes is primarily mediated by haem carrier protein 1 (HCP1) and possibly by haem responsive gene 1 (HRG1)[18–20]. Inside enterocytes, haem is catabolised by the enzyme haem oxygenase 1 (HO1) to elemental iron, biliverdin and carbon monoxide[20,21]. In turn, by means of iron reductase duodenal cytochrome b (DcytB) and divalent metal transporter 1 (DMT1), non-haem iron crosses the apical membrane of the intestinal epithelial cell to enter the cytoplasm[22]. After that, iron enters the labile iron pool (LIP) in the cytosol, a source of iron for haem synthesis and iron–sulphur cluster biogenesis. The cytoplasmic iron is subsequently incorporated into iron-containing proteins, stored in the iron storage protein ferritin, or exported into the circulation by the only known iron exporter ferroportin, with the participation of the copper-dependent ferroxidase hephaestin (or glycosylphosphatidylinositol-linked, membrane-bound ceruloplasmin, GPI-Cp in other cell types)[23–26]. In the bloodstream, iron is bound by transferrin and taken up by hepatocytes, erythroblasts and other cells through transferrin receptor 1 (TFR1)-mediated endocytosis[23]. The potential pathway of haem iron export from enterocytes involves the transfer of intact haem (not catabolised by HO1) across the basolateral membrane by feline leukaemia virus subgroup C cellular receptor 1 (FLVCR1) to the blood, where it is captured by haemopexin and delivered in the form of haem–haemopexin (Haem–Hx) via receptor CD91 to various sites in the body[20,27,28]. Other pathways that may contribute to early iron absorption include lactoferrin receptor-mediated process, pinocytosis and gut microbiota[29,30]. Iron recirculation by tissue macrophages, which phagocytose senescent erythrocytes and rapidly degrade the haem released from haemoglobin through the catabolic activity of HO-1, ensures a major part of daily iron supply to erythroid precursors[31]. Systemic iron homeostasis is regulated by hepcidin, a hormone secreted by the hepatocytes[32]. In cases of iron deficiency, low hepcidin facilitates the unimpeded ferroportin function of professional iron exporter cells[32]. When the organism is saturated with iron, the hepcidin blood level increases, and this peptide has a greater potential to interact with ferroportin and, consequently, to reduce iron transfer into the bloodstream[32]. In this process, bone morphogenetic protein 6 (BMP6) acts as an 'iron sensor'[33,34]. Its transcription in liver sinusoidal endothelial cells (LSECs) is induced by high iron levels[33,34]. BMP6 is then released from LSECs and has a stimulatory paracrine effect on hepcidin expression in hepatocytes by binding to BMP receptor (BMPR)[33,34]. Solid lines represent confirmed pathways, while dashed lines indicate proposed pathways that have yet to be confirmed.

(3) Iron losses. Anaemia of prematurity can also result from disorders that cause erythrocyte loss due to bleeding and/or haemolysis[48]. Recurrent phlebotomies for diagnostic purposes cause extra iron loss, particularly in small preterm infants who are the most critically ill and require the most frequent blood testing[48].

(4) Other factors. Factors such as delivery mode, ethnic and sex differences may influence iron status in neonates[49–51].

Factors affecting risk of iron overload

The mother's regulation of iron bioavailability may prevent embryonic iron overload[52]. In other words, iron overload is not common under normal conditions. However, newborns are still at risk for iron overload due to multiple erythrocyte transfusions and diseases.

(1) Multiple erythrocyte transfusions. This is a major cause of excessive iron accumulation in premature neonates[15]. Although no clinical signs of iron overload have been detected, both SF and hepatic iron concentration have been shown to be positively correlated with the volume of transfused blood in low-birth-weight preterm infants[15,53]. Erythrocyte transfusion also negatively impacts the survival of extremely low-birth-weight preterm infants, for example, by affecting retinopathy of prematurity development and late neurodevelopment[54]. The risk of iron overload appears to be higher in very low-birth-weight infants who receive more than two transfusions than in those who receive one or two transfusions[15].

(2) Diseases. Neonatal haemochromatosis frequently accompanied by extrahepatic siderosis is a disorder affecting foetuses and newborns[16]. Newborns with neonatal haemochromatosis typically develop symptoms within hours after birth, while some severe cases lead to stillbirth or neonatal demise[16]. Gestational alloimmune liver disease (GALD) has been established as the cause of foetal liver injury, leading to nearly all cases of neonatal haemochromatosis[16]. No more than 2% of neonatal haemochromatosis cases may arise from non-GALD diseases, such as perinatal infection, trisomy 21, mitochondrial DNA depletion due to deoxyguanosine kinase deficiency, bile acid synthetic defect delta 4-3-oxosteroid 5 beta-reductase deficiency (SRD5B1 mutations), mitochondrial DNA depletion due to deoxyguanosine kinase deficiency (DGUOK mutations), and GRACILE syndrome (growth retardation, aminoaciduria, cholestasis, iron overload, lactic acidosis and early death; BCS1L mutation)[16]. However, how these diseases cause iron overload is still unclear. In addition, iron overload occurs in 70% of neonates with haemolytic disease of the foetus and newborn due to red cell alloimmunisation at birth, but the incidence of iron overload gradually decreases during the first three months without iron supplementation[55]. Moreover, β-thalassaemias are heterogeneous, autosomal-recessive-inherited anaemias characterised by reduced or absent β-globin chain synthesis[56]. The disease can be associated with severe anaemia and the need for lifelong transfusion therapy (β-thalassaemia major, TM) or relatively less severe anaemia (non-transfusion-dependent thalassaemia, or β-thalassaemia intermedia, TI)[57]. Ineffective erythropoiesis and consequent extramedullary haematopoiesis, splenomegaly and systemic iron overload are major features of this disease[57].

Animal models of neonatal iron disorders

A list of animal models is presented in Table 1.

Animal models of iron deficiency and their features

Genetic models

Its genetic similarity to humans and its possibilities for genetic manipulation makes the rodent an attractive model. Two iron-deficient rodent models, microcytic anaemia (*mk*) mice and *b* rats, carry a glycine-to-arginine substitution at position 185 in the iron transporter gene *Slc11a2* (encoding DMT1)[38,58,59]. The G185R mutation in DMT1 affects targeting of the protein to the brush border of the intestinal epithelium, the normal physiological site for iron uptake. All *mk* mice and *b* rats exhibit systemic, severe microcytic, hypochromic anaemia, with impaired intestinal iron absorption and defective iron metabolism in peripheral tissues[38,58,59].

Donovan *et al.*[60] created two conditional knockout mouse models: *Meox2-cre;*$Fpn^{flox/flox}$ and *vil-Cre-*ER^{T2}; $Fpn^{flox/flox}$ mice. They found that Fpn-deficient animals suffer from IDA, but accumulate iron in enterocytes, macrophages and hepatocytes[60], consistent with the key role that Fpn plays in these cell types. The gut-specific inactivation of Fpn confirmed that it is critical for intestinal iron absorption. These studies have identified the main sites of Fpn activity and provide insight into haemochromatosis.

Effective iron export by Fpn requires the participation of two copper-dependent ferroxidases hephaestin (Heph) and ceruloplasmin (Cp). The mislocation of Heph in the sex-linked anaemia (*sla*) mouse leads to a defect in the release of iron from intestinal enterocytes into the circulation; in other words, higher cellular iron levels in intestinal enterocytes but lower systemic iron levels are present in these mice[61,62]. Despite a deletion removing 192 amino acids, *sla* mice still produce a truncated Heph protein, but its mislocation from the basolateral membrane makes it unable to interact with laterally located Fpn, which contributes to the observed iron transport defects in *sla* mice[62]. Newborn *sla* mice show IDA with low haemoglobin levels[63]. Recent studies have found that mice lacking Heph in the whole body ($Heph^{-/-}$) and in the intestine alone ($Heph^{int/int}$), mice with double knockout of Heph and Cp ($Heph^{-/-}Cp^{-/-}$) and mice with intestine-specific Heph deletion ($Heph^{int/int}Cp^{-/-}$) exhibit dietary iron absorption defects and disrupted iron homeostasis, with serious systemic iron deficiency[64,65].

The study by Nicolas *et al.*[66] was a milestone in research on the molecular basis of anaemia. They found that transgenic mice with overexpressed hepcidin develop severe microcytic hypochromic anaemia. These transgenic animals are born with decreased body iron levels, reduced body size, pallor, and hairless and crumpled skin, which are related to erythrocyte abnormalities[66]. Model animals that overexpress hepcidin[66] or that lack the peptide[67] (upstream stimulatory factor 2 (Usf2)-knockout mice accompanied by iron overload, as mentioned in the section 'Animal models of iron overload and their features') are valuable tools for studying iron homeostasis *in vivo* and for deciphering the molecular mechanisms of hepcidin's action as a major regulator of systemic iron homeostasis.

The lack of hepcidin repressor matriptase-2 (encoded by *Tmprss6*) results in increased hepcidin levels, triggering iron deficiency and anaemia[68,69]. $Tmprss6^{-/-}$ mice model the human disorder iron-refractory IDA. The anaemic phenotype of these mutant mice results from the blockage of intestinal iron export into plasma[68]. Based on these findings, the authors conclude that matriptase-2 activity represents the first significant step in hepcidin regulation and iron homeostasis.

The hypotransferrinaemic mouse is a model of inherited transferrin deficiency[70,71]. Two of its best-known phenotypes are severe IDA and massive tissue iron overload[70,71].

These rodents offer a unique model for studying both iron deficiency and excess. Although haematopoietic cells are severely iron deficient, most, if not all, other tissues show marked iron overload. These animals complement other model animals,

Table 1. Examples of neonatal animal models of iron deficiency and overload

Animal models	Animals	Procedure	Features	References
Iron deficiency models				
Term born				
Genetic model	Microcytic anaemia mice and Belgrade rats	Substitution at position 185 in *Slc11a2*	Systemic, severe microcytic, hypochromic anaemia; impaired intestinal iron absorption; defective iron metabolism in peripheral tissues	(38,58,59)
	Meox2-cre;Fpn$^{flox/flox}$ and *vil-Cre-ER*^{T2}*;Fpn*$^{flox/flox}$ mice	Conditional knockout in Fpn	IDA; accumulated iron in enterocytes, macrophages, hepatocytes	(60)
	Sex-linked anaemia mice	Mislocalisation of Heph	Low haemoglobin levels, IDA	(63)
	Heph$^{-/-}$, *Heph*$^{int/int}$, *Heph*$^{-/-}$ *Cp*$^{-/-}$ and *Heph*$^{int/int}$*Cp*$^{-/-}$ mice	Lacking Heph in the whole body and in the intestine alone, Heph and Cp double knockout, and intestine-specific Heph deletion	Defects in iron absorption, serious systemic iron deficiency	(64,65)
	Transgenic mice with overexpression of hepcidin	Overexpression of hepcidin	Severe microcytic hypochromic anaemia, decreased body iron level, reduced body size, pallor, hairlessness, crumpled skin	(66)
	Tmprss6$^{-/-}$ mice	Lack of matriptase-2	Increased hepcidin levels, IDA	(68)
	Hypotransferrinaemic mice	Inherited transferrin deficiency	Severe IDA, massive tissue iron overload	(70,71)
Dietary iron insufficiency	Chinchilla rabbits	Received mothers' milk only from PND 15 to PND 40	Decrease in plasma iron concentration, blood Hb, erythrocyte and MCV; developed a pure microcytaemia	(72)
	Wistar rats	Fed an iron-deficient diet (4 ppm Fe) for 32 d beginning at PND 0	Anaemia, morphological damage of myelin	(73)
	Lambs	No iron supplementation at 3 weeks postpartum	Low Hb, anaemia	(74)
	Piglets	Without iron supplementation or fed a low-iron diet	IDA; negative impact on brain development, peripheral immunity, serum metabolome, hepatic histology, development of the colonic microbiota and volatile fatty acids concentrations; decrease in blood Hb, HCT, MCV, and hepatic non-haem and haem iron content	(75–81)
	Sprague-Dawley rats	Fed an iron-reduced diet (3 ppm Fe)	Iron deficiency	(82)
	C57BL/6J mice	Mothers were fed a copper-deficient diet until the day of parturition, and newborn pups were fed a copper-deficient diet for 6 weeks	Systemic iron deficiency, low Heph and Cp ferroxidase activity and hepcidin expression, high enterocyte mRNA and protein levels of Fpn1 in pups	(83)
	Rats	Mothers were fed an iron-deficient diet (5.2 ppm Fe) for 6 weeks during pregnancy	40% reduction in iron level in pups' brain	(85)
	Sprague-Dawley rats	Pregnant rats were fed a 3–6 ppm Fe diet	Low iron concentrations in the brain, liver and heart; decreased brain-derived neurotrophic factor function in pups	(86,87)
	Guinea pigs	Mothers were fed an iron-deficient diet (11.7 ppm Fe)	Low Hb, HCT and MCV; a modified fatty acid profile of the offspring's brain	(88)
	Rhesus monkeys	Pregnant monkeys were fed a 225 ppm Fe diet	Low serum Hb, iron, TSAT and ferritin levels in pups	(91)
Phlebotomy	C57BL/6 mice	Subjected to timed phlebotomy between PND 2 and PND 10	A reduction in erythrocytes, Hb, MCV, MCH and MCHC; an increase in RET and RDW	(96)

(*Continued*)

Table 1. (*Continued*)

Animal models	Animals	Procedure	Features	References
	C57BL/6 mice	Subjected to phlebotomy from PND 3 to 14	Decreased HCT and brain iron concentration; altered hippocampal energy, phospholipid metabolism and gene expression	(97)
	C57BL/6 mice	Phlebotomised daily from PND 3 to 14	Low HCT and body weight; an altered hippocampal transcriptome	(98)
	Lambs	Underwent regular phlebotomy for 11 d beginning at PND 2–3	Negative iron balance, lower plasma iron	(7)
	Piglets	IDA in sows and offspring induced by blood removal combined with an iron-reduced diet during gestation	Low erythrocytes, HCT	(99)
Caesarean section	Rats and mice[1]	Performed on the day of birth	/	(101,103)
Preterm born				
Genetic model	E17.5 Tmprss6$^{-/-}$ mouse foetuses	Lack of matriptase-2	Iron deficiency, microcytic anaemia, dramatic increase in liver hepcidin 1 mRNA expression, decrease in body iron content	(69)
Maternal dietary iron insufficiency	E18.5 mouse foetuses	Pregnant mice were fed an iron-deficient diet (4 ppm iron), and foetuses were dissected at E18.5	Low foetal hepatic non-haem iron levels, drastic reductions in L-Ft levels, barely detectable hepcidin mRNA expression, decreased in hepatic Fpn levels	(92)
	E20 foetuses of Sprague-Dawley rats	Mothers were fed a low-iron diet (<6 ppm Fe), and foetuses were obtained at E20	Low foetal Hb levels and body weight	(95)
	E21 foetuses of Rowett Hooded Lister rats	Rats were fed reduced Fe diets (12.5 and 7.5 ppm Fe) for 4 weeks before mating. The female rats were maintained on the same diet throughout pregnancy and were sacrificed at day 21 of gestation to obtain foetuses	Decreased foetal HCT and liver iron	(93)
	E21 foetuses of Wistar and Rowett Hooded Lister rats	Rats were fed a low-iron diet (7.5 ppm Fe) for 4 weeks prior to and during pregnancy. Tissues were collected at day 21 of gestation	Low foetal liver iron content	(94)
	E12.5, E15.5 and E18.5 foetuses of C57BL/6 mice	Mothers were fed a low-iron diet (4 ppm Fe) 2 weeks prior to and throughout pregnancy, and foetuses were dissected at E12.5, E15.5 and E18.5	Decreased foetal serum iron and liver non-haem iron concentrations	(52)
Caesarean section	Mice [1]	Performed 1 d before birth	/	(102–104)
	Piglets	Delivered on 111th or 112th day of pregnancy	A marked decrease in Hb, erythrocytes, MCV, MCH and HCT	(107)
	Piglets	Delivered on 109th day of pregnancy	Low body weight, Hb, erythrocytes, HCT, RET, plasma iron level and TSAT	(108)
Iron overload models				
Term born				
Transfusion- genetic model	Preclinical transfusion-dependent humanised mouse model of TM	Generated by targeted gene replacement of the mouse adult globin genes in embryonic stem cells with a human $\gamma\beta^0$ globin gene-switching cassette, weekly hypertransfusion therapy beginning at PND 2	Inhibited erythropoiesis in the bone marrow, elevated hepatic iron stores	(109)
Genetic model	*Bcs1l*$^{G/G}$ mice	c.232A>G mutation in *Bcs1l*	Small amounts of iron in the liver	(117)
	Mice, rats, zebrafish[1]	DGUOK deficiency; DGUOK mutant	/	(119–122)

Table 1. (*Continued*)

	$Hbb^{th1/th1}$ and $Hbb^{th3/+}$ mice	A deletion eliminates one of the two mouse β-globin genes, β^{major}; a deletion eliminates both the β^{minor} and β^{major} genes in heterozygosity	Hepatosplenomegaly, anaemia, aberrant erythrocyte morphology	(124,125)
	Mice	Transplantation of haematopoietic foetal liver cells harvested from $Hbb^{th3/th}$ embryos at 14.5 d of gestation into lethally irradiated syngeneic adult recipients	Severe anaemia, massive splenomegaly, extramedullary haematopoiesis, hepatic iron overload	(125,129)
	Rabbits	CRISPR/Cas9-mediated genome editing	Marked iron deposits in the spleen, liver and kidney	(130)
Dietary iron overload	Piglets	Daily administration of 10, 15 or 50 mg Fe/kg BW/d from PND 2 to 21	Hepatic and hippocampal iron overload, a decrease in duodenal DMT1 mRNA expression, a drastic increase in hepatic hepcidin mRNA expression and H-Ft protein expression, deficits in social novelty recognition	(139,140)
	Wistar rats	Oral administration of 10 mg/kg Fe	Brain oxidative damage and memory deficits	(17)
	C57BL/6 mice	Pregnant mice were supplied with excessive dietary iron (9.9 g/kg FeSO4) starting from 1 week before giving birth, and their pups were fed the same diet after a weaning period of 3 weeks	Altered lipid metabolism	(143)
Preterm born				
Dietary iron overload	E18.5 foetuses of C57BL/6 mice	Mothers were fed a high iron supplementation diet (344 ppm Fe), foetuses were obtained at E18.5	Increased foetal iron	(142)

[1]The model has the potential to study iron metabolism regulation in early postnatal periods. Cp, ceruloplasmin; DMT, divalent metal transporter 1; E, embryonic day; Fpn, ferroportin; Hb, haemoglobin; HCT, haematocrit; Heph, hephaestin; H-Ft, ferritin heavy chain; IDA, iron deficiency anaemia; L-Ft, ferritin light chain; MCH, mean cell haemoglobin; MCHC, MCH concentration; MCV, mean corpuscular volume; PND, postnatal day; RDW, red cell distribution width; RET, reticulocyte; TSAT, transferrin saturation

including *mk* mice, *b* rats and *sla* mice, for the study of iron homeostasis. Although these models are generally studied on adult animals, in some cases the consequences of genetic mutations have been studied in early postnatal period, as we show in Table 1. We believe that these models have the potential to study iron metabolism and regulation in the neonatal period. However, future research is needed to focus on consequences in neonatal periods.

