doi:10.2533/chimia.2022.996 Chimia 76 (2022) 996–1004

New Insights into the Physiology of Iron Transport: An Interdisciplinary Approach

Jonas Zaugg[ab#], Jonai Pujol Giménez[ab#], Romina Silvia Cabra[ad#], Wilhelm Hofstetter*[ad], Matthias A. Hediger*[ac], and Christiane Albrecht*[ab]

Abstract: The TransCure project entitled 'Iron Transporters DMT1 and FPN1' took an interdisciplinary approach combining structural biology, chemistry and physiology to gain new insights into iron transport. Proteins studied included Divalent Metal Transporter 1 (DMT1, SLC11A2), enabling the import of Fe^{2+} into the cytoplasm, and the iron efflux transporter Ferroportin (FPN1, SLC40A1). The physiology and pathophysiology, and the mechanisms underlying iron transport in the gut, across the placenta and in bone were investigated. Small molecule high-throughput screening was used to identify improved modulators of DMT1. The characterization of DMT1 inhibitors have provided first detailed insights into the pharmacology of a human iron transport protein. In placental physiology, the identification of the expressional and functional alterations and underlying mechanisms in trophoblast cells clarified the association between placental iron transport by DMT1/FPN1 and gestational diabetes mellitus. In bone, iron metabolism was found to differ between cells of the monocyte/ macrophage lineages, including osteoclasts. Osteoclast development and activity depended on exogenous iron, the expression of high levels of the transferrin receptor (TFR) and low levels of FPN1 suggesting the expression of an 'iron storage' phenotype by these cells. The principles and main findings of the TransCure studies on transmembrane iron transport physiology are summarized in this review.

Keywords: Bone · DMT1 · FPN · Iron homeostasis · Placenta

Christiane Albrecht: MSc in Pharmacy at Ludwig-Maximilians-Universität, Munich, Germany. PhD in Clinical Pharmacology (supervisor Prof. Jürg Reichen) at the Department of Clinical Pharmacology, University of Bern. Postdoc at the University of Zurich (1 year) and in the Membrane Transport Biology Group at the MRC Clinical Sciences Centre, London, UK (4 years) with Prof. Christopher Higgins. Then, Principal Investigator (PI) at the Chair of Physiology, Technische Universität München, Campus Weihenstephan (3 years). Since 2006, Research Group Leader and Principal Investigator at the Institute of Biochemistry and Molecular Medicine (IBMM), University of Bern. Since 2010 Associate Professor at the University of Bern.

Romina Cabra: Currently working as a Medical Science Liaison for the neurology portfolio in an international biopharmaceutical company. She obtained a bachelor's and a master's degree in Biomedical Sciences from the University of Fribourg and the University of Bern, respectively. She has concluded her studies with a PhD in Biomedical Sciences in the Bone Biology & Orthopaedic Research group supervised by Prof. Dr. Willy Hofstetter. During her PhD she has studied and analyzed the role of iron in osteoclast and osteoblast lineage cells.

Matthias A. Hediger: Dr. sc. nat. (Biochemistry), ETH Zurich. Postdoc at the University of Connecticut, CT, USA. Postdoc at UCLA School of Medicine, Los Angeles, CA, USA. Assistant Professor, Harvard Medical School, Boston, MA, USA. Associate Professor, Harvard Medical School, Boston, MA, USA. Professor Ordinarius, Institute of Biochemistry and Molecular Medicine (IBMM), University of Bern. Director of the IBMM, University of Bern (2005–2017). Director of NCCR TransCure, University of Bern (2010–2014). Since 2019, Group Leader and Head of the Membrane Transport Discovery Lab, Department of Nephrology and Hypertension, Inselspital Bern & Department for BioMedical Research, University of Bern.

Willy Hofstetter: MSc in Biochemistry at ETH Zurich; PhD in Biochemistry (supervisor Prof. N. Herschkowitz) at the Children's Hospital, Inselspital, Bern. Postdoc at the University of Georgia, US, with Prof. D. Dervartanian. Then, he joined the Institute of Pathophysiology, University of Bern (Prof. H. Fleisch). Since 1997, he is the head of the Research Group for Bone Biology & Orthopaedic Research in the Department for BioMedical Research (DBMR). Since 2021, Research Group Leader in the Bone & Joint Program of the DBMR.

**Correspondence:* Prof. Dr. C. Albrecht[ab], E-Mail: christiane.albrecht@ibmm.unibe.ch; Prof. Dr. M. Hediger[ac], E-Mail: matthias.hediger@ibmm.unibe.ch; Prof. Dr. W. Hofstetter[ad], E-Mail: willy.hofstetter@dbmr.unibe.ch
[a]Swiss National Centre of Competence in Research (NCCR) TransCure, University of Bern, Switzerland; [b]Institute of Biochemistry and Molecular Medicine, Faculty of Medicine, University of Bern, Switzerland; [c]Membrane Transport Discovery Lab, Department of Nephrology and Hypertension and Department of Biomedical Research (DBMR), Inselspital, University of Bern; [d]Bone & Joint Program, Department for BioMedical Research (DBMR), University of Bern
[#]These authors share first authorship

Jonai Pujol-Giménez: Studied Biology and Biochemistry at the University of Navarra, Spain. He also obtained his Master's and PhD degrees there, both in the Department of Nutrition, Food Science and Physiology, under the supervision of Prof. MP Lostao. He then moved to the laboratory of Prof. Matthias Hediger at the University of Bern, Switzerland, where he is currently a senior member of the group. He conducts interdisciplinary research in the field of solute carriers (SLCs), developed novel functional transport assays, performed innovative studies on the structure and function of metal ion transporters, and developed pharmacological modulators.

Jonas Zaugg: Studied Molecular Life Sciences (MLS) in Bioanalytics, University of Applied Sciences (FHNW) and obtained his Master's degree in Biology, Molecular and Cellular Biology, Faculty of Science of the University of Zurich (UZH). He graduated at the Institute of Biochemistry and Molecular Medicine, Faculty of Medicine, University of Bern and continued as Early Postdoc in the research group of Prof. Christiane Albrecht. He investigated the function of placental transporters in the context of human gestational diseases. Member of the NCCR TransCure student and fellow committee; involved in TransCure projects SLC7 family amino acid transporters and iron transporters DMT1 and FPN.

1. Iron Homeostasis in Health and Disease

Human iron homeostasis is controlled through a tightly regulated process involving a number of cellular transport proteins and enzymes (Fig. 1A). Recent molecular and genetic studies have sparked a breakthrough in iron biology and understanding of iron-related diseases.[1–10]

Iron can donate or accept electrons in redox reactions, which makes it an important player in fundamental cellular processes such as DNA synthesis, nucleic acid repair, mitochondrial respiration, cell growth and death.[11] Moreover, iron is incorporated into heme, the main component of hemoglobin, which mediates oxygen transport and supply by erythrocytes. Thus, iron is an essential elemental for life, but it can also be toxic when it reaches elevated levels, allowing it to react with hydrogen peroxide and thereby generate reactive oxygen species (ROS) *via* the Fenton reaction.[10] ROS in turn can cause oxidative damage by lipid peroxidation leading to cell death and tissue damage. Therefore, due to its dual beneficial and deleterious properties, iron homeostasis needs to be firmly controlled, which is achieved through tightly regulated membrane transport processes. In the human body, most of the iron is part of the erythrocyte hemoglobin, while the rest is mainly stored in macrophages and hepatocytes. Much of the body's iron stores are required to fuel erythropoiesis in order to ensure sufficient red blood cell production to transport oxygen to all tissues.[12] The iron requirements are primarily met by recycling senescent red blood cells by macrophages, while the primary external iron source is absorption from the diet, which is mediated by the enterocytes of the small intestine. The main source of iron for humans is the absorption from the digested food, where it can be found as ferric iron (Fe^{3+}) or organic iron, known as heme (Fe-protoporphyrin IX).

Inorganic iron can be obtained from animal [Fe^{3+} bound to ferritin (FT)] and vegetable (*e.g.* cereals and legumes) sources. After digestion in the stomach, inorganic iron reaches the duodenum as free Fe^{3+}, where it is taken up by the enterocytes (Fig. 1B). In a first step, Fe^{3+} is reduced to ferrous iron (Fe^{2+}) by the brush border cytochrome B (DCYTB) ferrireductase. This is

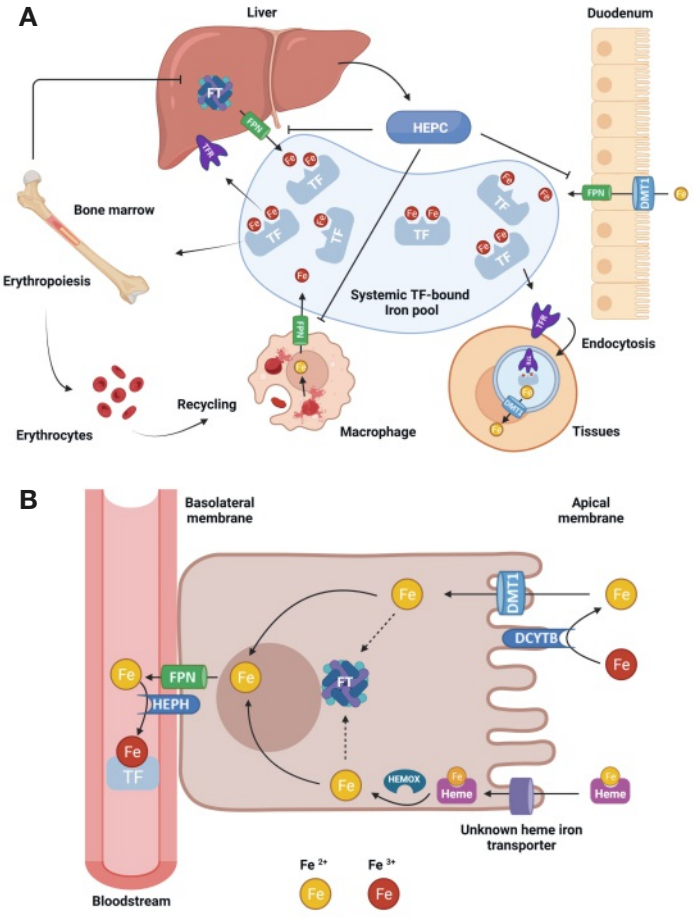

Fig. 1. Systemic iron metabolism. A. Iron metabolism is tightly regulated by membrane transport mechanisms and enzymes to meet the metabolic needs and minimize potential iron toxicity. The figure shows the major transport pathways of iron, the distribution of the transporters involved, regulation by hepcidin and iron recycling pathways (see text for details). B. Mechanisms of intestinal iron absorption. There are two main pathways of dietary iron absorption: one for inorganic iron *via* DMT1 and one for heme iron *via* an unknown mechanism. Intracellular heme degradation and the release of iron into the bloodstream are also illustrated. *Abbreviations*: TF, transferrin; TFR, transferrin receptor; DMT1, divalent metal transporter 1; FPN, ferroportin; FT, ferritin; HEPH, hephaestin; HEPC, hepcidin; HEMOX, heme oxigenase; DCYTB, duodenal cytochrome B. Created with BioRender.com.