Dietary iron insufficiency

A low-iron diet for the animals is typically required in this nutritional iron deficiency model. Suckling chinchilla rabbits receiving only their mothers' milk (without extra iron supplementation) from PND 15 to PND 40 showed a continuous decrease in plasma iron concentration, blood haemoglobin concentration, erythrocyte count and mean corpuscular volume (MCV) and subsequently developed pure microcytaemia[72]. An iron-deficient diet (4 ppm Fe) was given to Wistar rats for 32 d beginning at PND 0 to simulate anaemia[73]. The rat data indicate that iron is essential for maintaining optic nerve cell structure and that structural damage from iron deficiency is not easily reverted by iron repletion[73]. A large animal model found that 33% of housed lambs had haemoglobin values below 80 g/l (regarded as anaemia) at 3 weeks postpartum if no iron was supplemented[74].

In addition, pigs are promising animal models for iron disorder research. Owing to their lowest hepatic iron stores among all mammalian species, high iron requirements for fast growth, limited external supply caused by low iron content in the sow's milk, and immature mechanisms of iron absorption, suckling piglets easily develop systemic IDA without iron supplementation[75,76]. Research on neonatal piglets fed a low-iron diet demonstrated that iron deficiency had a negative impact on the development of brain white matter[77], peripheral immunity[78], the serum metabolome[79], hepatic histology[79], the development of colonic microbiota[80], and volatile fatty acids concentrations[80]. A 28-d study of anaemic piglets showed a significant decrease in their haemoglobin concentration, haematocrit level and MCV in blood and hepatic non-haem and haem iron content, along with higher mRNA expression levels of renal erythropoietin and erythroferrone in the bone marrow[81]. An interesting aspect of the study was that iron was found to be retained in the duodenum of anaemic piglets, with down-regulation of copper-related genes and decreased *FPN* and *HEPH* expression, which indicated that the regulation of intra-enterocytic iron absorption was probably related to the interaction between copper and iron metabolism proteins[81]. Similarly, results from studies of iron-deficient rats given an iron-reduced diet at various stages of postnatal development (at 8 and 21 d; 6, 9, 12 and 36 weeks; and 1 year of age) have also shown that copper plays important role in overall body iron homeostasis[82]. Chen *et al.*[83] also showed lower Heph and Cp ferroxidase activity and hepcidin expression but higher enterocyte mRNA and protein levels of Fpn1 in young (6-week-old) copper-deficient mice with systemic iron deficiency. This study indicates that copper is required for the proper processing and/or stability of Heph.

Neonatal iron imbalance due to maternal iron deficiency may also serve as a model. Pregnancy frequently leads to maternal iron deficiency in humans due to the increased demand for iron, which may have serious consequences for the offspring. Some studies have assumed that poor maternal iron status during pregnancy does not affect foetal or neonatal iron endowment, but severe iron deficiency does[46,84]. Infants from moderately and severely anaemic mothers have lower birth weights and iron stores, placing them at higher risk of iron deficiency at an early age[84]. In an experimental rat model, female rats were given an iron-deficient diet (5.2 ppm Fe) for 6 weeks to produce iron-deficient pups[85]. They found that these pups had a significant (40%) reduction of iron level in the brain compared with pups from control mothers[85]. Similarly, in pregnant Sprague-Dawley rats fed a 3–6 ppm Fe diet, the brain iron concentration in the offspring was 60% lower in PND 7 rats and 19% lower in PND 28 rats, and the iron concentrations were lower in the liver and heart of PND 7 rats[86]. This animal model also showed that iron deficiency decreased brain-derived neurotrophic factor function, which may be related to learning deficits[87]. Earlier studies used guinea pigs because their brain growth spurt and myelogenesis, like in humans, occur prenatally, that is, earlier than in rats and mice[88,89]. Leblanc *et al.*[88] showed that maternal iron deficiency led to lower levels of haemoglobin, haematocrit and MCV in guinea pig pups and that it was associated with a modified fatty acid profile in the offspring's brain. Rhesus monkeys are also an excellent model in that they mimic the developmental risk factors for IDA in humans, given their similarities to humans in gestational physiology and brain development. Compromised iron status was seen in newborn rhesus monkeys when their mothers suffered from iron deficiency, and the newborns easily developed iron-deficient anaemia by 6 months postpartum if iron supplementation did not meet their growth-related needs[90,91].

Foetuses dissected from the uterus can reflect the iron condition of infants in the prenatal period, and as such, they can be a model for premature infants. In a study from our laboratory, an experiment was performed using pregnant mice fed an iron-deficient diet containing 4 ppm Fe, and foetuses were dissected at embryonic day 18.5[92]. The foetuses from the anaemic mothers were found to have lower hepatic non-haem iron levels, drastic reductions in ferritin light chain levels, barely detectable hepcidin mRNA expression and a greater decrease in hepatic Fpn levels compared with controls[92]. Previous work has also reported different strains of rats, such as Rowett Hooded Lister[93,94], Wistar[94] and Sprague-Dawley[95], for studying foetal growth. Cornock *et al.*[94] compared differences in foetal and maternal iron homeostasis between Wistar and Rowett Hooded Lister rats fed a low-iron diet prior to and during gestation. It is worth noting that, although lower iron content was found in both rat strains, there were considerable differences between them[94]. Furthermore, understanding the mechanisms that govern iron homeostasis in the mother–placenta–foetus unit could aid in managing iron disorders during pregnancy. Sangkhae *et al.*[52] used female and male heterozygous iron-regulatory protein (IRP) 1/hepcidin mice to generate placentas and embryos lacking IRP1/hepcidin or not, and they investigated the effects of maternal iron status on placental and foetal iron homeostasis. They confirmed, from the iron deficiency induced by a low-iron diet, that placental iron homeostasis is mediated by the iron-responsive elements/IRP system. The homeostatic response of the metabolically active placenta is to retain iron, which protects the placenta from severe iron deficiency and consequent reduced oxidative phosphorylation. Although this placental iron retention comes at the cost of foetal iron deficiency, it may ultimately protect the foetus from the more serious adverse effects of wider placental dysfunction.

In general, scientific studies using low iron intake in the diet both during pregnancy and postpartum indicate the key role of

iron ions in physiological functions. The results underscore the importance of a careful approach when designing the route and regimen of iron supplementation for treating IDA in infants.

Phlebotomy

Taking blood samples can also cause a loss of total body iron and can induce anaemia (that is, phlebotomy-induced anaemia (PIA)). Animal models of PIA can be used to investigate the health status of full-term and preterm neonates who experience significant iron loss from frequent phlebotomy procedures. Serial-timed phlebotomy in neonatal mice led to abnormalities in the content and morphology of erythrocytes, as well as their haemoglobin, reticulocyte properties and immune cell responses – findings that are similar to those in humans[96]. Another study used a mouse model of PIA with adequate neurological development and haematocrit levels comparable to those found in premature infants in the neonatal intensive care unit to investigate the effect of anaemia on hippocampal neurochemistry and gene expression[97]. Once again, it was shown that, as haematocrit levels decrease in newborn mice with PIA, the level of phosphoethanolamine, the precursor of myelin, also decreases[97]. A recent study found the side effect of PIA in hippocampal function and showed that consequences of PIA may be based in sex-specific transcriptomic alterations[98].

In addition, negative iron balance was found in lambs with PIA[7]. The risk of neonatal brain iron deficiency due to repeated phlebotomy is real, as iron is prioritised for erythrocyte production over brain accretion[7]. Rydal *et al.*[99] developed an experimental model of IDA in sows and offspring induced by blood removal combined with an iron-reduced diet during gestation. Piglets from the blood-removal group had significantly lower erythrocyte counts and haematocrit, as well as a tendency to have lower haemoglobin levels[99]. Moreover, recent epidemiologic research indicates that severe anaemia may be an essential risk factor for the development of necrotising enterocolitis[100]. Using a preclinical neonatal murine model of PIA, Arthur *et al.*[100] showed that anaemia can enhance intestinal inflammation and barrier disruption, possibly by altering macrophage function, resulting in a type of predisposing intestinal injury that may increase the risk for necrotising enterocolitis. These observations may be the basis for further preclinical studies on the role of anaemia in common comorbidities.

Caesarean section

Caesarean section is a common surgical procedure for delivering full-term and preterm neonates. A systematic review and meta-analysis of full-term neonates showed that, compared with neonates born vaginally, those born by caesarean section had lower levels of haematocrit, haemoglobin and erythrocytes[51]. Similar results were observed in premature neonates[50], indicating that neonates delivered by caesarean section might be more likely affected by IDA in infancy. In rodent models, caesarean section is performed either at term (on the day of birth) or preterm (1 d before birth)[101–103]. It is important to note that premature mice could not survive if they were delivered 1 or 2 d earlier than spontaneous parturition[102,104].

Larger animals enable approaches that are not feasible in rodents and, thus, serve as important translational models to bridge the gap between rodent model studies and human clinical studies. Sheep have been a relevant and valuable species for modelling preterm birth. Not only is the sheep's gestation period closer to that of humans, but their relatively large size allows specific foetal or pregnant sheep surgery. However, the difference in the stomach between ruminant and monogastric species such as pigs and humans limits the relevance of findings from sheep studies with regard to digestive tract development. Piglets are becoming popular animals for research on preterm offspring. Unlike rodents and sheep, the main advantage of the preterm pig is its similarity to the human baby in terms of size and the impaired respiratory, nutritional, immunological and metabolic responses after preterm birth[105]. Preterm piglets present severe symptoms of prematurity, such as hepatic, digestive, locomotive and thermoregulatory immaturity[105,106]. Eiby *et al.*[106] established a pig model of the preterm neonate, in which piglets were delivered by caesarean section at six gestational ages, that is, at days 91, 94, 97, 100, 104 and 113 (term day 115). This experiment showed that the degree of organ development and maturation is proportionate to age and that only the late premature piglets had high survival rates[106]. An earlier study found that there was a markedly low level of haemoglobin, erythrocytes, MCV, mean cell haemoglobin and especially packed cell volume (also known as haematocrit) in piglets delivered by caesarean section on the 111th or 112th day of pregnancy until treatment with iron[107]. In addition, in our ongoing project, we are comparing the differences between premature piglets delivered at the gestational age of 109 d and full-term piglets; we found that premature piglets have lower body weights, erythrocyte parameters, plasma iron levels and transferrin saturation (TSAT)[108]. However, the most important aspect of producing a caesarean section piglet model is ensuring that it best represents human newborns. The pig model, thoroughly validated in terms of iron metabolism, can be used for diagnostic purposes, as well as to test new drugs or supplements. So far, however, few studies have focused on iron deficiency using larger animal models because working with these animals is time-consuming and expensive; feeding is a major limiting issue; and most importantly, the premature animals die easily.

Animal models of iron overload and their features

Transfusion

Due to extravascular intramedullary haemolysis, people with TM require regular erythrocyte transfusions to survive, leading to systemic iron overload[56]. For neonatal studies, a preclinical transfusion-dependent humanised mouse model of TM was generated by targeted gene replacement of the mouse adult globin genes in embryonic stem cells with a human $\gamma\beta^0$ globin gene-switching cassette[109]. The fully humanised TM mice were born alive, surviving solely upon human HbF and minor amounts of HbA2, and became progressively anaemic upon completion of the foetal-to-adult Hb switch; the majority died before weaning age. Weekly hypertransfusion therapy starting 2 d after birth inhibited erythropoiesis in the bone marrow, but elevated hepatic iron stores[109]. In addition, different adult animal models have been applied for transfusion studies, including mouse[110], rat[111], dog[112], sheep[113], pig[114] and marmoset[115]. However, to date, few studies have been conducted on early postnatal iron metabolism using neonatal models. Given the differences between adults and newborns, it is imperative that relevant neonatal animal models of transfusion be developed to investigate adverse transfusion reactions.

Genetic model

GRACILE syndrome is a neonatal, lethal metabolic disorder with iron overload[116]. Affected infants present severe growth

retardation and iron overload signs, including hepatic haemosiderosis, increased SF concentrations, hypotransferrinaemia with increased TSAT, and free plasma iron[116]. A transgenic mouse model with a c.232A>G mutation in *Bcs1l* has been developed to mimic GRACILE syndrome[117]. However, only small amounts of iron are detected in the liver of $Bcs1l^{G/G}$ mice, while liver iron accumulation is observed both at gestation and at birth stages in individuals with GRACILE syndrome[117]. Levéen *et al.*[117] speculated that this disparity may be related to differences in placental iron transport between mice and humans.

DGUOK deficiency is the most common genetic factor of human mitochondrial DNA depletion syndromes, a group of severe, phenotypically heterogeneous, recessively inherited disorders. Patients with DGUOK mutations show hepatic iron overload, which may progress to liver failure[118]. Thus far, several animal models of these syndromes have been described, including DGUOK-deficient strains of mouse[119], rat[120] and zebrafish[121], in addition to a DGUOK mutant mouse line with a phenylalanine-to-serine substitution of residue 180[122], but no iron disorder studies have been conducted with these models. Guo *et al.*[123] established an *in vitro* liver disease model of liver organoids and hepatocytes developed from the pluripotent stem cells of patients with DGUOK mutation who had died at 2 months of age, along with associated controls and isogenic cell lines corrected by the CRISPR/Cas9 system. They used ferric ammonium citrate to induce iron overload and found the DGUOK mutant model to be more sensitive to iron overload-induced ferroptosis – which is mediated by nuclear receptor co-activator 4-dependent degradation of ferritin in lysosome and cellular labile iron release – than control[123].

Patients affected by TI do not require chronic blood transfusions for survival. However, non-transfused patients show hyperabsorption of iron in the gut due to dysregulation of hepcidin transcription[56]. Two mouse models of TI are available: $Hbb^{th1/th1}$[124] and $Hbb^{th3/+}$[125]. Adult $Hbb^{th1/th1}$ and $Hbb^{th3/+}$ mice exhibit signs of severe disease, such as hepatosplenomegaly, anaemia and aberrant erythrocyte morphology, which are comparable to those of patients affected by TI[125]. Using the $Hbb^{th3/+}$ model, researchers have found that ablating the hepcidin regulator erythroferrone[126] or the oral Fpn inhibitor vamifeport[127] and injecting the mice with the haem oxygenase inhibitor tin protoporphyrin IX[128] can ameliorate anaemia, ineffective erythropoiesis and dysregulated iron homeostasis. Unfortunately, mice completely lacking adult β-globin genes ($Hbb^{th3/th3}$) die late in gestation. A mouse model of TM was established by transplanting haematopoietic foetal liver cells harvested from $Hbb^{th3/th3}$ embryos at 14.5 d of gestation into lethally irradiated syngeneic adult recipients[125,129]. In addition, as noted above, the humanised murine model of TM is also useful for the development of new transfusion and iron chelation regimens[109], as well as preclinical trials of new drugs that could improve the quality of life of patients. Recently, Yang *et al.*[130] generated a β-thalassaemia rabbit model using CRISPR/Cas9-mediated genome editing and found marked iron deposits in the rabbit spleen, liver and kidney.

Hereditary haemochromatosis (HH) is a common autosomal recessive disorder and the most common cause of iron overload in adults[131]. Most people do not experience symptoms until old age, because patients need many years to build up the excess iron that causes the disease[131,132]. Although the classic haemochromatosis phenotype has been rarely reported early in life, neonatal screening for HH offers an opportunity for early clinical intervention, preventing the morbidity and mortality associated with HH. Four types of HH have been identified: type I haemochromatosis due to homeostatic iron regulator (HFE) mutation; type II haemochromatosis due to haemojuvelin (HJV) and hepcidin mutations (juvenile haemochromatosis); type III haemochromatosis related to transferrin receptor 2 (TfR2) mutation; and type IV haemochromatosis (Fpn disease). The most common HH subtype, highly prevalent in the white population, presents with an adult-onset and chronic phenotype and is caused by the HFE p.C282Y mutation[133]. The non-HFE HH subtypes are relatively rare and typically manifest during the juvenile period[133]. In juvenile HH, symptoms may appear in the second decade of life and are associated with mutations in the genes encoding hepcidin, HJV and phosphatidylinositol glycan anchor biosynthesis class A (PIGA), an enzyme involved in glycosylphosphatidylinositol (GPI)-anchor biosynthesis[133]. The lack of GPI synthesis results in improper processing of HJV and neuronal Cp[133]. Lack of a GPI anchor causes diminished HJV on the cell surface and reduced hepcidin synthesis[133]. Most importantly, signs of PIGA-mediated juvenile haemochromatosis have been observed as early as 2 years of age[133]. There are currently many mouse models for HH, including $HFE^{-/-}$[134], $HJV^{-/-}$[135], $TfR2^{Y245X}$[136], $TfR2^{-/-}$[137], Fpn^{ffe}[138] and $USF2^{-/-}$[67].

Dietary iron overload

Iron loading also potentially results from excessive intake of dietary iron, although this is uncommon in human infants. However, elevated iron supplementation has been widely used in animal models to imitate human iron overload. Excessive iron supplementation in early life causes hepatic and hippocampal iron overload, a decrease in duodenal DMT1 mRNA expression and drastic increases in hepatic hepcidin mRNA and ferritin heavy chain protein expression, as well as deficits in social novelty recognition in nursing term piglets[139]. Despite high hepcidin expression, researchers have found that both mRNA and protein expression of Fpn1 in the duodenal mucosa and liver were unaffected by iron provision or birth weight[139,140]. Authors speculated that hepcidin-induced Fpn1 degradation is hyporesponsive to iron excess in a nursing pig model[139,140]. In a Wistar rat model used for investigating the effect of iron on the brain, the results showed that postnatal iron administration induces brain oxidative damage and memory deficits[17,141]. Guo *et al.*[142] designed dietary high-iron, medium-iron and low-iron groups of pregnant female mice and found that maternal high-iron supplementation affected reproductive performance and increased foetal iron levels at day 18.5. In another experiment, pregnant mice starting at 2 weeks of gestation and their offspring up to 30 weeks were supplied with excessive dietary iron[143]. Their data suggested that prolonged exposure to high iron levels from the foetal stage to adulthood alters lipid metabolism[143].

Diagnosing neonatal iron deficiency and overload

Biochemical and haematological tests are used to diagnose iron deficiency or overload. Iron deficiency is accompanied by low SF, TSAT, reticulocyte haemoglobin concentration (CHr) and hepcidin, as well as a high ratio of zinc protoporphyrin to haem (ZnPP/H ratio) and a high level of serum TfR1[46,144,145]. Decreased haemoglobin and MCV values are late signs of iron deficiency, which occur in severe IDA but not in mild iron deficiency[46,144,145]. The World Health Organization recommends a SF value of <12 μg/l to define depleted iron stores, and a haemoglobin level

of <110 g/l to present anaemia for children less than 5 years of age[146]. Iron overload shows normal haemoglobin, MCV and CHr levels; increased SF, TSAT and hepcidin levels; and a reduced serum TfR1 level and reduced ZnPP/H ratio[46,145]. In humans and piglets, a newer biomarker, urinary hepcidin, accurately reflects changes in iron status and is a promising non-invasive diagnostic tool[147,148]. In addition, magnetic resonance imaging is a non-invasive technique for assessing tissue iron deposition[149,150] but poses several potential hazards for infants, such as adverse effects related to sedation, radiofrequency electromagnetic field effects and noise exposure[151]. The use of this technology in paediatric examinations remains limited, especially outside clinical settings. Therefore, blood tests are still the most common method for evaluating neonatal iron status.

It should be noted that there are large differences between these parameters at different gestational ages. A review summarised that gestational age was negatively correlated with MCV and the ZnPP/H ratio and positively correlated with haemoglobin, SF and TSAT[43]. As mentioned above, hepcidin testing has shown inconsistent results. In summary, combining multiple biomarkers will likely provide better information on iron status during the neonatal period than relying on only a single biomarker.

Conclusions

Maintaining physiological iron status during early stages of life is a complex process, with iron regulatory mechanisms and requirements differing from those in adults. For healthy newborns, breast milk or iron-fortified infant formula is recommended to provide necessary iron until their birth weight doubles (approximately 4–6 months), at which point iron-rich complementary foods should be introduced. For neonates with confirmed iron deficiency, iron supplements can be administered appropriately, but they must be safe, effective and thoroughly tested. In addition, it is also important to avoid iron excess, as neonatal iron metabolism may be disturbed due to underdeveloped mechanisms for regulating iron homeostasis. Overall, further research efforts are warranted to explore the mechanisms underlying neonatal iron metabolism, to understand various aspects of iron disorder pathogenesis, to develop suitable animal models and to investigate reliable methods for diagnosing and effectively treating iron-deficient and -overloaded neonates.

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Preterm Piglets Born by Cesarean Section as a Suitable Animal Model for the Study of Iron Metabolism in Premature Infants

Xiuying Wang [1], Małgorzata Lenartowicz [2,*], Rafał Mazgaj [1], Magdalena Ogłuszka [3], Dominika Szkopek [4], Kamil Zaworski [5], Zuzanna Kopeć [1], Beata Żelazowska [1], Paweł Lipiński [1], Jarosław Woliński [4,5] and Rafał Radosław Starzyński [1,*]

1 Laboratory of Iron Molecular Biology, Department of Molecular Biology, Institute of Genetics and Animal Biotechnology, Polish Academy of Sciences, 05-552 Jastrzębiec, Poland; x.wang@igbzpan.pl (X.W.); r.mazgaj@igbzpan.pl (R.M.); z.kopec@igbzpan.pl (Z.K.); b.zelazowska@igbzpan.pl (B.Ż.); p.lipinski@igbzpan.pl (P.L.)
2 Laboratory of Genetics and Evolutionism, Institute of Zoology and Biomedical Research, Jagiellonian University, 30-387 Kraków, Poland
3 Department of Genomics and Biodiversity, Institute of Genetics and Animal Biotechnology, Polish Academy of Sciences, 05-552 Jastrzębiec, Poland; m.ogluszka@igbzpan.pl
4 Laboratory of Large Animal Models, The Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences, 05-110 Jabłonna, Poland; d.szkopek@ifzz.pl (D.S.); j.wolinski@ifzz.pl (J.W.)
5 Department of Animal Physiology, The Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences, 05-110 Jabłonna, Poland; k.zaworski@ifzz.pl
* Correspondence: malgorzata.lenartowicz@uj.edu.pl (M.L.); r.starzynski@igbzpan.pl (R.R.S.)

Abstract: Preterm infants are most at risk of iron deficiency. However, our knowledge of the regulation of iron homeostasis in preterm infants is poor. The main goal of our research was to develop and validate an animal model of human prematurity to assess iron status in preterm infants. We performed a cesarean section on sows on the 109th day of pregnancy, which corresponds to the last trimester of human pregnancy. Preterm piglets showed decreased body weight, red blood cell indices, plasma iron level and transferrin saturation. Interestingly, higher hepatic and splenic non-heme iron content and plasma and hepatic ferritin levels were found in premature piglets compared with term ones. In addition, premature piglets showed higher mRNA levels of iron-regulatory hormone hepcidin in the liver than term animals, which have not been reflected in higher plasma hepcidin-25 levels. We also showed changes in hepcidin regulators, including hepatic bone morphogenetic protein 6, plasma erythroferrone and growth differentiation factor 15 in preterm piglets. Consequently, no difference was observed in iron-exporter ferroportin levels in the spleen and liver. Overall, it seems that premature piglets show a pattern of iron metabolism characteristic of functional iron deficiency and iron accumulation in the tissue.

Keywords: piglet; cesarean section; animal model; iron; preterm

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1. Introduction

Due to its redox properties, iron is an essential micronutrient involved in many key biochemical processes and cellular functions. Iron deficiency (ID) is one of the most widespread nutritional deficiencies and affects all age groups. Children up to 5 years of age are the most affected due to high iron requirements in this period of rapid growth. Perinatal perturbations in iron homeostasis may result in alterations in cognitive functions and neurodevelopment [1]. Preterm infants are prone to develop ID anemia in the first 4 months of life due to lower iron stores at birth compared with term infants because most fetal iron stores are built up as a result of iron transfer from the mother during the third trimester of gestation. This transfer may be interrupted by gestation shortening. During development, ID negatively affects the growth and function of many organs, including the liver, heart and brain [2].

Designing strategies for iron supplementation in neonates is complicated. Detailed investigations of this issue during the neonatal period after normal and shortened pregnancies, as well as the search for suitable animal models for neonatal iron supplementation studies, are urgently required. A proper animal model of human preterm physiology should include viable neonates similar to preterm human infants and have a body size that allows comparative monitoring, blood sampling and clinical interventions. Large animals such as rhesus monkeys [3], sheep [4] and dogs [5] have been used for modeling human physiology and nutrition. However, the availability of these animals is very restricted, and their cost is very high, thus limiting the feasibility of experiments using these models. In contrast, pigs are available and relatively cheap to maintain animals, and their usefulness in studies on human prematurity has been proven by Eiby et al. [6]. Piglets provide a clinically relevant model of preterm neonatal physiology where the maturation of multiple organ systems is similar to human early preterm infants [6]. In particular, although the human placenta is hemochorial and the pig placenta is epitheliochorial, the key proteins involved in iron transport function similarly across species despite these structural differences [7]. The results of several studies [8,9], including our own [10–13], indicate that term newborn piglets are a suitable model with which to explore iron metabolism in the neonatal period. Here, we attempt to validate piglets born by cesarean section as a pig model of prematurity, reflecting the mechanisms of ID in human premature infants as much as possible. Moreover, the piglets obtained by cesarean section and kept in incubators can be used in follow-up studies during the neonatal period.

2. Results

2.1. Substantial Decrease in Body Weight in Premature Piglets

Premature piglets were delivered at 95% gestation (109 d of 115.4 d gestation) by cesarean section to mimic human late preterm birth. Compared with full-term delivery, premature birth by cesarean section led to a drop in body weight by 44% ($p < 0.01$; Figure 1). Premature piglets presented spontaneous respiration upon delivery and were hemodynamically stable.

(**a**) (**b**)

Figure 1. Term (left) and preterm (right) piglets appearance (**a**) and their body weight (**b**). (**a**) The photos were taken just after birth. Premature piglets need heating pads to maintain body temperature. (**b**) Data are presented as the mean ± SEM ($n = 6$). ** asterisk denotes a statistically significant difference at $p < 0.01$ between term and preterm piglets.

2.2. Changes in Red Blood Cell (RBC) and Reticulocyte Indices in Premature Piglets

Hemoglobin level, RBC count, hematocrit value and reticulocyte count in preterm piglets were significantly reduced compared with term animals by 20, 24, 16 and 30%, respectively ($p < 0.01$; Figure 2). On the other hand, mean corpuscular volume (MCV) and mean cell hemoglobin values were higher in preterm piglets compared with full-term piglets by 11 and 6%, respectively ($p < 0.05$; Figure 2). Overall, RBC indices indicate the occurrence of anemia in preterm piglets.

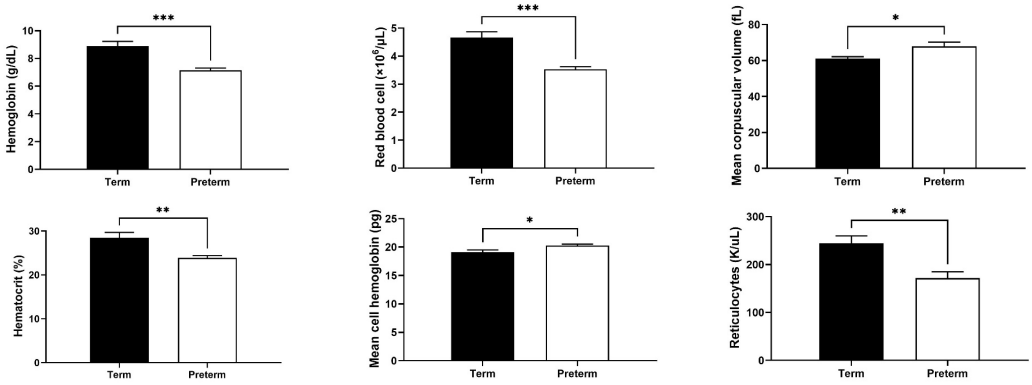

Figure 2. Red blood cell and reticulocyte indices. Data are presented as the mean ± SEM (n = 6). *, ** or *** asterisks denote statistically significant differences at $p < 0.05$, $p < 0.01$ or $p < 0.001$, respectively.

2.3. Strong Decrease in Biochemical Plasma Iron Parameters in Premature Piglets

Premature piglets showed dramatically lower plasma iron levels (reduction by 86%), total iron binding capacity (TIBC) (reduction by 71%) and transferrin saturation (TSAT) values (reduction by 49%) than full-term piglets ($p < 0.05$; Figure 3). These results attest to a severe ID in preterm piglets.

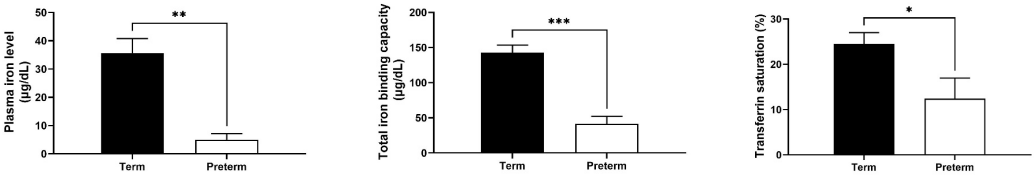

Figure 3. Blood plasma biochemical iron parameters. Data are presented as the mean ± SEM (n = 6). *, ** or *** asterisks denote statistically significant differences at $p < 0.05$, $p < 0.01$, or $p < 0.001$, respectively.

2.4. Increased Hepatic Iron Status in Premature Piglets

In preterm piglets, we noted that hepatic non-heme iron content was twice as high as that in term piglets ($p < 0.01$; Figure 4a). Hepatic ferritin level reflects iron abundance in the liver [14]. The assessment of protein levels of ferritin light chain (L-ferritin) and ferritin heavy chain (H-ferritin) in piglets' livers by Western blotting largely confirmed the results of direct measurement of iron content. Preterm piglets showed substantial increases in levels of L- and H-ferritin compared with term animals ($p < 0.05$; Figure 4b). Blood plasma ferritin concentration, an indirect indicator of hepatic iron stores, was also elevated by 50% ($p < 0.001$; Figure 4c). Finally, microscopic analysis of liver sections stained for non-heme iron with Perls' staining shows strong iron deposits in preterm piglets, whereas, in term animals, weaker staining of non-heme was found (Figure 4d).

2.5. Splenic and Bone Marrow Iron Status in Preterm and Term Piglets

Similar to the liver, a significant increase in non-heme iron content in the spleen was observed in preterm piglets ($p < 0.05$; Figure 5a). However, no increase in L- and H-ferritin protein levels was detected in these piglets compared with term animals (Figure 5b). Splenic non-heme iron was not detected by Perls' staining either in preterm or in term piglets (Figure 5c). Similarly to the spleen, no iron deposits were detected in the bone marrow of either preterm or term piglets (Figure S1).

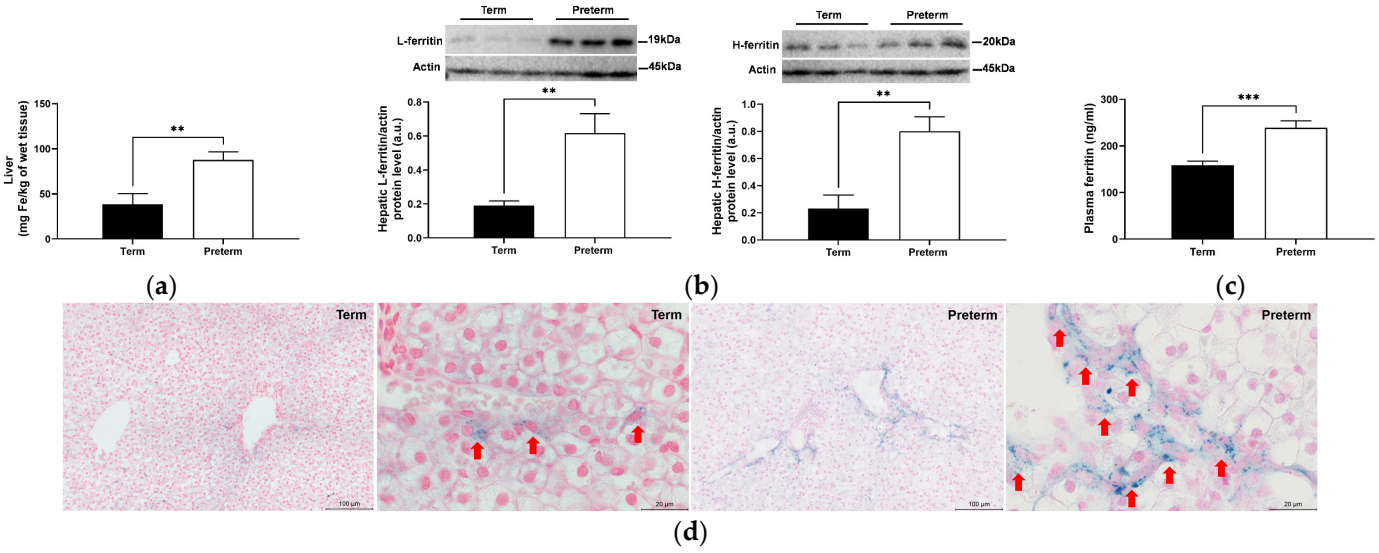

Figure 4. Hepatic non-heme iron content (**a**) and ferritin protein level (**b**), plasma ferritin level (**c**), and hepatic histological examination of iron loading (**d**). Data are presented as the mean ± SEM (n = 6). ** or *** asterisks denote statistically significant differences at $p < 0.01$ or $p < 0.001$, respectively. (**b**) Representative Western blot images and relative densitometric bar graphs of hepatic ferritin. Actin was used as a protein loading control. a.u., arbitrary units; H-ferritin, ferritin heavy chain; L-ferritin, ferritin light chain. (**d**) Non-heme iron deposits were detected by staining with Perls' staining (blue stain) (original magnification: 200×. Scale bars = 100 μm). Iron deposits at high magnification are shown by red arrowheads (original magnification: 1000×; scale bars = 20 μm).