followed by absorption *via* the divalent metal ion transporter 1 (DMT1/SLC11A2, also known as DCT1 and NRAMP2) by enterocytes.[9,13] Iron is then transported to the circulation through ferroportin (FPN1/SLC40A1, also known as IREG1 and MTP1),[14–16] where it is immediately oxidized to Fe^{3+} *via* the membrane-bound multi-copper oxidase hephaestin (HEPH),[17] followed by binding to the main iron plasma carrier transferrin (TF).

In terms of organic iron absorption, in mammals most iron is found as part of heme, which forms the prosthetic group of myoglobin and hemoglobin. Therefore, the breakdown of these proteins, which are present in red meat, provides an important source of iron for human nutrition. Indeed, about 2/3 of total iron absorbed by the intestine is incorporated in heme.[18] Although heme absorption by the intestinal enterocytes was described as early as 1955 and despite its relevance in iron homeostasis, the exact absorptive pathway of heme in the intestine remains elusive (Fig. 1B).[18,19] A heme carrier protein called HCP1 (SLC46A1) was proposed to serve as the small intestinal apical heme transporter.[20] However, subsequent studies demonstrated that its main function is to mediate absorption of folate rather than heme,

which is transported with a much lower affinity.[21] Absorbed heme is metabolized by heme oxygenases, resulting in the release of iron, carbon monoxide (CO) and biliverdin. Free iron can then exit into the blood stream *via* FPN1, as described above.

Normally, all the iron present in the circulating blood is bound to TF. However, under certain conditions, when the TF binding capacity is exceeded, non-transferrin bound iron (NTBI) can also be found in the circulating blood.[22] TF-bound iron is taken up by erythrocyte precursors in the bone marrow *via* receptor-mediated endocytosis. Iron-TF first binds to the transferrin receptor 1 (TFR1) and enters the cell through clathrin-mediated endocytosis. As specified below, similar processes occur, for instance, also in placental trophoblasts and bone-derived osteoclasts (Figs 2 and 3). Acidification of the endosomes facilitates the release of TF bound ferric iron, which is then reduced to ferrous iron *via* the endosomal ferrireductase (*i.e.* STEAP3[23]) and released into the cytosol *via* the endosomal variant of DMT1, while TFR1 is recycled back to cell surface.[24] Once in the cytosol, iron joins the labile iron pool and/or is incorporated into heme, or it is stored bound to FT. FT is the main iron storage protein complex in cells and iron release from FT is regulated by ferritinophagy, the degradation process of the complex in the autolysosome.[10] Liver hepatocytes play a major role in the regulation of iron metabolism. Hepatocytes synthesize large amounts of FT, due to their role as a major storage site of absorbed iron.[25] Moreover, the liver is responsible of producing most of the TF, along with hepcidin (HEPC), a peptide hormone that serves as a key regulator of iron homeostasis.[25,26] HEPC is secreted into the circulating blood where it inhibits the release of iron mediated by FPN1 expressed on the basolateral side in enterocytes, as well as in macrophages and hepatocytes.[25] HEPC inhibits FPN1 by promoting its internalization and degradation and by directly blocking the iron export.[27] The synthesis and release of HEPC is regulated by the serum iron concentration. At high serum iron concentrations, HEPC is upregulated, whereas at low serum ferritin levels, hepcidin production is reduced, leading to increased intestinal iron absorption and blood iron availability.