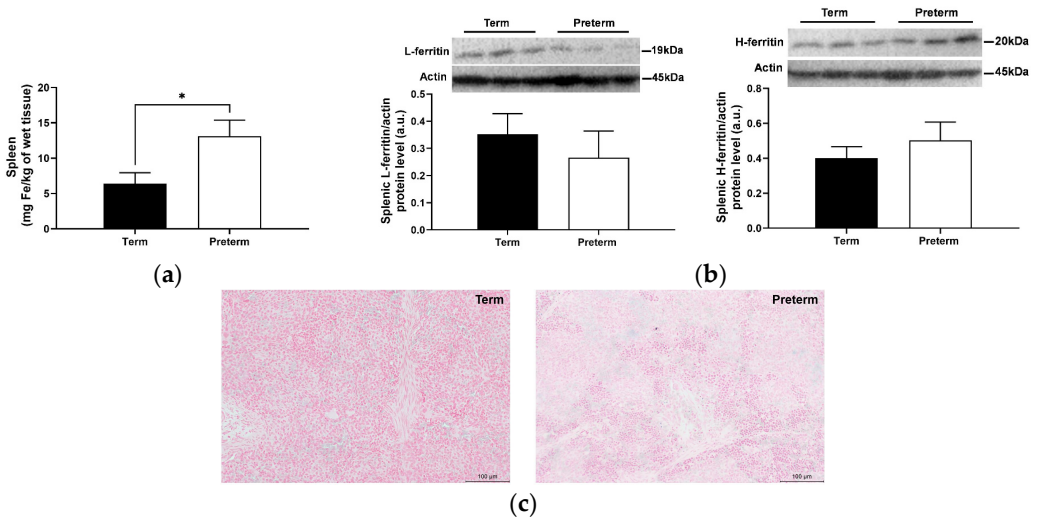

Figure 5. Splenic non-heme iron content (**a**), ferritin protein level (**b**), and histological examination of iron loading (**c**). Data are presented as the mean ± SEM (n = 6). * asterisk denotes a statistically significant difference at $p < 0.05$ between term and preterm piglets. (**b**) Representative Western blot images and relative densitometric bar graphs of splenic ferritin. Actin was used as a protein loading control. a.u., arbitrary units; H-ferritin, ferritin heavy chain; L-ferritin, ferritin light chain. (**c**) Non-heme iron deposits were detected by staining with Perls' staining (original magnification: 200×. Scale bars = 100 μm).

2.6. Changes in Levels of Hepcidin and Its Regulators in Premature Piglets

Hepcidin, a peptide produced mainly by the liver in response to high iron content, is considered the main regulator of systemic iron homeostasis [15]. Hepatic hepcidin mRNA expression in premature piglets was nearly eight times that of term ones ($p < 0.05$), but no difference was found in the blood plasma hepcidin-25 concentration (Figure 6a). Relative to term piglets, the expression level of mRNA encoding for bone morphogenetic protein 6 (BMP6), a key endogenous inducer of hepcidin synthesis [16] in premature animals, was also significantly higher ($p < 0.01$; Figure 6b). In parallel, we found in the blood plasma decreased levels of erythroid hepcidin suppressors, such as erythroferrone (reduction by 52%) and growth differentiation factor 15 (GDF15) (reduction by 13%) ($p < 0.05$; Figure 6b). Furthermore, assessment of erythropoietin blood plasma concentration in preterm piglets revealed a substantial decrease compared with term ones ($p < 0.05$; Figure 6b). Finally, preterm and term piglets showed no difference in the levels of interleukin-6 and tumor necrosis factor α (Figures 6b and S2), the inflammatory cytokines responsible for the induction of hepcidin during inflammation [17].

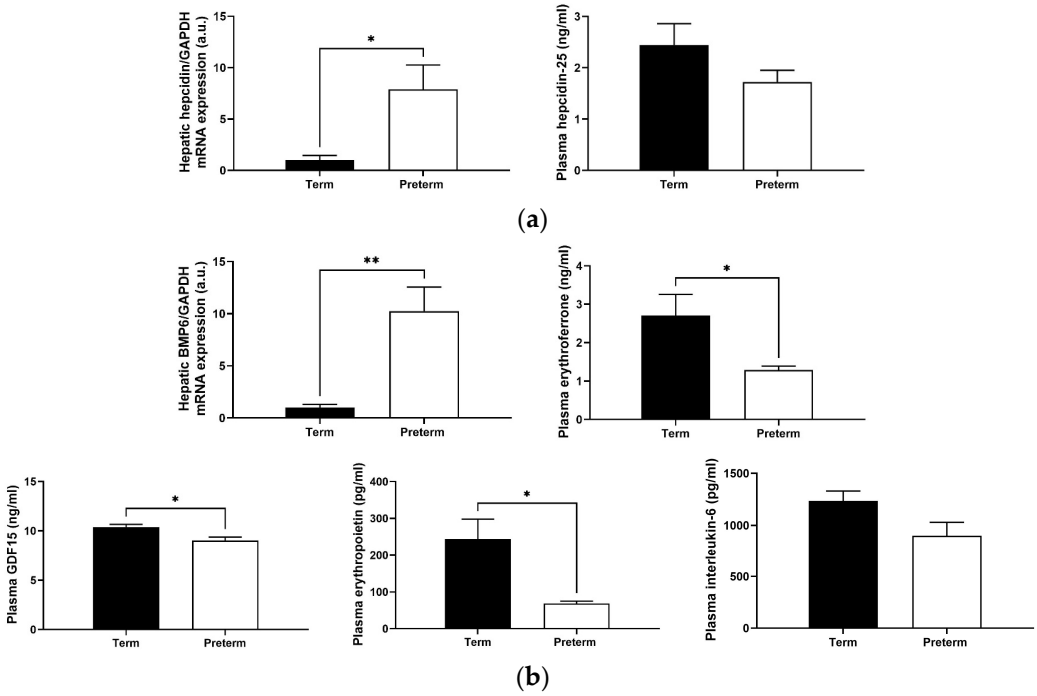

Figure 6. Hepcidin level (**a**) and its regulators (**b**). Data are presented as the mean ± SEM ($n = 6$). * or ** asterisks denote statistically significant differences at $p < 0.05$ or $p < 0.01$, respectively. a.u., arbitrary units; BMP6, bone morphogenetic protein 6; GDF15, growth differentiation factor 15.

2.7. Similar Hepatic and Splenic Ferroportin Levels in Term and Preterm Piglets

Ferroportin, the only iron exporter known in mammalian cells that transfers iron to plasma apo-transferrin, is a molecular target of hepcidin, which binds to this protein to induce its degradation, thus inhibiting iron release from exporting cells [18]. Analysis of both ferroportin mRNA and protein levels in the liver and spleen showed no difference between preterm and term piglets (Figure 7), which is consistent with similar blood plasma hepcidin levels in piglets from both investigated groups.

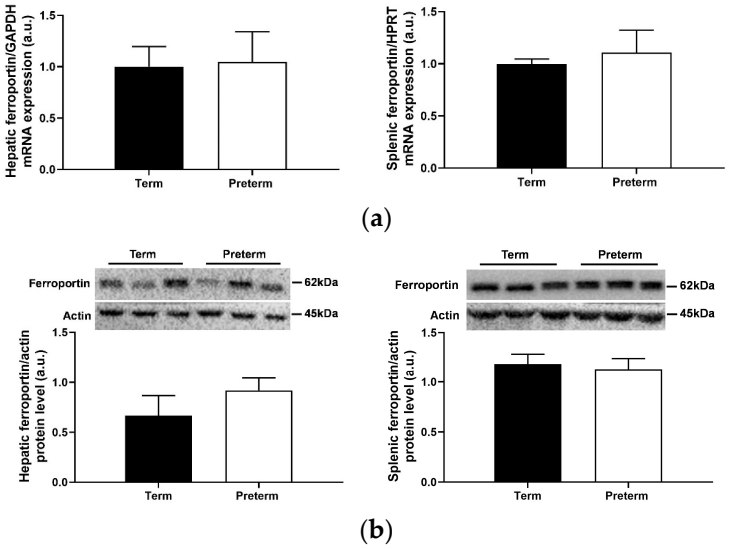

Figure 7. Ferroportin mRNA expression (**a**) and protein level (**b**) in the liver and spleen. Data are presented as the mean ± SEM (n = 6). (**b**) Representative Western blot images and relative densitometric bar graphs of hepatic and splenic ferroportin. Actin was used as a protein loading control. a.u., arbitrary units.

3. Discussion

The cesarean section is a common way to harvest preterm piglets. The first attempts were made by Eiby et al. [6], who developed a porcine model of preterm infants delivered by cesarean section at six gestational ages from day 91 to day 113. This experiment focused on anthropometric studies and showed that the degree of organ development and maturation is proportional to advanced age [6]. However, the regulation of iron metabolism in preterm piglets was not considered. In our case, piglets born by cesarean section on the 109th day of gestation started spontaneous breathing and did not show signs of hypoxemia or hemodynamic instability. It is worth noting that the shortening of pregnancy by an average of 6% translated into a significant decrease in the birth weight of premature piglets by 44% in our studies. Similarly, in humans, weight loss is observed depending on the degree of prematurity [19,20].

In order to validate our pig model of ID in prematurity, we first analyzed the RBC status in the blood plasma. We found that RBC count, hemoglobin level and hematocrit value were significantly lower in preterm piglets compared with full-term piglets. Similar results in piglets born by cesarean section after pregnancy lasting 111–112 days were reported by other authors [21]. Regarding human preterm infants, a similar hematologic pattern was reported in umbilical cord blood [22]. Our results also showed higher MCV and mean cell hemoglobin values in preterm piglets than in term animals. High MCV is characteristic of fetal erythrocytes and decreases with the duration of pregnancy [23]. As it was demonstrated by Alur et al. [20], hemoglobin and hematocrit values decreased, and MCV increased with the degree of human prematurity, which was in accordance with our results obtained in the piglet model of prematurity. Assuming that our pig model reflects human late prematurity, the analysis of umbilical cord blood of late preterm infants in the study of Rolim et al. [24] aligns perfectly with our results in preterm piglets.

Biochemical analysis of iron in the blood plasma or serum is important for the evaluation of both ID and anemia. Our results show that both plasma iron level and TIBC value were down-regulated in preterm piglets relative to term animals, consistent with results from studies on human preterm infants [25,26]. In both studies, a statistically significant increase in blood iron concentration and TIBC was documented with the advancement of pregnancy. In addition, lower TSAT was shown in preterm piglets compared to term ones in our study. ID is accompanied by low TSAT [27,28]. It has been suggested that TSAT values of <17% can attest to ID [29].

Examination of the hepatic and splenic iron content usually provides important information about iron status in the organism. Here, we have undertaken a comprehensive analysis of iron status in the liver of piglets, and surprisingly, we found that it is definitely higher in preterm piglets. In line with our results, data obtained by Georgieff et al. [30] indicated that the content of non-heme iron in the liver is higher in premature infants compared with term ones. Contrary to our results, Siddappa et al. [31] reported that in humans, umbilical cord serum ferritin concentration (an indirect indicator of hepatic iron stores) increases with gestational age, from an average of 63 to 171 μg/L at week 23 and week 41 of gestation, respectively. Similar to the liver, we found higher iron levels in the spleen of preterm piglets compared to term animals. However, this difference did not translate into decreased protein levels of both ferritin subunits. It is worth noting that splenic non-heme iron content is much lower than hepatic iron in either preterm or term piglets. Therefore, we suggest that changes occurring at a low iron level in the spleen are not reflected in fluctuations of ferritin protein level. We were unable to show changes in the level of non-heme iron in bone marrow smears, probably due to the very low level of iron in erythroid cells of the bone marrow. Similar results were obtained in preterm infants using Turnbull staining immediately after birth, clearly indicating low levels of non-heme iron in the bone marrow [32].

The lower non-heme iron content in term piglets compared to preterm piglets is somewhat puzzling. A possible explanation for this phenomenon could be an intensive mobilization of iron from hepatic iron stores to satisfy iron needs for increased production of RBCs (driven by increased erythropoietin levels, as shown in our study) at the end of pregnancy. Indeed, during mammalian prenatal development, definitive erythropoiesis occurs mainly in the fetal liver [33]. The liver is the main extramedullary erythropoietic tissue of the human fetus at midterm and continues to produce blood cells through the first week of life [34,35]. Furthermore, our data showing a considerable difference in the body weight between preterm and term piglets (about 700 g) strongly indicate that during the last 6 days of pregnancy, iron is intensively taken up from the liver to meet the demand for this microelement from other tissues. In summary, we propose that decreased non-heme iron levels in the liver in term piglets are due to exhaustive iron utilization in erythropoiesis accompanied by the shift of this iron fraction to the heme compartment in erythroid cells and intensive growth of fetuses during the very last period of pregnancy.

The next step in our study was to measure the expression of hepcidin, a small peptide hormone produced mainly by hepatocytes that orchestrate body iron fluxes by adjusting iron supply to body iron requirements [15,36]. Hepcidin binds to ferroportin to induce its degradation, thus inhibiting iron release from exporting cells [18]. We showed a statistically significant upregulation of hepcidin mRNA level in preterm versus term piglets in the liver. Considering that expression of hepcidin is transcriptionally controlled by elevated liver iron [37], this observation is consistent with high hepatic iron content in preterm animals.

It is known that BMP6 is the main transcriptional activator of hepcidin, which functions as an "iron sensor" whose transcription in liver sinusoidal endothelial cells is induced by high iron levels [16]. Here, we found that BMP6 is strongly induced in the liver of preterm piglets, which may explain the high mRNA expression of hepcidin in these animals. Contrary to iron, hepcidin expression can be suppressed by secreted erythroid factors, such as erythroferrone [38] and GDF15 [39]. We examined plasma levels of these factors and found their decline in preterm piglets. This implies that high hepcidin levels in these animals may also result from the attenuation of erythroid hepcidin suppressors. Kautz and Nemeth [38] described how erythroferrone synthesis is promoted by the release of erythropoietin, which then contributes to expanded erythropoiesis. Accordingly, blood plasma erythropoietin level was much lower in preterm piglets than in term animals, likely explaining low erythroferrone expression in the former. It is also worth noting that anemia of prematurity is characterized by reduced erythropoietin production [40,41]. A low plasma erythropoietin level is an important reason that nadir hematocrit values of preterm infants are lower than those of term infants [41], which is consistent with our results. In addition,

some authors show that fetal serum hepcidin is likely triggered by the inflammatory effect of labor and delivery and that the level of hepcidin in preterm infants after cesarean section may be higher due to inflammation [17]. Apparently, this is not the case in our study, as the levels of inflammatory cytokines were similar in preterm and term piglets.

To our surprise, the measurement of bioactive hepcidin-25 concentration in piglet blood plasma showed very low levels of this peptide, with no statistically significant difference between animals from both experimental groups. In humans, fetal hepcidin levels in cord blood from preterm births were comparable to that of full-term births, suggesting a rather low level of hepcidin in the fetal stage [42]. Serum hepcidin values were also comparable and very low in both preterm infants with ID and preterm infants without ID [43]. In another of our studies, we also found that RNA levels of hepatic hepcidin increased, while blood hepcidin-25 levels did not change in 28-day-old piglets [44]. It should be emphasized that hepcidin is synthesized in the liver as an 84-aa pre-pro-hormone containing a typical N-terminal 24 amino acid endoplasmic reticulum targeting signal sequence and maturated by proteolysis through a consensus furin cleavage site (which is conserved in mammals) [45] to generate the bioactive 25-aa peptide secreted in the circulation [46]. We hypothesize that in the perinatal period, the control of hepcidin processing and release may be impaired, and that is why circulating hepcidin levels do not reflect its hepatic mRNA expression. Altogether, the analysis of blood plasma hepcidin in preterm and term piglets strongly suggests that this parameter may not be useful in the assessment of the perinatal systemic iron status of pigs.

As mentioned above, hepcidin achieves its function by binding to its cell membrane receptor, ferroportin, the sole iron exporter, which results in the endocytosis and degradation of ferroportin and consequently reduces iron release from macrophages, hepatocytes and enterocytes [18]. Here, we showed unchanged levels of ferroportin both in the liver and spleen of term and preterm piglets. This result is consistent with the stable concentration of hepcidin-25 in the blood plasma of piglets from both experimental groups. In the study by Tabbah et al. [47], ferroportin was localized postmortem in the liver of premature and full-term human neonates. The authors showed that hepatic ferroportin staining in term newborns was more prominent around the portal veins, while lower levels were observed around the central vein. In contrast, in the livers of newborns that died from early-onset neonatal sepsis, the ferroportin signal shifted to a diffuse pattern around the central vein [47].

Our study is the first attempt to characterize an animal model of prematurity based on the Polish Landrace breed. The data from the present study suggest that the validated pig model of prematurity largely mirrors systemic iron metabolism in preterm human infants. This model allows for in-depth biochemical and molecular analysis of tissue samples that are understandably impossible to obtain from humans. Obviously, we are aware of the limitations of our model, namely an extremely rapid growth rate of pig fetuses, which is not observed in human newborns. Furthermore, despite many similarities between pigs and humans, there are also some inconsistencies between pig and human organs. However, we believe that these differences do not undermine our evaluation of the pig model of maturity in the context of iron metabolism studies. Our results prove preterm piglets to be a promising and competitive model for the investigation of iron metabolism in premature infants.

4. Materials and Methods

4.1. Animals and Experimental Design

The experiment was carried out in the Laboratory of Large Animal Models (The Kielanowski Institute of Animal Physiology and Nutrition of the Polish Academy of Sciences, Jabłonna). In the experiment, we used 3 Polish Landrace sows. The animals, 35–40 days after insemination (confirmed by ultrasound examination), were transported to a pig farm in Jabłonna. The animals were housed in individual pens and fed a balanced diet twice daily according to the nutritional requirements of the animals and stage of gestation.

The animals had constant access to water ad libitum. Naturally, abortion does not occur in pigs, and pharmacologically induced delivery can result in fetal death due to large litters. For this purpose, healthy cull sows were selected from the herd. Due to the age and number of litters, the sows would still be sold to the slaughterhouse after giving birth (reduced breeding value). Cesarean section was manipulated on the 109th day of gestation to obtain premature piglets. After cesarean sections, 24 healthy premature piglets of both sexes were obtained. Six premature piglets were weighed and sacrificed for sampling immediately after birth. As a control, 6 naturally born Polish Landrace piglets (an average gestation period of 115.4 days) from 3 sows and of both sexes were sampled immediately after birth. The rest of the premature piglets were housed in special cages equipped with dry bedding and heating pads and fed via an intragastric tube for several consecutive days until they began to take food from a bottle with a teat. These piglets were used to observe postnatal growth and physiology.

4.2. Biological Sample Collection

Blood was collected immediately after full-term and preterm birth directly from the heart (under isoflurane anesthesia, 1.28%) at slaughter into tubes with heparin. Following an overdose of sodium pentobarbital by intravenous injection (100 mg/kg bw), liver and spleen samples were rapidly dissected and flushed with PBS. Samples of tissues were immediately dissected and fixed in a 4% formaldehyde solution (#1004968350, Sigma-Aldrich, St. Louis, MO, USA). The remaining portions were frozen in liquid nitrogen prior to storage at −80 °C until used for analysis. Bone marrow cells for Perls' staining were rinsed from femurs with ice-cold Hanks' balanced salt solution, then smeared onto slides and fixed with methanol.

4.3. Measurement of RBC Indices and Blood Plasma Iron Parameters

RBC indices were determined using IDEXX ProCyte Dx, an automated hematology analyzer (IDEXX Laboratories, Westbrook, ME, USA). The colorimetric measurement of an iron–chromazurol complex (absorbance at 630 nm) was used to measure iron concentration in the blood plasma (#1-420-0200, Biomaxima, Lublin, Poland) and TIBC (#1-421-0060, Biomaxima, Lublin, Poland). The percentage of TSAT was then calculated according to the following formula: TSAT = (plasma iron/TIBC) × 100. The plasma ferritin concentration was evaluated by a porcine ELISA kit (#EP0255, Fine Biotech, Wuhan, China).

4.4. Measurement of Iron Content in Tissues

The tissular non-heme iron content 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 562 nm, Beckman DU-68, Beckman Coulter, Brea, CA, USA) [48].

4.5. Perls' Staining

After a 24 h fixation, segments of the liver and spleen were dehydrated, embedded in paraffin, and cut into 5 µm sections with a Hyrax M25 rotary microtome (Zeiss, Oberkochen, Germany). After mounting on glass slides, sections were deparaffinized, stained with Perls' Prussian blue for 30 min, counterstained with nuclear red (#1001210500, Sigma-Aldrich, St. Louis, MO, USA) for 2 min and analyzed under a light microscope (Eclipse E200, Nikon, Amsterdam, The Netherlands). The same procedure of staining with Prussian Blue was performed with bone marrow smears prepared from piglets.

4.6. Measurement of Plasma Hepcidin, Erythropoietin, Erythroferrone, and GDF15 Level

For quantification of plasma hepcidin, erythropoietin, erythroferrone and GDF15 level, we used commercial ELISA kits (human Hepcidin 25, #EIA-5782, DRG Instruments GmbH, Marburg, Germany; porcine erythropoietin, #ABIN6955643, Antibodies-online, Aachen, Germany; porcine erythroferrone, #E07E0518, BlueGene Biotech, Shanghai, China; and porcine GDF15, #E07G0127, BlueGene Biotech, Shanghai, China) according to the

manufacturer's guidelines. The human Hepcidin 25 ELISA kit was validated for use in pigs in our previous study [49].

4.7. RNA Isolation and Real-Time Quantitative RT-PCR Analysis

Total RNA was isolated from about 30 mg of wet tissue using the SV Total RNA Isolation System (#Z3105, Promega, Madison, WI, USA). mRNA levels were measured by a real-time quantitative RT-PCR of cDNA derived from specific transcripts in the LightCycler® 96 Instrument (Roche Diagnostics, Basel, Switzerland), using the pair of primers shown in Table S1. The amplified products were detected using SYBR Green I (#06924204001, Roche Diagnostics, Basel, Switzerland) according to the manufacturer's guidelines. To confirm amplification specificity, the PCR products were subjected to melting curve analysis and agarose gel electrophoresis. Transcript levels were normalized relative to those of control reference genes selected using NormFinder software (MOMA, Aarhus, Denmark) (https://www.moma.dk/software/normfinder (accessed on 13 February 2023)). The expression of target genes versus housekeeping genes (GAPDH or HPRT) was calculated by the formula $2^{-\Delta\Delta CT}$ [50].

4.8. Western Blot Analysis

To determine protein levels of L-ferritin, H-ferritin and ferroportin, 40 µg of membrane and cytosolic extracts were prepared and separated by electrophoresis on either 9% or 16% SDS-PAGE gels depending on the molecular weight of the protein [51]. Electroblotting of the resolved proteins onto the PVDF transfer membrane (#88518, Thermos Scientific, Waltham, MA, USA), blocking, and incubation with primary and secondary antibodies were performed according to previously described methods [51]. Table S2 shows the details of the primary and secondary antibodies. For quantitative analysis of protein content, reactive bands were quantified relative to actin using a Molecular Imager with Quantity One 4.6 software (Bio-Rad, Hercules, CA, USA).

4.9. Statistical Analysis

The results were statistically analyzed using independent samples t-tests using SPSS Statistics 26 (IBM, Armonk, NY, USA). Figures were prepared using GraphPad Prism 9.1.1 (GraphPad Software, San Diego, CA, USA). $p < 0.05$ was considered significant. Data are presented as mean values ± SEM.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms252011215/s1.

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Informed Consent Statement: Not applicable.

Data Availability Statement: Data will be available upon request.

Conflicts of Interest: The authors declare no conflicts of interest.

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Oral supplementation with Sucrosomial® Iron improves the iron status of preterm piglets delivered by cesarean section†

Xiuying Wang, [a] Paweł Lipiński,[a] Magdalena Ogłuszka,[b] Rafał Mazgaj,[a] Jarosław Woliński,[c,d] Dominika Szkopek, [c] Kamil Zaworski,[d] Zuzanna Kopeć,[a] Beata Żelazowska,[a] Germano Tarantino,[e] Elisa Brilli[e] and Rafał Radosław Starzyński *[a]

Premature infants are more likely to develop iron deficiency caused by an inadequate iron storage due to shortened pregnancy. Sucrosomial® Iron (SI) is an oral iron formulation of ferric pyrophosphate with high bioavailability and tolerability. This research compared the iron status of preterm and full-term piglets and evaluated the effects of SI on iron homeostasis in the early postnatal period. Eighteen preterm piglets (born *via* cesarean section on gestation day 109) and twelve full-term piglets (natural birth) were divided into five groups (n = 6 piglets per group): full-term/preterm piglets without iron supplementation, full-term/preterm piglets supplemented with SI (2 mg Fe per piglet per day, days 4–10), and preterm piglets supplemented with ferrous sulfate (2 mg Fe per piglet per day, days 4–10). Samples were collected on day 11. Preterm piglets showed poor growth and low total body iron content, and they developed iron deficiency anemia, as indicated by decreased red blood cell indices and plasma iron parameters. The iron deficiency was partially improved by SI supplementation. Interestingly, higher hepatic and splenic non-heme iron content, accompanied by increased tissue and plasma ferritin, were found in preterm piglets compared to full-term piglets. SI also contributed to tissue iron accumulation in preterm piglets. Functional iron deficiency and iron accumulation in tissues make the regulation of iron metabolism in preterm piglets different from that in full-term ones. SI can alleviate the negative effects of iron imbalances caused by premature birth by regulating the hepcidin–ferroportin axis. In addition, SI did not induce inflammatory or oxidative responses, and its effects are comparable to those of the classic iron supplement, ferrous sulfate. These results indicate that SI is a promising iron supplement for improving the iron status of premature infants.

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1. Introduction

Iron, as a crucial trace element, is involved in many metabolic functions that are important for normal cellular growth and development. Factors that have been suggested to negatively affect neonatal iron status include premature delivery and inappropriate iron supplementation. Based on statistical data from the World Health Organization, 1 in 10 babies is born prematurely, and prematurity is the leading cause of death in children under the age of 5.[1,2] Considering that most iron is transferred from mother to fetus during the third trimester of pregnancy, premature infants have lower total body iron (TBI) content at birth compared to full-term infants.[3] Premature infants also have high iron requirements due to increased postnatal growth, with rapid blood volume expansion and increased hemoglobin demand.[3] Therefore, premature infants are at particular risk of developing iron deficiency. Early iron deficiency not only causes anemia in neonates but more importantly, also leads to the abnormal function of multiple organs, including the heart,[4] intestine,[5] and brain.[6] In addition, long-term motor, cognitive, and socio-emotional behavioral deficits are observed in children and young adults who experienced a period of iron deficiency early in life.[6] For these reasons, iron supplementation is recommended for premature infants with iron insufficiency to maintain their iron status and meet the iron demands of catch-up postnatal growth.

[a]*Laboratory of Iron Molecular Biology, Department of Molecular Biology, Institute of Genetics and Animal Biotechnology, Polish Academy of Sciences, 05552 Jastrzębiec, Poland. E-mail: r.starzynski@ighz.pl*
[b]*Department of Genomics and Biodiversity, Institute of Genetics and Animal Biotechnology, Polish Academy of Sciences, 05552 Jastrzębiec, Poland*
[c]*Laboratory of Large Animal Models, The Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences, 05110 Jabłonna, Poland*
[d]*Department of Animal Physiology, The Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences, 05110 Jabłonna, Poland*
[e]*Scientific Department, Pharmanutra S.p.A., 56122 Pisa, Italy*

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Several professional societies have published recommendations for iron supplementation in premature infants, with most recommended doses falling within the range of 1–3 mg per kg per day.[7] Orally administered iron, as a simple and affordable intervention, has been used to reduce iron deficiency for centuries. Typically, iron is orally administered in a ferrous sulfate ($FeSO_4$) tablet, which is considered to be a first-generation iron supplement. However, orally administered iron salts are usually poorly absorbed, and unabsorbed iron may result in adverse effects, *e.g.*, nausea, vomiting, and diarrhea.[8] Sucrosomial® Iron (SI) is a new oral iron formulation of ferric pyrophosphate, which is covered by a phospholipid bilayer membrane and sucrester matrix.[9] SI is characterized by increased bioavailability and reduced toxicity.[9] Its special structure allows SI to be gastro-resistant and to pass through the intestinal tract without causing side effects due to the interaction between iron and the intestinal mucosa.[10] Data from Caco-2 cell experiments showed that SI-treated cells displayed higher ferritin iron accumulation than cells incubated with $FeSO_4$, Lipofer (a water-dispersible micronized iron) and Sunactive Iron (a micronized dispersible ferric pyrophosphate).[11] This result indicates that more iron is bioavailable to cells after SI incubation, thus SI can be substituted for common oral iron salts to improve body iron status. In clinical scenarios, oral SI is used to treat iron deficiency induced by chronic kidney disease, inflammatory bowel disease, cancer, surgery and other conditions.[9] Our previous research showed that SI could counteract iron deficiency in the anemic full-term piglet model.[12,13] However, until now, there have been no studies on the effectiveness of SI on premature infants.

We hypothesized that SI would improve the iron status of preterm piglets. In this study, preterm piglets delivered at 95% gestation (109 d of 115 d gestation) by cesarean section were used as an animal model for late preterm human infants (approximately 35 weeks of gestational age). Studies by Sangild *et al.*[14] and Eiby *et al.*[15] find that preterm piglets suffer from many of the same organ immaturities as premature infants. Moreover, piglets easily develop systemic iron deficiency anemia without iron supplementation because they have the lowest hepatic iron stores among all mammalian species and high iron requirements for fast growth; thus, piglets are a promising animal model for exploring iron metabolism in the neonatal period.[16,17] Moreover, our own data clearly show that supplementing healthy pregnant sows with SI during the third trimester does not improve their own iron status nor that of their offspring.[18] Therefore, instead of trying to improve the iron status of the piglets by supplementing the sow, we explored the possibility of directly supplementing the preterm piglets with SI.

2. Materials and methods

2.1. Animal care and experimental design

All animal procedures were performed in accordance with the guidelines of EU Directive 2010/63/EU for animal experiments and were approved by the 2nd Local Ethics Committee in Warsaw (WAW2/125/2021). The animal experiments were conducted in the Laboratory of Large Animal Models (The Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences, Jabłonna, Poland). We used 3 Polish Landrace sows to obtain preterm piglets. Artificial insemination was performed using semen from Polish Landrace boars. Pregnancy was confirmed by ultrasound examination at 35–40 days after insemination. These sows were housed in individual pens, and given free access to water and a diet formulated to meet their nutritional requirements according to their stage of gestation.

Eighteen preterm piglets of both sexes were delivered by cesarean section on day 109 of gestation, which is 6 days before full gestation (115 days). Within 3 to 4 hours after parturition, an intragastric feeding tube was inserted into each preterm piglet. The surgically placed feeding tube avoids possible tissue damage and the stress of repeated feeding tube insertions or of the misplacement of the tube into the trachea. The feeding tube was used to feed the preterm piglets until they learned how to use a nursing bottle on approximately day 2 or 3. Twelve Polish Landrace piglets born by normal vaginal delivery from 3 sows were used as controls. Newborn piglets were assigned to one of five groups (n = 6 piglets per group): (1) full-term piglets without iron supplementation; (2) full-term piglets supplemented with SI (PharmaNutra, Pisa, Italy; 2 mg Fe per piglet per day) from day 4 to day 10 after birth; (3) preterm piglets without iron supplementation; (4) preterm piglets supplemented with SI (2 mg Fe per piglet per day) from day 4 to day 10 after birth; (5) preterm piglets supplemented with $FeSO_4$ (Gambit, Kutno, Poland; 2 mg Fe per piglet per day) from day 4 to day 10 after birth. The dosage of iron supplementation for preterm piglets is primarily based on the daily iron requirements for full-term piglets,[19] adjusted for body weight to estimate the needs of preterm piglets. It is also guided by the iron supplementation recommendations for human premature infants.[7] Day 4 is chosen to start iron supplementation because piglets are born with limited iron reserves, sufficient only for the first 3–4 days.[20–22] In modern pig production, iron supplementation is routinely given at this stage to prevent iron deficiency anemia.[20–22]

The piglets were housed in special cages equipped with dry bedding and heating pads to maintain a temperature of between 35 and 37 °C. During the experiment, all piglets were given every hour with warm (37 °C) human milk (in collaboration with Milk Bank, Saint Family Hospital, Warsaw, Poland). They were allowed to move freely within the cages and had visual and tactile contact with each other, but no contact with the sow. Piglets were weighed every morning before iron was administered.

2.2. Sample collection

On day 11, blood samples were obtained directly from the heart (under isoflurane anesthesia, 1.28%) into tubes with heparin. Plasma was isolated by centrifugation (3500*g*, 10 min) and then stored at −80 °C until used. After blood collection,

the piglets were euthanized by intravenous injection with 0.5 mL per kg b.w. of Morbital (133.3 mg mL^{-1} of sodium pentobarbital + 26.7 mg mL^{-1} of pentobarbital, Biowet, Puławy, Poland), and liver and spleen samples were taken. One portion of each tissue sample was fixed in 4% paraformaldehyde (#1004968350, Sigma-Aldrich, St Louis, MO, USA) for at least 24 h and then embedded in paraffin for histological examination. The remaining portions were divided into smaller pieces and immediately frozen in liquid nitrogen and stored at −80 °C for further analysis.

2.3. Red blood cell (RBC) and reticulocyte indices, plasma iron parameters and TBI content

The complete blood count test was detected using the ProCyte Dx Hematology Analyzer (IDEXX Laboratories, Westbrook, ME, USA). According to the nutrient requirements for swine outlined by the National Research Council of the United States, a hemoglobin level of ≥10 g dL^{-1} is considered adequate, while 8 g dL^{-1} indicates borderline anemia, and ≤7 g dL^{-1} signifies anemia.[23]

Plasma iron concentration (#1-420-0200, Biomaxima, Lublin, Poland) and total iron binding capacity (TIBC; #1-421-0060, Biomaxima, Lublin, Poland) were determined using colorimetric methods based on the formation of a chromazurol B-cetrimide–iron complex, with absorbance recorded at 630 nm. Percent of transferrin saturation (TSAT) was then calculated according to the following formula: TSAT = (plasma iron/TIBC) × 100. The plasma ferritin concentration was assessed by a porcine ELISA kit (#EP0255, Fine Biotech, Wuhan, China). The TBI content was calculated indirectly using hemoglobin concentration, plasma ferritin level and body weight, as previously described:[24] TBI = (0.2776 × hemoglobin (g L^{-1}) × body weight (kg)) + [($\log_{10}$ plasma ferritin (μg L^{-1}) − 1.345)/0.0439 × body weight (kg)].

2.4. Tissue non-heme iron content

Non-heme iron content in the liver and spleen was measured 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 562 nm (Beckman DU-68, Beckman Coulter, Brea, CA, USA).[25]

2.5. Tissue iron staining

Following fixation, hepatic and splenic segments were dehydrated, embedded in paraffin, and cut into 5 μm sections using a Hyrax M25 rotary microtome (Zeiss, Oberkochen, Germany). The sections were then mounted on glass slides, deparaffinized, stained with Perls' Prussian blue for 30 min, counterstained with nuclear red (#1001210500, Sigma-Aldrich, St Louis, MO, USA) for 5 min, and analyzed under a light microscope (Eclipse E200, Nikon, Tokyo, Japan).

2.6. Protein abundance analysis

As previously described,[12] crude membrane extracts from the liver and spleen were prepared to analyze ferroportin protein abundance, and cytosolic extracts were obtained to measure ferritin light chain (L-ferritin) and ferritin heavy chain (H-ferritin) protein levels. Depending on the molecular weight of the protein, 40 μg of membrane extracts were separated by electrophoresis on either 9% or 16% SDS-PAGE gels. Electroblotting of the resolved proteins onto a PVDF membrane (#88518, Thermos Scientific, Waltham, MA, USA) was performed, followed by blocking and incubating with primary and secondary antibodies (ESI Table 1†). For quantitative analysis of protein abundance, reactive bands were quantified relative to actin using the Image Lab image acquisition and analysis software (Bio-Rad, Hercules, CA, USA).