Given the vital function of iron in human physiology, it is expected that systemic or cellular imbalances in iron homeostasis have the potential to develop into serious health issues. Since iron is crucial for the production of the oxygen carrier hemoglobin, insufficient iron supply leads to a shortage of hemoglobin production that results in iron-deficiency anemia.[28] Conversely, in patients with red blood cell disorders such as β-thalassemia, iron release into the bloodstream is promoted to compensate the reduced lifespan of the defective red blood cells, which leads to iron overload in different organs.[29] Apart from these hematological disorders, systemic iron homeostasis imbalances are involved in the development of many other human pathologies. Mutations of genes controlling iron regulation, as in hereditary hemochromatosis patients, promote iron release into the bloodstream and hence provoke iron-overload in different organs, which ultimately leads to severe complications such as liver failure, myocardial diseases or diabetes. In chronic inflammatory disease, increased expression of pro-inflammatory cytokines, such as interleukin 6 (IL-6), promotes HEPC expression, which limits iron absorption and availability leading to the development of anemia.[30] Likewise, imbalances in iron homeostasis that occur at cellular level can develop into severe diseases as well. A classic example is Friedreich ataxia, a devastating genetic disorder in which reduced levels of mature frataxin (FXN), a nuclear gene (FXN)-encoded mitochondrial iron chaperone, which is required for iron–sulfur cluster biogenesis in mitochondria, results in progressive neurodegeneration and hypertrophic cardiomyopathy.[31,32] In cancer cells, dysregulation of iron homeostasis is associated with tumor progression, and over-expression of iron-related genes are inversely correlated with patient survival.[33] In addition to all these iron homeostasis-related diseases, there are many other human pathologies in which iron homeostasis is imbalanced. However, whether this is cause or a consequence is not yet clear. In this regard, many studies have implicated cellular iron overload and related oxidative-stress in the development of neurological disorders such as Parkinson's disease, Alzheimer's disease, Huntington's disease or multiple sclerosis, among others.[34,35] In atherosclerotic plaques, iron is found at much higher concentrations than in healthy tissues.[35] Similarly, in several ageing-related diseases, such as type 2 diabetes mellitus, osteoporosis, osteoarthritis, macular degeneration and liver fibrosis, increased levels of iron have been linked to disease severity.[35] Altogether, these findings highlight that alterations in iron metabolism are linked to a wide variety of highly relevant human diseases including those associated with placental dysfunction during pregnancy or impaired bone metabolism (see specific chapters below). Therefore, the development of molecular tools that can rescue or prevent further dysregulation of iron homeostasis at both systemic and cellular levels have a great potential for future therapeutic applications.

2. Iron Transport across the Placenta

During the TransCure studies the Albrecht group investigated the mechanisms of iron homeostasis in the human placenta. The placenta is a temporary but vital organ during pregnancy maintaining the balance between nutrition and fetal growth control through a selective and regulated supply of macronutrients and critical micronutrients such as iron. In humans, the iron requirement during pregnancy is significantly higher compared to the nonpregnant state in order to fulfill fetal and placental iron demands. All materno-fetal and feto-maternal exchange processes take place across the placental barrier of the chorionic villi which are surrounded by syncytiotrophoblasts (STB), a multinuclear epithelial surface layer that is in direct contact with the maternal blood (Fig. 2). Iron is transferred unidirectionally from the mother to the fetus across the blood–placenta barrier. Therefore, placenta-mediated fetal iron homeostasis is predominantly controlled by regulating iron uptake.

2.1 *Principles of Transplacental Iron Transport*

Fig. 2 summarizes the current knowledge on maternal-fetal iron transfer across the placenta. TF-mediated iron uptake by the STB is initiated by TFR1-binding of di-ferric transferrin (2 Fe^{3+}-TF) followed by clathrin-dependent endocytosis at the maternal side of the STB (microvillous membrane, MVM) into endosomes. Due to endosomal acidification, Fe^{3+} (red) is released from TF, followed by reduction to Fe^{2+} (yellow) presumably by the metalloreductases STEAP3/STEAP4. DMT1 is supposed to transport Fe^{2+} following a proton-dependent mechanism from endosomes into the cytosol. TF and TFR1 return to the apical MVM to be used for further cycles. In contrast to the transfer in the gut and red blood cells, cytosolic iron is transferred to the fetal circulation through the iron exporter FPN1 or stored intracellularly in oxidized form bound to FT. After crossing the basal membrane (BM), Fe^{2+} is oxidized by either ceruloplasmin (CP), HEPH or zyklopen (ZP/HEPHL1). Finally, Fe^{3+} can be bound by TF and further distributed *via* the fetal circulation towards the fetus. Human poly (rC) binding protein 2 (PCBP2) is an iron chaperone that may modulate iron release across the BM and may protect fetal tissue by delivery of oxidative ferrous iron from DMT1 to FPN1 or ferritin (Fig. 2, dotted line). Besides DMT1, there are also alternative ferrous iron transporters that mediate endosomal iron export from the endosome into the cytosol. *In vivo* studies propose DMT1 as non-essential iron transporter in the placenta since in two DMT1 mutant animal models carrying the same DMT1 missense mutation iron-deficient but viable animals were born.[36–38] However, as the *Dmt1*-null mice were severely anemic, the role of DMT1 in placental iron transport requires further experimental confirmation.[39] Other potential endosomal iron transporters with high expression in the human placenta are ZIP8/