2.7. mRNA expression analysis

Isolation of total RNA (SV Total RNA Isolation System, #Z3105, Promega, Madison, WI, USA), cDNA synthesis (#K1632, RevertAid H Minus First Strand cDNA Synthesis Kit, Thermo Fisher Scientific, Waltham, MA, USA), and real-time quantitative PCR (#06924204001, SYBR Green I, Roche Diagnostics, Mannheim, Germany) were conducted following the manufacturers' instructions. Primer sequences are shown in ESI Table 2.† Housekeeping genes were selected using NormFinder software (MOMA, Aarhus, Denmark). The expression of the target gene *versus* the housekeeping gene (GAPDH or HPRT) was calculated by the formula $2^{-\Delta\Delta CT}$.[26]

2.8. Analysis of plasma hepcidin, erythropoietin, erythroferrone, growth differentiation factor 15 (GDF15), and interleukin 6 (IL6) levels

To quantify the plasma levels of these parameters, commercial ELISA kits (Hepcidin 25, #EIA-5782, DRG Instruments GmbH, Marburg, Germany; Erythropoietin, #E07E0002, BlueGene Biotech, Shanghai, China; Erythroferrone, #E07E0518, BlueGene Biotech; GDF15, #E07G0127, BlueGene Biotech; IL6, #E07I0006, BlueGene Biotech) were used following the manufacturer's instructions.

2.9. Tissue malondialdehyde content

Malondialdehyde content in the liver and spleen was measured using an ELISA kit (ELK9850, ELK Biotechnology, Denver, CO, USA) according to the manufacturer's instructions.

2.10. Statistical analysis

Data from full-term and preterm piglets (excluding the preterm piglets supplied with $FeSO_4$) were analyzed by two-way ANOVA, with delivery mode (full-term normal vaginal delivery and premature delivery by cesarean section), diet (with or without SI supplementation), and the interactions between them as sources of variables. If there was a significant interaction or an interaction trend, a *post hoc* test was conducted using Fisher's LSD, comparing: full-term piglets with and without SI supplementation; full-term and preterm piglets without iron supplementation; preterm piglets with and without SI supplementation; and full-term and preterm piglets with SI supplementation. To compare the effects of SI and $FeSO_4$ supplementation on preterm piglets, results among the

preterm piglets without iron, with SI, and with $FeSO_4$ were statistically analyzed using one-way ANOVA with Fisher's LSD. Statistical analysis was performed and figures were prepared using GraphPad Prism v.9.1.1 (GraphPad Software, San Diego, CA, USA). All data are presented as the mean ± SEM. A *P*-value of ≤ 0.05 was considered statistically significant, while *P*-values between 0.05 and 0.10 were considered as trending towards significance.

3. Results

3.1. Preterm piglets showed poor growth and low TBI

The daily weight line graph in Fig. 1 shows that full-term piglets weigh approximately twice as much as preterm piglets at all stages. Preterm piglets exhibit lower average daily gain and TBI compared to full-term piglets ($P < 0.01$). Although no significant difference was observed, SI supplementation appears to result in negative weight gain in preterm piglets.

3.2. Premature birth decreased levels of plasma biochemical iron parameters in piglets

The TSAT, a biomarker that takes into account both plasma iron and its main transport protein transferrin, is clinically used to detect the status of iron in the body.[27] The plasma iron level and TSAT were lower in preterm piglets compared to the full-term piglets ($P < 0.05$; Fig. 2). No significant change was observed between the iron-supplemented and non-supplemented groups.

3.3. Premature birth and oral SI altered the RBC and reticulocyte indices in piglets

Erythropoietic cells are the primary users of iron in the body. For this reason, iron homeostasis and erythropoiesis are tightly intertwined. A delivery mode × diet interaction was observed for RBC count, hemoglobin level, reticulocyte count and percentage, and reticulocyte hemoglobin equivalent (RET-He) level ($P < 0.05$; Fig. 3). Without iron supplementation, preterm piglets showed lower RBC count and RET-He level, and higher reticulocyte count and percentage than full-term piglets ($P < 0.05$). Dietary supplementation of SI increased RBC count, hemoglobin level and RET-He level, but decreased reticulocyte count and percentage in preterm piglets ($P < 0.05$). In contrast, this iron supplement showed an opposite effect for RBC count, and did not affect hemoglobin level, reticulocyte count and percentage or RET-He level in full-term piglets. With regard to the effects of SI on full-term *vs.* preterm piglets, higher RBC count and hemoglobin level were observed in the preterm piglets than in the full-term piglets ($P < 0.05$). No delivery mode × diet interaction was observed for mean corpuscular volume (MCV), mean cell hemoglobin (MCH), or mean corpuscular hemoglobin concentration (MCHC). Compared with full-term delivery, premature delivery by cesarean section led to higher MCV and MCH, and lower MCHC ($P < 0.05$).

3.4. Premature birth and oral SI increased hepatic and splenic iron deposits in piglets

Iron is stored within the liver, the spleen, and other organs of the reticuloendothelial system. Iron distribution and storage are typically assessed by quantifying the total iron content and by

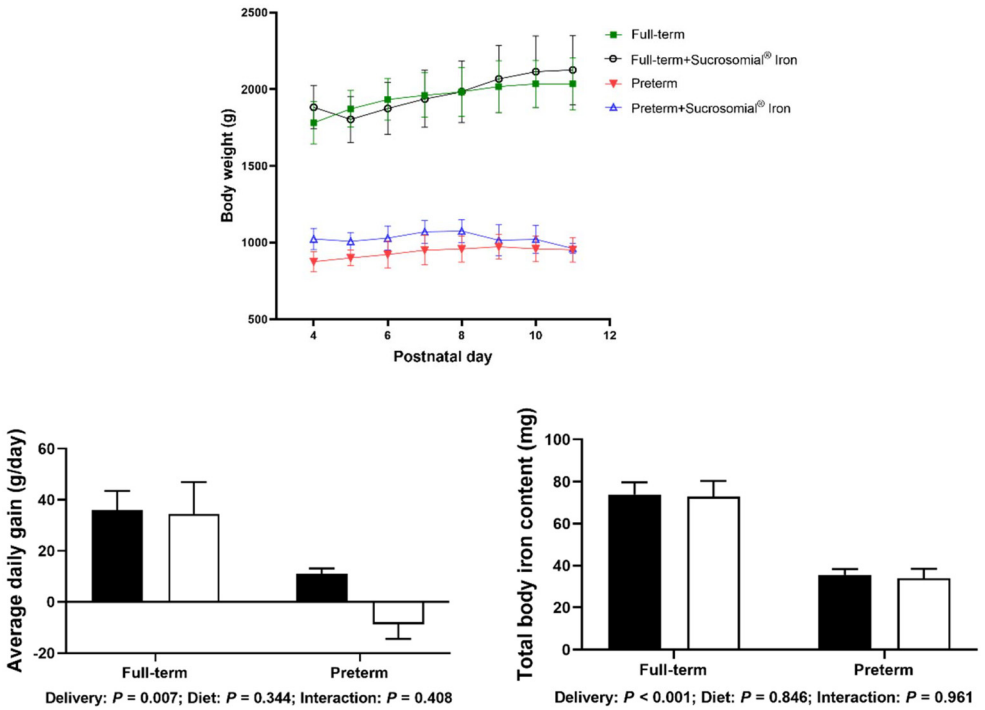

Fig. 1 Growth performance and total body iron content. Piglets without iron supplementation (■); piglets with Sucrosomial® Iron supplementation (□). Data are presented as the mean ± SEM ($n = 6$).

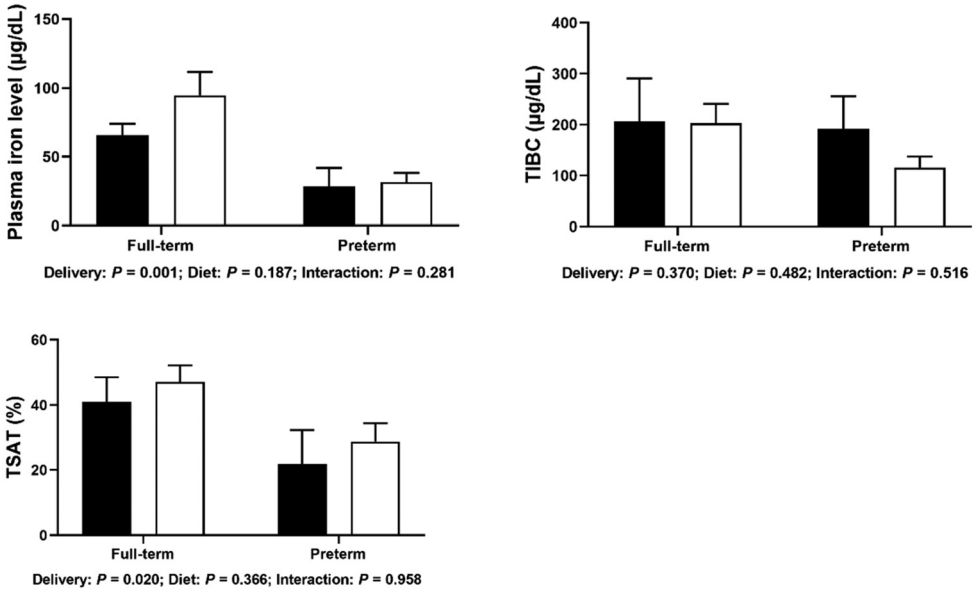

Fig. 2 Blood plasma biochemical iron parameters. Piglets without iron supplementation (■); piglets with Sucrosomial® Iron supplementation (□). Data are presented as the mean ± SEM (n = 6). TIBC, total iron binding capacity; TSAT, transferrin saturation.

measuring iron storage protein levels in the target tissues. In the liver, an interaction between delivery mode and diet was found for non-heme iron content (P = 0.01; Fig. 4A). Regardless of iron supplementation, a significant increase in hepatic non-heme iron content was shown in preterm piglets compared with full-term piglets (P < 0.05). In addition, SI supplementation enhanced hepatic non-heme iron content in preterm piglets (P < 0.05) but not in full-term ones. No delivery mode × diet interaction was observed for ferritin concentration. Plasma ferritin (P < 0.05) and hepatic L- (P = 0.076) and H-ferritin (P < 0.05) levels in preterm piglets were elevated relative to full-term piglets (Fig. 4B and C). SI tended to increase the protein levels of hepatic L- (P = 0.096) and H-ferritin (P = 0.085). Microscopic analysis of liver sections stained for non-heme iron with Perls' Prussian blue showed localized iron deposits in preterm piglets (Fig. 4D).

As in the liver, large iron deposits were found in the spleen of preterm piglets. A trend for delivery mode × diet interaction was discovered for splenic non-heme iron content (P = 0.083; Fig. 5A). In the preterm groups, a significant increase in splenic non-heme iron content was observed in the piglets supplemented with SI compared to those without iron supplementation (P < 0.05). When comparing the full-term and preterm piglets given SI, the preterm piglets had higher non-heme iron content than the full-term piglets (P < 0.05). No delivery mode × diet interaction was observed for ferritin protein level in the spleen, while the preterm piglets showed a high ferritin protein level (P < 0.05; Fig. 5B). Perls' Prussian blue staining also shows localized iron deposits in the preterm piglets (Fig. 5C).

3.5. Preterm piglets had high ferroportin protein levels in the liver and spleen

Ferroportin, a protein expressed on the surface of absorptive enterocytes, macrophages, and hepatocytes, represents the only known iron exporter that transfers iron to plasma apo-transferrin.[28] No delivery mode × diet interaction was observed for ferroportin protein levels in the liver and spleen. Piglets delivered by cesarean section had higher hepatic and splenic ferroportin protein levels than those delivered vaginally (P < 0.05; Fig. 6). SI tended to reduce the splenic ferroportin protein level (P = 0.054).

3.6. Delivery mode and oral SI affected hepcidin and its regulators

Hepcidin, a small peptide secreted by hepatocytes in response to high iron content, is a key regulator of system iron homeostasis.[29] A trend for the delivery mode × diet interaction was shown for hepatic hepcidin mRNA expression (P = 0.055) and plasma hepcidin-25 concentration (P = 0.089; Fig. 7A). In the preterm groups, both hepatic hepcidin mRNA expression and plasma hepcidin-25 concentration were higher in piglets supplemented with SI than in those without iron supplementation (P < 0.05). When given SI, preterm piglets had higher hepcidin levels both in the liver and blood than full-term piglets (P < 0.05).

Hepcidin expression can be suppressed by secreted erythroid factors.[30,31] There was an interaction observed for plasma erythroferrone concentration (P < 0.05), and an interaction trend for erythropoietin (P = 0.067; Fig. 7B). Without iron supplementation, preterm piglets showed higher erythroferrone (P < 0.05) and erythropoietin (P < 0.10) concentrations compared to full-term piglets. Among the preterm piglets, low erythroferrone and erythropoietin concentrations were found in the piglets given SI (P < 0.05). No delivery mode × diet interaction was found for plasma GDF15 concentration (Fig. 7B). Preterm piglets had lower plasma GDF15 concentration than full-term piglets (P < 0.05). In addition, the synthesis of hepcidin is induced by IL6.[32] Preterm piglets had low plasma IL6 concentration (P < 0.05), which were unaffected by oral SI (Fig. 7B).

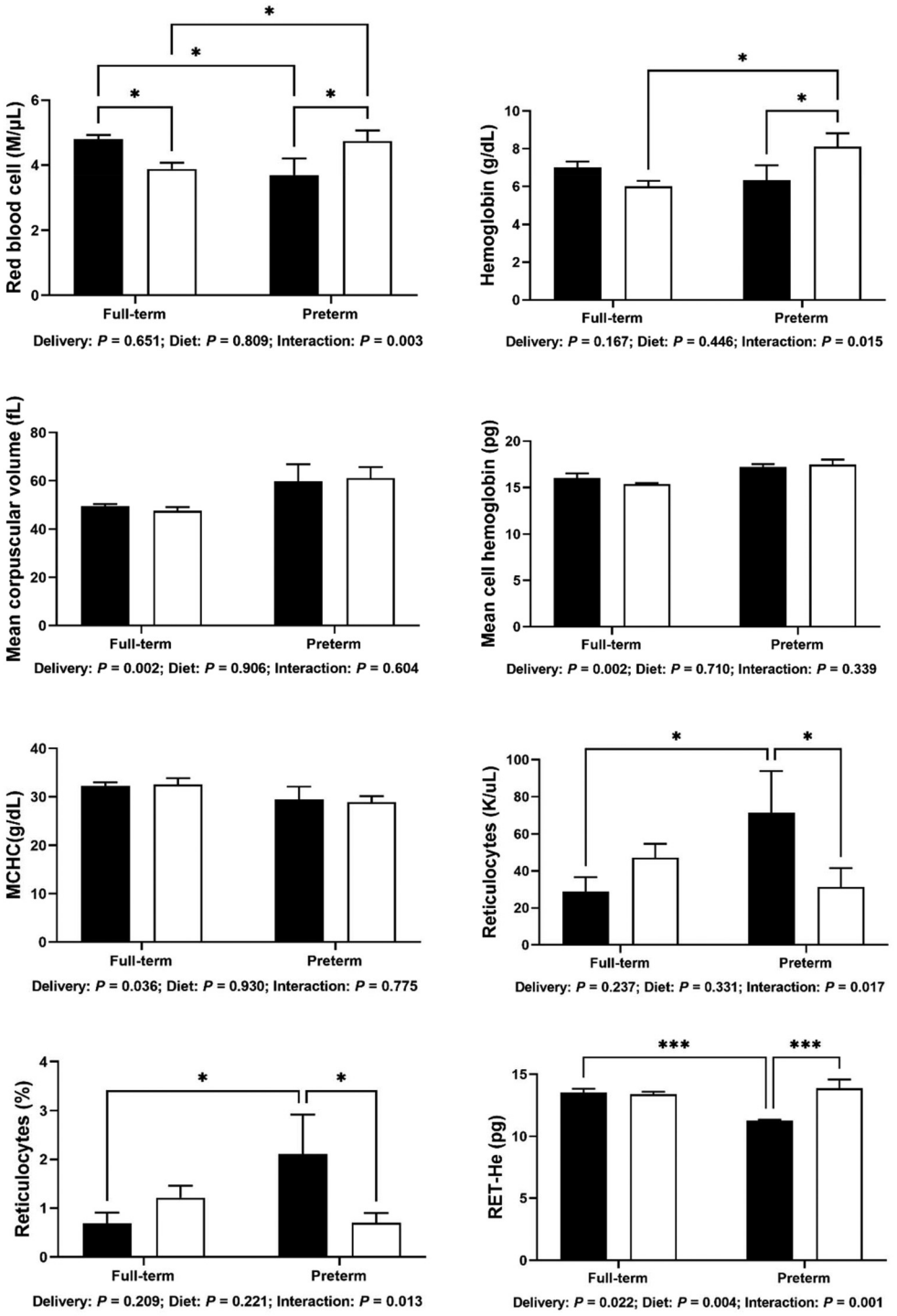

Fig. 3 Red blood cell and reticulocyte indices. Piglets without iron supplementation (■); piglets with Sucrosomial® Iron supplementation (□). Data are presented as the mean ± SEM (n = 6), * and *** asterisks denote statistically significant differences at $P < 0.05$ and $P < 0.001$, respectively. MCHC, mean corpuscular hemoglobin concentration; RET-He, reticulocyte hemoglobin equivalent.