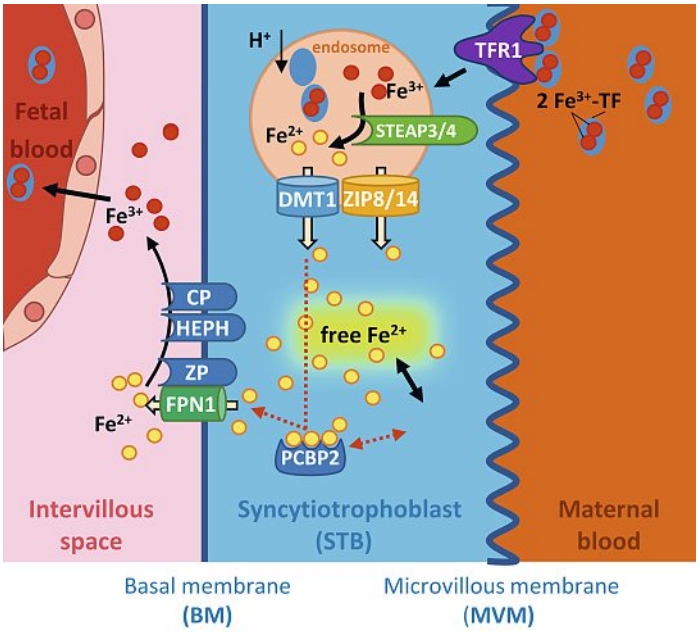

Fig. 2. Mechanisms and regulation of materno-fetal iron transfer across the placenta. Iron (Fe) is transferred unidirectionally from the maternal to fetal circulation across the blood-placenta barrier (right to left). Syncytialized trophoblast cells (blue) are in direct contact with the maternal blood (orange). Details on transferrin (TF)-mediated iron uptake are found in the text. Hypotheses on non TF-mediated iron uptake in the placenta are not shown. The iron chaperone human poly (rC) binding protein 2 (PCBP2) may modulate iron release across the BM and may protect fetal tissue by delivery of oxidative ferrous iron from DMT1 to FPN1 or ferritin (dotted line). *Abbreviations*: Fe^{3+} (red), ferric iron; Fe^{2+} (yellow), ferrous iron; STEAP3/STEAP4, metalloreductases; CP, ceruloplasmin; ZP, zyklopen; further abbreviations see Fig. 1.

SLC39A8 and ZIP14/*SLC39A14*, both members of the SLC39 zinc transporter family that also transport Fe^{2+}.[40] ZIP14 has been shown to mediate plasma membrane uptake of non-TF-bound iron[41] as well as TF-iron from endosomes (Fig. 2).[42] Targeted Zip14 mutants have no abnormal birth phenotype except lower birth weight,[43] while deletion of Zip8 in mice leads to mortality before birth.[44] Despite different pH-dependence, ZIP14 and ZIP8 together with DMT1 probably play redundant roles in placental endosomal iron export into the cytosol.

Iron export from the STB to the fetal stroma is mediated by FPN1, the only currently known iron exporter. FPN1 is abundantly expressed along the BM of human STB.[45] As a prerequisite for placental iron export towards the fetus, intracellular iron must be re-oxidized to Fe^{3+} so that it can be bound by Tf on the fetal side of the placental barrier (Fig. 2). The three ferroxidases CP, HEPH, and ZP have been detected in human placental tissue. *Cp*-null animals exhibit a normal phenotype at birth, suggesting that CP is not essential for placental iron transfer.[46] Moreover, a placenta-specific ferroxidase has been identified in association with sex-linked anemia (SLA)-mice harboring a mutation in Heph1l.[47] ZP has approximately 50% protein identity with CP and HEPH and is abundantly expressed in the placenta while being absent in liver and intestine.[47] Furthermore, the intracellular iron chaperone protein poly(rC)-binding protein 2 (PCBP2) was suggested to act as a recipient of iron from DMT1 and as a donor of iron to FPN1.[48] Iron can have toxic effects because it induces the generation of reactive oxygen species. Thus, the carry-over of iron by chaperons like PCBP2 may be relevant for protection of fetal tissues from putative oxidative ferrous iron and for iron release at the placental barrier across the BM (Fig. 2).[49] Though there is evidence that all three ferroxidases and the chaperon PCBP2 are expressed in the human placenta, experimental evidence regarding their functions in the iron transport mechanism across the placenta is mostly lacking.

2.2 *Regulation of Placental Iron Transport*

The relative resilience of fetal hemoglobin levels to maternal anemia highlights the ability of the placenta to respond to altered maternal iron supply. Stable isotope data in human pregnancies have demonstrated that more iron is transferred from the maternal diet to the fetus when maternal stores are low.[50] This is likely a consequence of upregulation of intestinal and placental iron transporters and TFR1.[51,52] However, the mechanisms underlying this regulation are not well characterized and may involve placental iron regulatory proteins 1 and 2 (IRP1/IRP2) and intracellular free iron. The iron regulatory hormone HEPC is expressed not only in human liver, but also in placental tissue[27] where it negatively regulates cellular iron export *via* FPN1 across the BM towards the fetal circulation. Furthermore, HEPC may play a role in fetal sensing of the placental iron status and hence in signaling fetal demand to the mother.[49] The anemic, iron-deficient phenotype of transgenic mouse embryos overexpressing *Hepc* further supports this notion,[53] though the exact role of HEPC in controlling placental iron levels is still a matter of debate.[54–57] Moreover, different studies suggested that the hemochromatosis factor HFE is involved in the regulation of placental iron metabolism.[58–62]

2.3 *Association of Placental Iron Homeostasis with Pregnancy Diseases*

The TransCure network was particularly interested in translational aspects and investigated the potential involvement of iron transporters in human diseases. Generally, most investigations on maternal iron status during pregnancy in relation to child health outcomes focused on maternal iron deficiency, while iron excess was significantly under-investigated.[63] The considered studies suggested deleterious effects on infant growth, cognition,[64,65] neonatal sepsis after premature rupture of membranes,[66] adverse birth outcomes like stillbirth, preterm- or small for gestational age[67] and childhood type 1 diabetes.[68] In the severe pregnancy disease preeclampsia (PE), inadequate invasion of extravillous trophoblasts into spiral arteries causes oxidative stress, high blood pressure and intrauterine growth restriction in 2–8% of all pregnancies.[69,70] In the clinical context, iron levels positively correlate with an elevation of blood pressure and the severity of PE.[71–73] Overall PE has been associated with several markers of disturbed iron homeostasis and ferroptosis (recently reviewed in ref. [74]).

Within the TransCure project the Albrecht group investigated the effect of disturbed iron homeostasis in gestational diabetes mellitus (GDM). GDM is one of the most common pregnancy complications and has a prevalence of 4.8% in Switzerland[75] and 9.2% in the US,[76] respectively. The etiology of GDM is multifactorial, but interestingly FT and HEPC levels showed in several clinical studies a positive association between maternal iron status and the risk to develop GDM.[77–83] Only few studies measured other parameters, such as serum iron[84] or heme iron intake,[85,86] but confirmed the positive relationship between increased maternal iron status and the risk for GDM. These findings raise potential concerns for the recommendation of routine iron supplementation among iron-replete pregnant women especially for those with a high risk to develop GDM.

In the NCCR TransCure placenta studies, the Albrecht group revealed altered expression of placental iron transporters and iron-regulatory proteins in placental tissue of GDM patients compared to healthy controls. Additionally to this clinical approach, hyperglycemic trophoblast cell models mimicking GDM conditions in three grades of severity were designed. Within these *in vitro* studies, reduced iron uptake into placental trophoblasts was detected by mechanisms involving alterations in autophagy and oxidative stress pathways.[87] The almost complete

reversion of hyperglycemic effects on placental iron homeostasis gene expression in trophoblasts after antioxidant treatment suggested beneficial effects of antioxidant supplementation in pregnant women with increased risk to develop GDM.[87] On the other side, excessive iron supplementation might expose women to increased lipid peroxidation and protein carbonylation by intracellular generation of reactive oxygen species.[88,89] To further elaborate the effects of iron depletion or increased antioxidative potential by treatment with antioxidants *in vivo*, the Albrecht group worked with a GDM-related mouse model. In collaboration with Dr. Sferrruzzi-Perri, University of Cambridge, they investigated a mouse model with wildtype mice receiving a high fat high sugar (HFHS) diet which resulted in compromised maternal glucose tolerance and insulin sensitivity in association with dysregulated lipid metabolism, thereby mimicking typical GDM symptoms.[90,91] Interestingly, placental tissues from HFHS fed mice and human GDM showed highly comparable expression patterns of iron homeostasis genes. Similar to human GDM, the placentae from HFHS mice also seem to protect the fetus from excessive oxidative iron levels by expressional reduction of placental iron-transporters and iron-regulatory proteins.[92]

3. Iron Homeostasis and Bone Metabolism

Iron and the skeleton has been a second focus of the TransCure studies on iron. This interest arose from the original finding that osteoclasts (OC), the cells resorbing bone, have an 'iron uptake' phenotype as compared to macrophages (MΦ), which have an 'iron export' phenotype.[93] This interest was further strengthened by reports showing that disturbances in iron homeostasis affect skeletal and joint development.[94,95] In the adult organism, the skeleton is continuously turned over to repair microfractures, to adapt to mechanical needs and to assure mineral homeostasis.[96,97] To assure maintenance of bone mass and microarchitecture the two major processes of bone metabolism, resorption by OC and formation by osteoblasts, are in equilibrium.[98] Deviation from this equilibrium leads to bone disease, either a loss of bone, leading to osteopenia and osteoporosis[99] or a gain of bone, leading to osteosclerosis and osteopetrosis.[100]

As outlined above, faulty iron homeostasis will lead to bone diseases. Iron deficiency will cause anemia and iron surplus hemochromatosis leading to a decrease in bone mass and osteoporosis.[101,102] There is a large number of factors contributing to the development of osteoporosis, and excess of iron was defined as an independent risk factor for the disease.[103] Iron overload conditions such as hereditary hemochromatosis, thalassemias, sickle cell disease are associated with osteopenia/osteoprosis and an increase in the frequency of bone fractures.[104] Also, in post-menopausal osteoporosis, it was suggested that annual bone loss correlates to plasma ferritin levels.[105] The mechanisms by which iron overload or deficiency affect bone metabolism are not yet clear. In particular, since excess iron deposits mainly in pancreas, liver, pituitary and heart, while iron deficiency leads to anemia, direct and indirect mechanisms may lead to the observed alterations in bone.

The two main cell lineages of bone are the mesenchymal osteoblast lineage that comprises osteoblasts, osteocytes and lining cells and is responsible for bone formation and mechanical sensing within the bone matrix,[106] and the haematopoietic osteoclast lineage,[107] which is resorbing the bone by dissolving the calcium phosphate (CaP) mineral and digesting the organic part of the bone matrix. Little is known, however, about the mechanisms by which iron is impairing bone metabolism.