3.7. Oral SI did not induce inflammatory and oxidative responses in the liver and spleen

No delivery mode × diet interaction was observed for the mRNA expression of toll-like receptor 4 (TLR4) and proinflammatory cytokines in the liver and spleen (Fig. 8A). Low mRNA expression of hepatic *TLR4*, *IL1β*, *IL6* and tumor necrosis factor α (TNFα), as well as splenic *IL1β* and *TNFα*, were observed in the preterm piglets ($P < 0.05$). SI decreased the mRNA expression of splenic *TNFα* ($P < 0.05$). Malondialdehyde

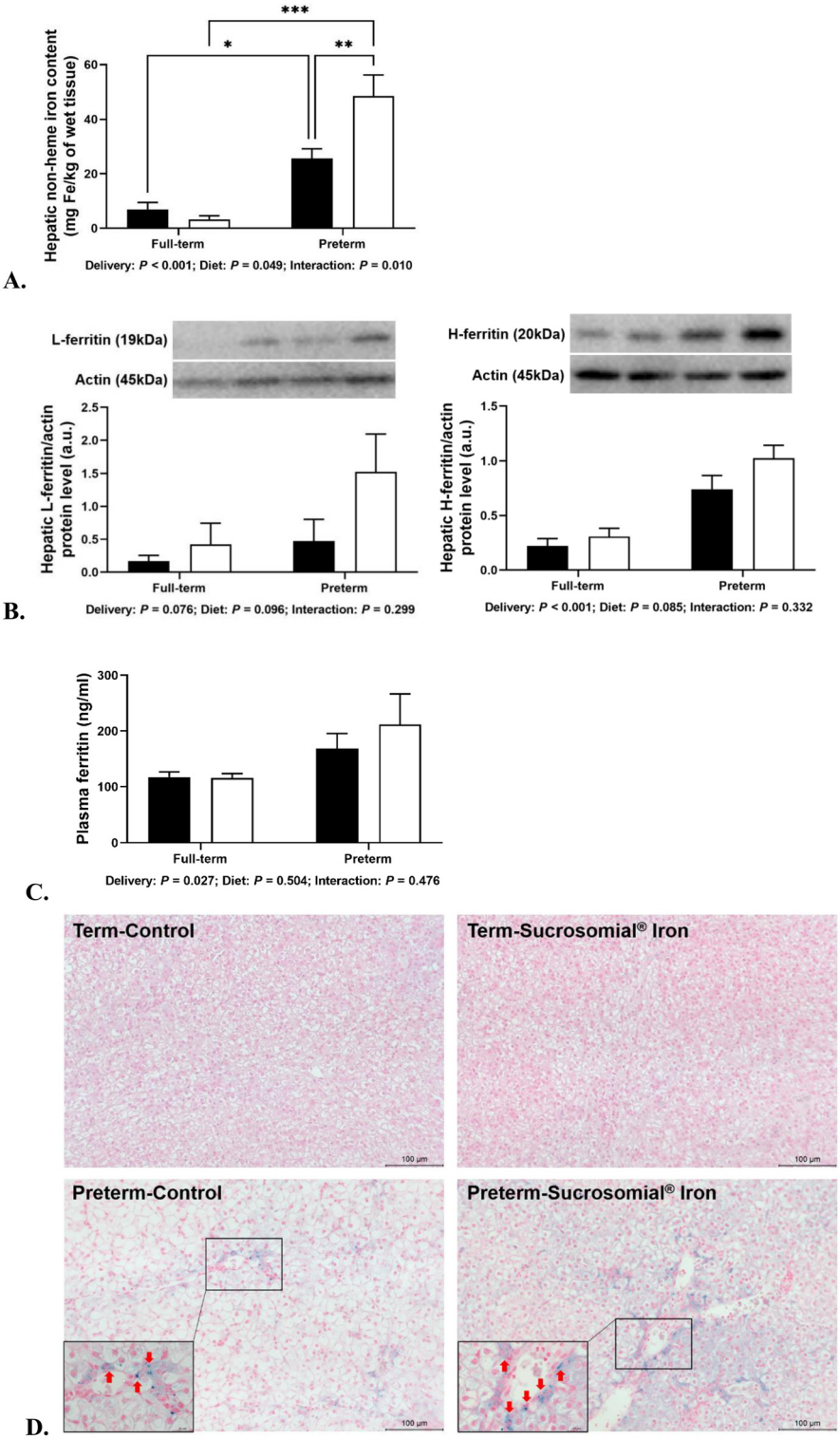

Fig. 4 Hepatic non-heme iron content (A) and ferritin protein level (B), plasma ferritin level (C), and hepatic histological examination of iron loading (D). Piglets without iron supplementation (■); piglets with Sucrosomial® Iron supplementation (□). Data are presented as the mean ± SEM (n = 6), *, ** and *** asterisks denote statistically significant differences at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively. B: Representative western blot images and relative densitometric bar graphs of hepatic ferritin. Actin was used as protein loading control. a.u., arbitrary units; H-ferritin, ferritin heavy chain; L-ferritin, ferritin light chain. D: Non-heme iron deposits were detected by staining with Perl's Prussian Blue (blue stain) (Original magnification: 200×. Scale bars = 100 μm). A high-magnification image of tissue showing iron deposits in preterm piglets is indicated by red arrowheads (inset in the bottom left-hand panel) (Original magnification: 1000×. Scale bars = 20 μm).

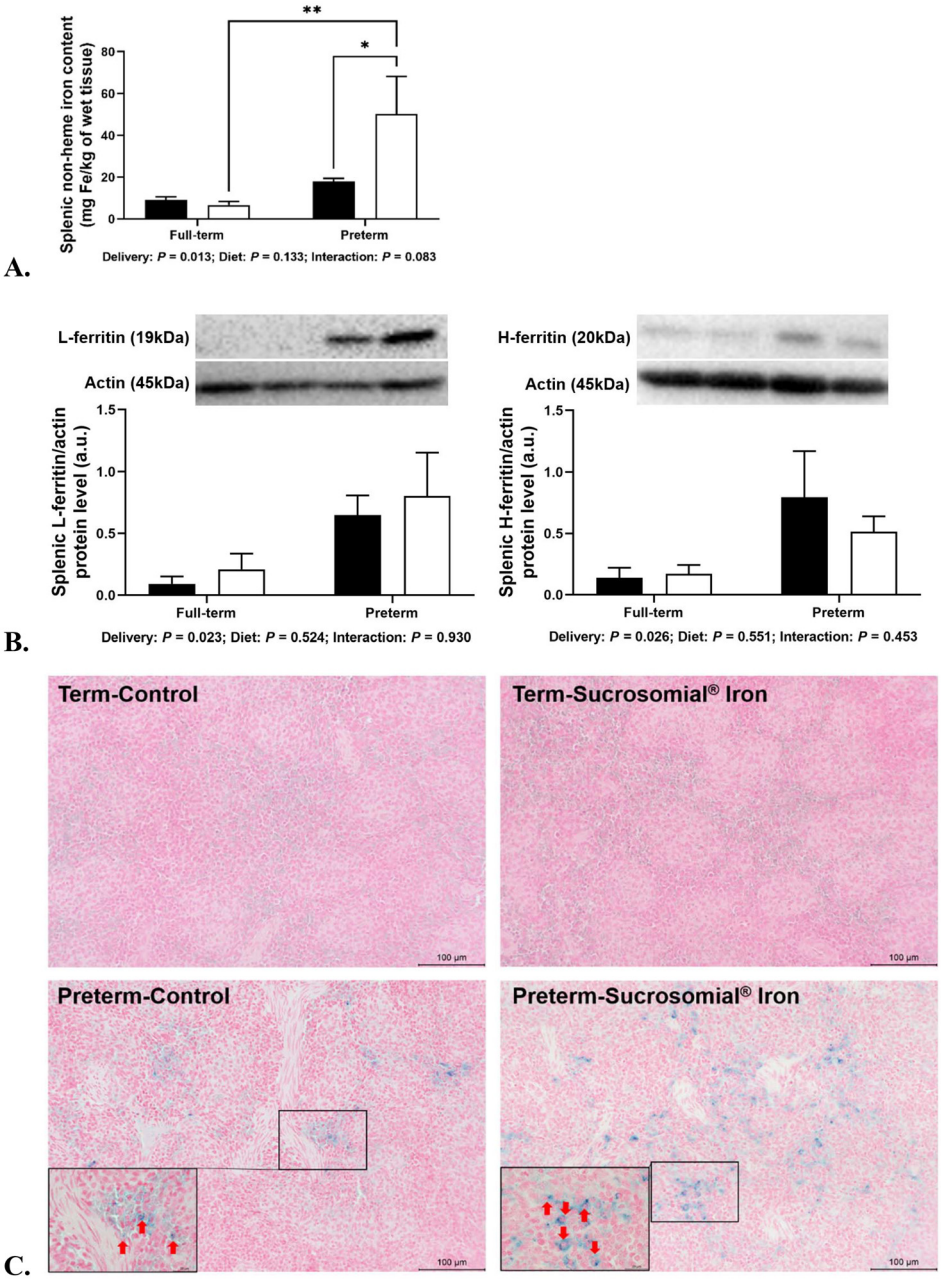

Fig. 5 Splenic non–heme iron content (A), ferritin protein level (B), and histological examination of iron loading (C). Piglets without iron supplementation (■); piglets with Sucrosomial® Iron supplementation (□). Data are presented as the mean ± SEM (n = 6), * and ** asterisks denote statistically significant differences at $P < 0.05$ and $P < 0.01$, respectively. B: Representative western blot images and relative densitometric bar graphs of splenic ferritin. Actin was used as protein loading control. a.u., arbitrary units; H-ferritin, ferritin heavy chain; L-ferritin, ferritin light chain. C: Non-heme iron deposits were detected by staining with Perl's Prussian Blue (blue stain) (Original magnification: 200×. Scale bars = 100 μm). A high-magnification image of tissue showing iron deposits in preterm piglets is indicated by red arrowheads (inset in the bottom left-hand panel) (Original magnification: 1000×. Scale bars = 20 μm).

content in the liver of both full-term and preterm groups was below the minimum detectable threshold. A trend for the delivery mode × diet interaction was shown for malondialdehyde content in the spleen (P = 0.088; Fig. 8B). Splenic malondialdehyde content was higher in preterm piglets than in full-term piglets, regardless of SI supplementation ($P < 0.05$).

3.8. Comparison of the effects of SI and $FeSO_4$ supplementation on iron status in preterm piglets

The plasma iron and RET-He levels were higher in preterm piglets supplemented with $FeSO_4$ than in those without any iron supplementation ($P \leq 0.05$; Table 1). Yet, aside from

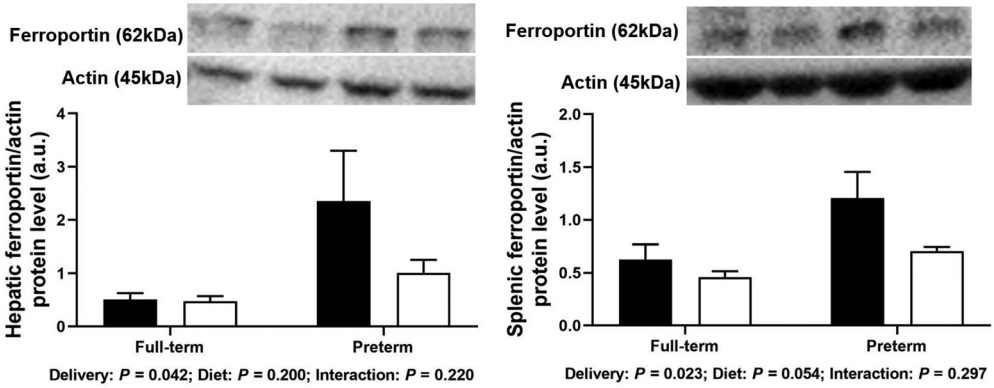

Fig. 6 Ferroportin protein levels in the liver and spleen. There are the representative western blot images and relative densitometric bar graphs of ferroportin. Piglets without iron supplementation (■); piglets with Sucrosomial® Iron supplementation (□). Data are presented as the mean ± SEM (n = 6). Actin was used as protein loading control. a.u., arbitrary units.

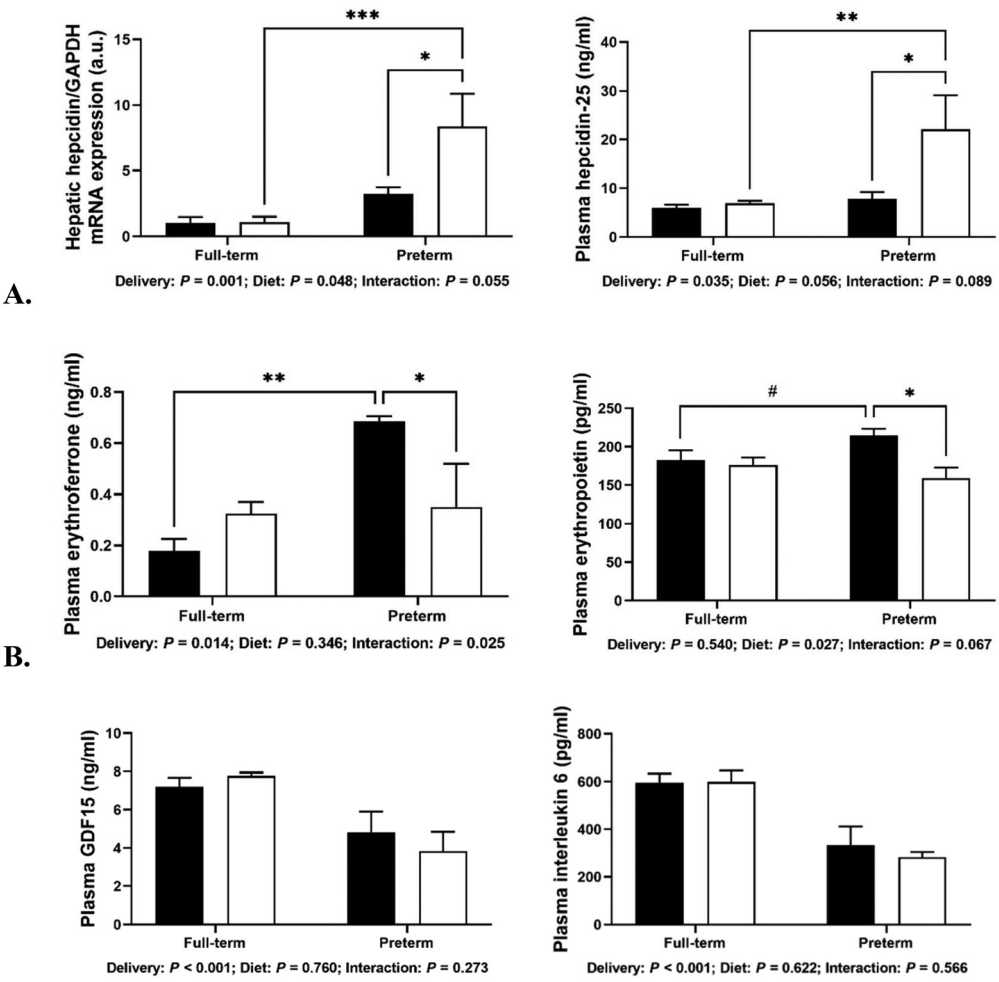

Fig. 7 Hepcidin level (A) and its regulators (B). Piglets without iron supplementation (■); piglets with Sucrosomial® Iron supplementation (□). Data are presented as the mean ± SEM (n = 6), #, *, ** and *** asterisks denote statistically significant differences at $0.05 < P < 0.10$, $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively. a.u., arbitrary units; GDF15, growth differentiation factor 15.

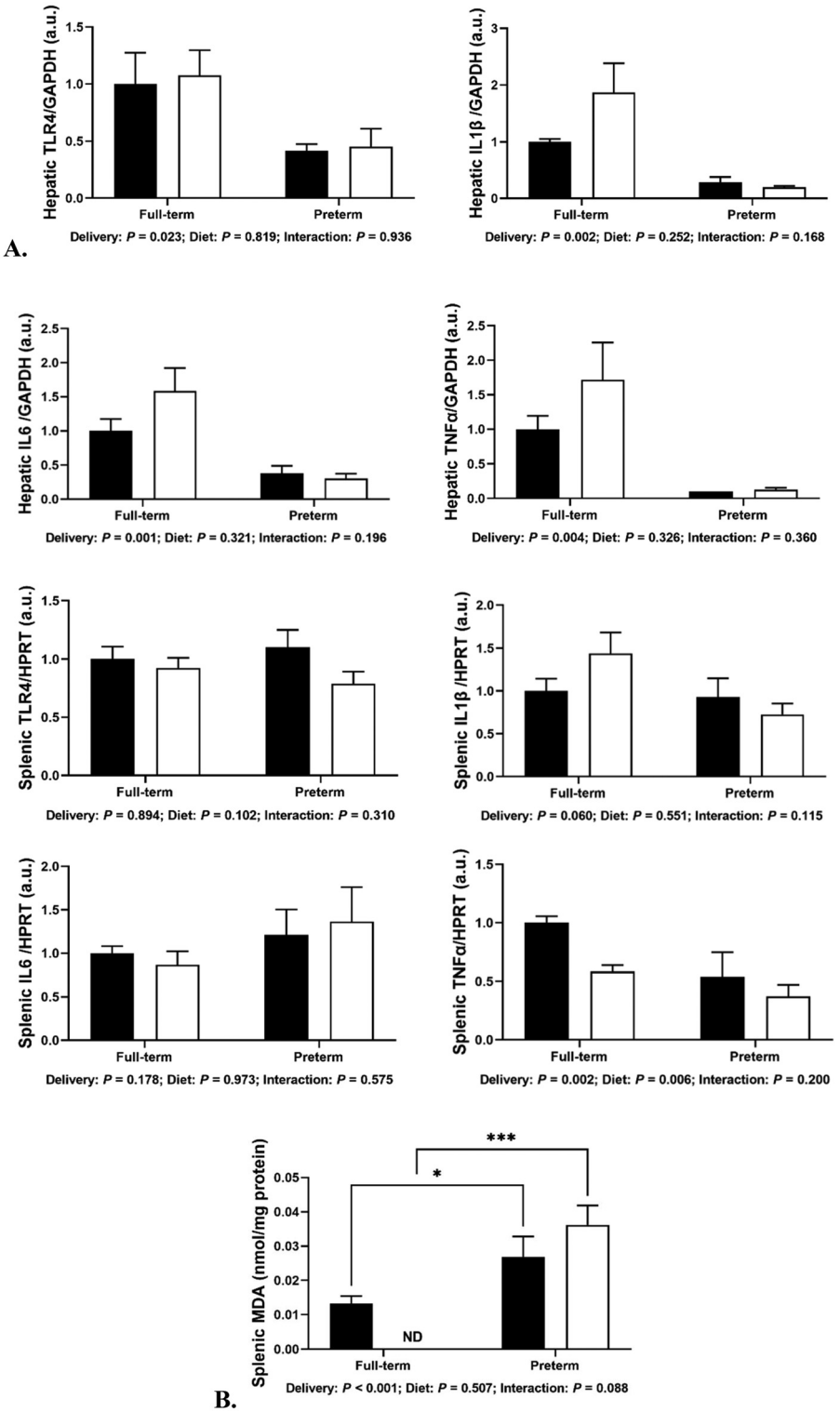

Fig. 8 Inflammatory (A) and oxidative (B) responses in the liver and spleen. B: Malondialdehyde (MDA) content in the liver of both full-term and preterm groups was below the minimum detectable threshold. Piglets without iron supplementation (■); piglets with Sucrosomial® Iron supplementation (□). Data are presented as the mean ± SEM (n = 6). * and *** asterisks denote statistically significant differences at $P < 0.05$ and $P < 0.001$, respectively. a.u., arbitrary units; IL, interleukin; ND, not detectable, below the minimum detectable threshold of MDA; TNFα, tumor necrosis factor α; TLR4, Toll-like receptor 4.