When investigating the effects of iron on bone cell lineages, the cells of the monocyte/macrophage lineages and the OC were of major interest. The two cell lineages originate from a common progenitor, Macrophage Colony-Forming Unit, (MCFU).[108] Depite this common origin, iron transport mechanisms in MΦ and OC differ, due to their different physiological roles. Besides being part of the innate immune system, one of the major functions of MΦ consists in the recycling of heme iron from old and damaged erythrocytes.[109,110] Recycled iron from heme covers approx. 75% of the daily requirement of iron.[110] Therefore, FPN1, the only known iron export protein,[15,111] is expressed at high levels in MΦ. OC on the other hand expend large amounts of energy to fulfill their basic function – bone resorption.[107] The resorption of bone can be divided into two steps – the acid dissolution of the hydroxyapatite mineral within the resorption lacuna and the release of lysosomal enzymes to dissolve the organic part of the bone matrix. For the acidification of the resorption lacuna, protons are transported against a concentration gradient of approx. 1000-fold by a vacuolar H^+-ATPase.[112]. The protons originate from the spontaneous decay of carbonic acid (H_2CO_3), the formation of which is catalyzed by Carbonic Anhydrase II (CAII) from CO_2 and H_2O (Fig. 3) to H^+ and bicarbonate (HCO_3^-). The osteoclastic anion exchanger SLC4A2 exchanges HCO_3^- with Cl^-,[113] which is further transported through the Cl^- channel ClC-7[114] into the resorption lacuna. Thus, it is the extrusion of hydrochloric acid into the resorption lacuna that enables the cell to generate and maintain a pH around 4.5 in this organelle. To assure the availability of the high levels of energy spent during resorption, OC are characterized by a high abundance in mitochondria.[115] In OC, iron is taken up *via* TFR1 and is supplied to mitochondrial heme proteins and iron-sulfur clusters. The increase in iron uptake has two functional consequences in OC development, (i) it increases mitochondrial respiration, and (ii) it increases transcription of PGC-1β (peroxisome proliferator-activated receptor-γ coactivator 1β), a driver of mitochondrial biogenesis, through ROS production.[116]

Within the TransCure project on iron transport and metabolism, the Hofstetter group focused on the role of iron transport in bone cell lineages, the bone-forming osteoblasts and the bone-resorbing OC. A low density array gene expression approach revealed iron transport proteins to be highly regulated in OC developing from monocyte/ macrophage progenitor cells.[93] No similar regulation of protein expression was observed in osteoblast lineage cells.[117]

During differentiation of osteoclast progenitor cells (OPC) *in vitro* (cultured with macrophage colony-stimulating factor M-CSF, and receptor activator of NF-κB ligand RANKL),[118] expression of TFR1 was increased, while expression of FPN1 was attenuated in comparison to expression levels in MΦ. Iron in the culture medium dose dependently increased proliferation

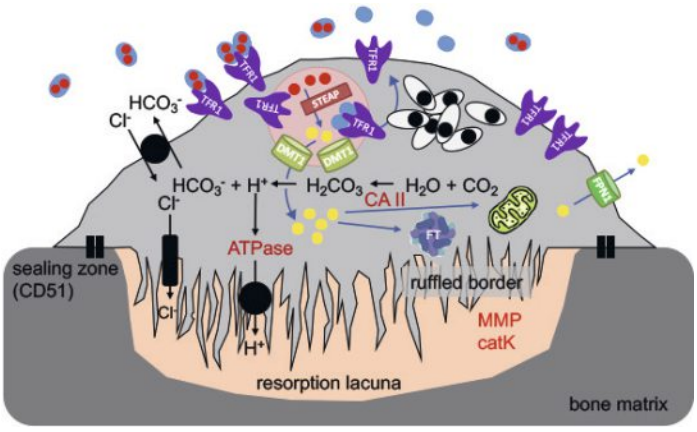

Fig. 3. Iron transport in osteoclasts. Dissolution of the calcium phosphate (CaP) mineral and the organic matrix of bone require high levels of energy. Energy production in the mitochondrial respiratory chain requires iron and the iron transport system of osteoclasts is tailored towards an iron uptake phenotype with only low capacity to extrude iron via ferroportin (FPN). *Abbreviations*: CA II, carbonic anhydrase II; MMP, metallo-proteinases; catK, cathepsin K; further abbreviations see Fig. 1.

of OPC while inhibiting the differentiation into osteoclast like cells (OCL). Furthermore, iron deficiency reduced the capacity of OCL to dissolve CaP, without affecting survival of the cells.

Deprivation of cultures of OCL of iron leads to an increase in the expression of TFR1, while levels of transcripts encoding FPN1 were not affected. Iron excess on the other hand led to attenuation of TFR1 expression, which is accompanied by an increase in the levels of transcripts encoding FPN1 and TFH1 (Transferrin Heavy Chain). Furthermore, iron excess redirects cell differentiation to the MΦ lineage, inhibiting osteoclast development, as characterized by an increase in the expression of the pan-MΦ marker F4/80 and a decrease in the OC marker tartrate resistant acid phosphatase (TRAP).[119] This shift is accompanied by the expression of an iron storage and release phenotype (TFH1, FPN1) in MΦ vs. an iron uptake phenotype (TFR1, DMT1) in OC.

Cellular uptake of iron by OC and intracellular trafficking is not yet fully elucidated. As outlined above, iron loaded holoTF binds to TFR1 (TFR2 not being found to be expressed by OC lineage cells) and subsequently is endocytosed *via* clathrin coated vesicles. Endosomal pH is lowered to allow for the release of ferric iron from holoTF and reduction to ferrous Fe^{2+}, which in turn is transported to the cytoplasm *via* DMT1 to form the labile iron pool (LIP). Cytoplasmic iron can be released from the cells *via* FPN1, stored in ferritin or mitochondria or can be incorporated into heme or iron-sulfur clusters.[24] In iron-deprived OC, after 2 h of iron uptake, two iron pools can be differentiated upon cellular fractionation on a density gradient, pool I localized in the cytoplasmic fractions, containing also early endosomes (characterized by the marker EEA1) and lysosomes (characterized by DMT1). Pool II was co-localizing with membrane vesicles that are characterized by TFR1, CD51 (integrin α_v) and TF. After another 4 h, in the absence of exogenous iron, pool I disappeared, while pool II proved to be stable. Furthermore, no cellular TF could be detected, the iron binding protein being recycled as described above. The storage mode of iron in osteoclasts remains, however, a mystery, since iron was not found to accumulate either in FT particles or in mitochondria, nor was iron 'stored' as part of the labile iron pool.