Table 1 Effects of iron supplementation in prematurely born piglets

Item	PC	PS	PF	*P*-Value		
				PC *vs.* PS	PC *vs.* PF	PS *vs.* PF
Growth performance						
Initial body weight (g)	875 ± 66	1023 ± 70	953 ± 39	0.121	0.352	0.403
Final body weight (g)	952 ± 80	961 ± 32	1060 ± 47	0.919	0.204	0.239
Average daily gain (g day^{-1})	11.0 ± 2.1	−8.9 ± 5.5	15.2 ± 4.9	0.023	0.533	0.007
Total body iron content (mg)	35.6 ± 2.7	33.9 ± 4.5	40.6 ± 1.1	0.735	0.322	0.167
Plasma biochemical iron parameters						
Plasma iron (μg dL^{-1})	28.6 ± 13.3	31.7 ± 6.6	55.4 ± 4.4	0.789	0.050	0.049
Total iron binding capacity (μg dL^{-1})	191 ± 64	116 ± 22	173 ± 39	0.206	0.756	0.292
Transferrin saturation (%)	21.8 ± 10.5	28.8 ± 5.6	36.2 ± 7.0	0.527	0.226	0.465
Red blood cell and reticulocyte count						
Red blood cell (M μL^{-1})	3.69 ± 0.51	4.74 ± 0.33	4.2 ± 0.32	0.108	0.373	0.344
Hemoglobin (g dL^{-1})	6.33 ± 0.79	8.10 ± 0.72	7.08 ± 0.48	0.108	0.435	0.290
Mean corpuscular volume (fL)	59.8 ± 7.1	61.1 ± 4.6	54.7 ± 1.4	0.846	0.455	0.318
Mean cell hemoglobin (pg)	17.2 ± 0.3	17.5 ± 0.5	16.9 ± 0.5	0.705	0.656	0.383
Mean corpuscular hemoglobin concentration (g dL^{-1})	29.4 ± 2.7	28.9 ± 1.3	30.9 ± 0.3	0.811	0.523	0.351
Reticulocyte (K μL^{-1})	71.6 ± 22.3	31.3 ± 10.2	56.1 ± 13.4	0.099	0.493	0.250
Reticulocyte (%)	2.11 ± 0.81	0.70 ± 0.20	1.29 ± 0.21	0.048	0.214	0.318
Reticulocyte hemoglobin equivalent (pg)	11.3 ± 0.1	13.9 ± 0.7	13.8 ± 0.5	0.011	0.008	0.955
Plasma ferritin level (ng ml^{-1})	169 ± 27	212 ± 55	149 ± 11	0.507	0.769	0.301
Tissue non–heme iron content (mg Fe per kg of wet tissue)						
Liver	25.7 ± 3.5	48.6 ± 7.6	36.7 ± 4.3	0.036	0.287	0.199
Spleen	18.0 ± 1.5	50.3 ± 17.9	22.2 ± 5.5	0.141	0.846	0.161

PC, preterm piglets without iron supplementation; PF, preterm piglets supplemented with ferrous sulfate; PS, preterm piglets supplemented with Sucrosomial® Iron.

average daily gain and plasma iron level, there were no significant differences for other plasma and tissue iron indicators between preterm piglets given $FeSO_4$ and SI.

4. Discussion

In humans, healthy, full-term newborns have adequate iron stores at birth and then remain iron-replete until 6 months of age; however, over 6 months of age, infants are at increased risk of developing iron deficiency if not given an iron supplement.[33,34] In contrast, premature infants have overall greater nutritional needs, including higher iron requirements, than healthy, full-term infants due to a shortage of iron stores caused by premature delivery.[34] Premature infants are at risk of exhausting their body iron stores much earlier than healthy, full-term infants. In this study, we used a porcine model of prematurity by cesarean section, as it mimics human late preterm birth. We found that preterm piglets showed poor growth and low TBI content. Similarly, in humans, weight loss and a decrease in total iron storage are observed, depending on the degree of prematurity.[35–37] In addition, results from biochemically analyzing iron in the blood showed that plasma iron levels and TSAT were reduced in preterm piglets relative to full-term piglets, consistent with results from studies on human premature infants.[38–40] This phenomenon of decreased plasma iron levels and TSAT is commonly observed in iron deficiency.[17] These findings indicate that premature neonates have smaller iron stores than neonates born at term.

In this experiment, oral SI had minimal effects on the average daily gain of full-term piglets, while preterm piglets exhibited negative growth, though without statistical significance. This suggests that preterm piglets, due to their incomplete development, may be more sensitive to iron supplementation. Although iron supplementation effectively improves iron status and prevents iron deficiency anemia, it may also inhibit neonatal weight gain and negatively affect growth and development.[41,42] The mechanisms underlying this growth-limiting effect remain unclear but may involve gastrointestinal distress, competition with essential minerals (*e.g.*, zinc and copper), pro-oxidative stress, and alterations in gut microbiota.[43–45] However, it is worth noting that the effects of iron supplementation on growth remain inconsistent across studies, with some reporting only limited benefits or no effect at all.[41,42] Further research is needed to investigate the effects of SI on growth performance, particularly in premature infants, and its underlying mechanisms.

Given the close connection between iron and erythropoiesis, a complete blood count test was used to diagnose iron deficiency and iron deficiency anemia in full-term and preterm piglets. In our study, hemoglobin levels in non-iron-supplemented preterm piglets were <7 g dL^{-1}. According to the National Research Council's standard for iron deficiency anemia in pigs,[23] a hemoglobin level of ≤7 g dL^{-1} indicates anemia. Our result also showed that premature birth led to a decrease in RBC count and MCHC, and an increase in MCV and MCH values relative to full-term delivery, which is consistent with the findings of human studies during early life.[46–48] High MCV is characteristic of fetal erythrocytes and decreases

with gestational age.[40,46,49] A survey of the impact of delivery mode on hematological indices in premature infants reported that infants born by normal vaginal delivery generally had higher RBC count and hemoglobin level than those born by cesarean section, possibly due to a greater amount of placental blood transfused to vaginally delivered infants.[50] We also found high reticulocyte count and percentage and low Ret-He level in prematurely delivered piglets. The reticulocyte count serves as an indicator of the rate of effective erythropoiesis in the bone marrow.[48,49] It has been previously reported that erythropoietic activity in premature infants is faster than in full-term infants.[48] Ret-He has been used to assess iron sufficiency because it reflects the amount of functional iron available for erythropoiesis during the preceding 3–4 days.[51] According to a retrospective study by Sriranjan *et al.*,[51] low Ret-He level and high reticulocyte count may indicate effective erythropoiesis, but not enough iron available for normal hemoglobinization. Taken together, these data suggest that preterm piglets delivered by cesarean section might be more susceptible to iron deficiency anemia than full-term piglets delivered vaginally.

Prematurely born infants require a high supply of iron to meet their needs for robust erythropoiesis and rapid catch-up postnatal growth. The postnatal drop in hemoglobin level in full-term neonates is well tolerated; this decline is faster in premature neonates.[52,53] Note that iron delivery for RBC production is prioritized at the expense of iron transport to the brain in severe neonatal iron deficiency.[54] In this study, SI increased RBC count, hemoglobin level and Ret-He level, and it decreased reticulocyte count and percentage in preterm piglets but not in full-term piglets. Similar results were reported in a recent study showing that daily administration of SI upregulates hemoglobin levels in iron-deficient mice but has no effect on healthy, non-anemic mice.[55] Our previous study also found that supplementation with SI is inefficient for the sows and their offspring when pregnant sows are iron-replete or show slightly decreased iron status.[18] It seems therefore that SI is an efficient iron supplement only in iron-deficient subjects.

We undertook a comprehensive analysis of iron status in the iron storage organs, *i.e.*, the liver and spleen. In this study, non-heme iron content in the liver and spleen of preterm piglets was higher than that in full-term animals; the results of Perls' staining confirmed this finding, showing strong iron deposits localized in preterm piglets' tissues. In general, intracellular ferritin is important for physiological iron accumulation,[56] and the amount of circulating ferritin reflects the size of iron stores in the liver.[57] In preterm piglets, we found increased levels of tissue L- and H-ferritin protein and plasma ferritin—an indirect measurement of iron content—that again confirmed the results of our evaluation of non-heme iron content. This phenomenon is observed not only in 11-day-old preterm piglets but also in newborn preterm piglets, as reported in our previous research.[58] Similar to our findings, Georgieff *et al.*[59] found that premature infants have higher non-heme iron content in the liver compared to full-term infants. In another study, the non-heme iron content in the liver and spleen of fetuses whose gestational ages ranged from 33 to 36 weeks were found to be slightly higher than those of fetuses at ≥37 weeks of gestation, though this difference was not statistically significant.[35] Cases of higher serum ferritin concentrations in premature infants compared to full-term infants have also been reported by others.[60,61] However, it is worth noting that values for these parameters vary greatly among individuals. Some human studies have found that both the concentration of circulating ferritin[62,63] and iron content in the liver[35,64] and spleen[35] increase with increasing gestational age. It seems that preterm piglets delivered by cesarean section show a pattern of iron metabolism characteristic of functional iron deficiency, as well as iron accumulation in the tissue. In our case, considering that full-term piglets grow much faster than preterm ones, we speculate that intensive mobilization of iron from hepatic and splenic stores occurs in full-term piglets to support their rapid growth, which is associated with blood volume expansion and increased RBC production.

Our finding that SI induced iron accumulation only in anemic preterm piglets, again demonstrates that SI has different effects on full-term and preterm animals. Similarly, Asperti *et al.*[55] observed that daily administration of SI for 2 weeks (and even extended to 4 weeks) did not induce iron accumulation in healthy mice, but increased body iron in anemic mice. Asperti *et al.*[65] also reported that supplementation with SI enhanced iron content in the spleen, duodenum and brain in a mouse model of iron–refractory iron deficiency anemia. This effect of SI on increasing iron content in tissues was also observed in iron-deficient C57BL/6 mice.[66] Remarkably, the efficacy of SI has been shown not only in animal models but also in some patients with clinical iron deficiency conditions. For example, in anemic patients with celiac disease, inflammatory bowel disease, or chronic kidney disease, long-term oral administration of SI can elevate serum ferritin concentrations.[67–69]

To explore the molecular mechanism(s) by which SI improves the iron status of preterm piglets, we examined the role of the hepcidin–ferroportin axis. We found that hepatic and splenic ferroportin protein levels were increased in preterm piglets. Under conditions of iron deficiency, the high ferroportin expression favors iron export from the tissue into the plasma to provide iron mainly for erythropoiesis. In addition, high hepatic and splenic ferroportin expression may alleviate the burden of too much iron in these tissues. In preterm piglets given SI, ferroportin expression was not as high as in the non-supplemented preterm piglets, probably because the SI had already provided iron to the body, and thus reduced the need to export iron from iron storage tissues. However, these phenomena were not found in full-term piglets, and ferroportin remained at the same level regardless of iron supplementation.

At the systemic level, ferroportin activity is negatively regulated by hepcidin.[28] Hepcidin binds to ferroportin to induce its degradation and internalization, thus restricting iron release from exporting cells.[28] We found that the basal levels

of hepcidin were different in preterm *vs.* full-term piglets. In the full-term piglets, low levels of hepcidin and ferroportin (relative to the preterm piglets) were sufficient to maintain body iron levels. In contrast, the preterm piglets showed higher hepcidin levels, suggesting that there are differences in iron regulation mechanisms between preterm and full-term piglets. In preterm piglets, SI supplementation induced higher levels of hepcidin, which was consistent with the reduced ferroportin expression. In humans, studies have shown that iron supplementation acutely elevates circulating hepcidin within 24 hours, with levels returning to baseline upon cessation.[70,71] Sustained hepcidin elevation has been observed in adults and low-birth-weight infants receiving continuous daily iron supplementation.[72,73] In our study, hepcidin elevation in the SI-supplemented preterm group may be attributed to the acute effects of continuous daily iron supplementation and the higher baseline liver iron stores in preterm piglets compared to full-term piglets.

The synthesis of hepcidin in hepatocytes is influenced by various factors, such as iron status, erythropoiesis, and inflammation. Increased intracellular iron in the liver promotes hepcidin transcription,[29] which is probably the main reason for the high hepcidin levels observed in preterm piglets. During erythropoiesis, the increased erythropoietin level induces secretion of the erythroblast-produced erythroid factor erythroferrone, which is a main inhibitor of hepcidin synthesis in the liver.[31] Soluble mediator GDF-15 produced by erythroid progenitors has been shown to have the same effect.[30] In our study, preterm piglets had lower plasma GDF15 than full-term ones. Lower plasma erythroferrone and erythropoietin concentrations were found in preterm piglets given SI relative to non-supplemented animals, consistent with high levels of hepcidin. Moreover, inflammatory cytokines, in particular IL6, can upregulate hepcidin by switching on latent transcription factor-signal transducer and activator of transcription 3.[32] Apparently, this was not the case in our study, as the IL6 levels were reduced in preterm piglets. In summary, SI can reduce the negative effects of low systemic iron levels caused by premature birth by increasing the body's iron levels and regulating the hepcidin–ferroportin axis.

Finally, we investigated whether SI induces inflammatory and oxidative responses, and compared the effectiveness of SI and $FeSO_4$ in improving the iron status of preterm piglets. First, SI did not induce an inflammatory response, and mRNA expression of tissue TLR4 and inflammatory cytokines was low in preterm piglets, regardless of SI supplementation. This expression pattern of inflammatory factors in preterm piglets is consistent with the human findings of Dembinski *et al.*[74] The underlying cause may be that the innate and adaptive immune systems of premature infants are underdeveloped, with limited immune cell function, leading to a weakened inflammatory response.[74–78] This may result in defects in TLRs, important sensors of the innate immune system, and insufficient production of certain proinflammatory cytokines, preventing premature infants from mounting an inflammatory response comparable to that of full-term infants and adults.[74–78] Therefore, decreased TLR4 expression and reduced cytokine secretion may indicate the immune vulnerability of premature infants. Second, we analyzed tissue malondialdehyde content. Malondialdehyde, a product of lipid peroxidation, is used widely as a marker of oxidative stress and tissue injury.[79,80] Iron overload was significantly correlated with oxidative stress markers.[79] In an experiment on iron overload, malondialdehyde content increased nearly sevenfold in the liver and spleen.[80] In our study, although oral SI increased iron accumulation in the liver and spleen, it did not elevate malondialdehyde content in these tissues. At last, we found that SI is equally as effective as the classic iron supplement $FeSO_4$ in overcoming iron deficiency in the preterm piglets. Thus, SI could be considered a viable alternative to iron salts for individuals who are unable to tolerate them or when iron salts prove ineffective.

Our study is the first to use a preterm piglet model based on the Polish Landrace breed to investigate iron metabolism and the effects of iron supplementation in premature infants. We acknowledge certain limitations: (1) This study did not include data on SI absorption in the intestines of preterm piglets. Currently, in a separate experiment, we are investigating intestinal iron absorption mechanisms and microbial community composition in preterm piglets, and the effects of SI on the intestine. (2) We did not examine SI's effects on other iron-consuming organs, such as the brain. To our knowledge, research on SI's impact on the brain remains limited, with only two studies reporting that SI improved motor and cognitive performance and alleviated severe depression and anxiety symptoms.[69,81] Given that neonatal iron deficiency can lead to long-term neurodevelopmental impairments—such as deficits in attention, memory, visual and auditory function, and alterations in social-emotional behavior[82–87]—exploring SI's effects on these aspects could provide valuable insights into its safety for brain development. (3) The impact of long-term SI supplementation was not investigated. In this study, short-term SI supplementation (1 week) led to increased hepcidin level and tissue iron deposition. However, no immediate adverse effects, such as inflammation or oxidative stress, were observed. Whether long-term SI supplementation could result in severe iron accumulation and adverse effects requires further investigation.

In conclusion, this experiment confirmed the poor iron status in premature infants and the differences in systemic iron regulation mechanisms between preterm and full-term infants. Oral SI may prove to be a valuable iron supplementation option in premature infants. Nevertheless, before SI can be used clinically to treat iron deficiency in premature infants, appropriately scaled trials are needed to confirm its safety.

Author contributions

X.W: data curation, formal analysis, investigation, methodology, validation, visualization, writing – original draft, writing – review and editing. P.L.: investigation, writing – review and

editing. M.O.: investigation, methodology, validation. R.M.: investigation, methodology, validation. J.W.: investigation, methodology, resources. D.S.: investigation. K.Z.: investigation. Z.K.: investigation. B.Ż.: investigation. G.T.: resources. E.B.: resources. R.R.S.: conceptualization, funding acquisition, investigation, methodology, project administration, supervision, writing – review and editing.

Abbreviations

$FeSO_4$	Ferrous sulfate
GDF15	Growth differentiation factor 15
H-ferritin	Ferritin heavy chain
IL	Interleukin
L-ferritin	Ferritin light chain
MCH	Mean cell hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean corpuscular volume
RBC	Red blood cell
RET-He	Reticulocyte hemoglobin equivalent
SI	Sucrosomial® Iron
TBI	Total body iron
TIBC	Total iron binding capacity
TLR4	Toll-like receptor 4
TNFα	Tumor necrosis factor α
TSAT	Transferrin saturation

Data availability

The data supporting this article have been included in the article and its ESI.†

Conflicts of interest

There are no conflicts to declare.

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