The energy required to set up the resorptive pH in the form of ATP is generated in the respiratory chain of the mitochondria. Osteoclasts are particularly rich in these organelles and mitochondrial biogenesis has been suggested to be linked to osteoclast activation and bone metabolism.[116] It is noteworthy, however, that the studies described above do not suggest iron trafficking and storage in mitochondria, even though the OCL have been starved for iron for 24 h before uptake. Nevertheless, iron deprivation causes a decrease in the activity of OCL to dissolve CaP mineral, suggesting that one of the essential steps in the establishment of the pH gradient is affected under these culture conditions.

An interesting aspect of iron regulation is the modulation of cell development in conditions of iron excess. Iron excess results in an inhibition of OC development and a stimulation of MΦ differentiation. Particularly noteworthy are the downregulation of TFR1 and upregulation of FPN1. Furthermore, TFH1 was upregulated as well in MΦ, providing the MΦ with two mechanisms to handle iron, by releasing it *via* FPN1 and by storing it in ferritin particles.

In conclusion, the cells of the monocyte/macrophage and osteoclast lineages fulfill fundamental physiological functions. They are part of the innate immune system and, on the other hand, are the cells resorbing bone. While OC mainly need iron to provide the necessary energy in the process of bone resorption, monocytes/macrophages are crucial for iron homeostasis. Excess iron in the cellular environment attenuates OC differentiation and facilitates monocyte/macrophage development, the first expressing an iron 'uptake' phenotype with high levels of TFR1, the second expressing a 'release and storage' phenotype with high levels of FPN1 and FTH1.

4. Development of Modulators Targeting DMT1

As part of the NCCR TransCure project on iron transport and metabolism, the Hediger and Reymond groups used small molecule high-throughput screening in an attempt to identify improved modulators of DMT1. Previous efforts to identify modulators of DMT1 led to the identification of two distinct scaffolds classes with moderate efficiency, represented by 1) bis-cationic isothioureas such as dibenzofurans and mesitylene,[120] and 2) pyrazolyl-pyridine.[121] To identify more potent DMT1 modulators, we used these molecules as seeds for a 3D ligand-based virtual screening, which allowed us to identify new bis-isothiourea and pyrazolyl-pyrimidone molecules as additional DMT1 inhibitors.[122] Next, we tested a variety of analogues of these compounds and performed studies to provide mechanistic details on their pharmacological activity. In this context, we characterized the interaction of aromatic bis-isothiourea-substituted compounds with DMT1 and its prokaryotic homologue EcoDMT. Our work revealed that these compounds follow a competitive inhibition mechanism with potencies in the low micromolar range. Moreover, working with the group of Raimund Dutzler as part of this NCCR TransCure project, we could solve the crystal structure of EcoDMT in complex with brominated derivatives and validated the observed binding mode by mutagenesis and structure-activity relationship experiments. Altogether, this work provided the first detailed mechanistic insight into the pharmacology of members of the SLC11/NRAMP family of metal ion transporters.[123]

On the other hand, pyrazolyl-pyrimidone, the small size and better drug-like properties of which made it an attractive drug candidate, acted as a non-competitive inhibitor with a potency in the low micromolar range. Surprisingly, our subsequent work to understand its mechanism of action revealed that it acts as a metal chelator rather than a specific DMT1 inhibitor.[124]

To identify new small-molecule modulators of DMT1, we recently developed a fragment-based drug discovery approach. This approach consists in testing a small number of structurally diverse fragments with the goal to find weak interactions, in order to identify hits that can later be optimized. We assembled a fragment library based on the GDB17 database containing all possible organic molecules up to 17 atoms and screened for DMT1 and ZIP8 modulators.[125] The screening led to the identification of novel DMT1 and ZIP8 inhibitors,[126] (Fig. 4).

5. Conclusions

In addition to the structural characterization of prokaryotic SLC11 transporters (described in the article by Manatschal & Dutzler in this special issue[127]), the NCCR TransCure research in the framework of the Iron Transporter Project has focused on the identification, improvement and characterization of DMT1 inhibitors as well as on the study of iron transport physiology in placenta and bone. Interdisciplinary work on existing DMT1 pharmacology provided important information for understanding the true potential of the most promising molecular modulators described to date. In addition, our screening efforts uncovered novel modulators of transporters for divalent metals, opening new avenues for the development of therapeutic strategies to combat diseases related to the deregulation of iron homeostasis. Physiological studies addressed specific aspects of iron homeostasis in placenta and bone. The data suggest crucial local effects of iron on cell and tissue development and function, in addition to the systemic effects of chronic inflammation in response to iron deposition in tissues or iron deficiency anemia and the corresponding secondary effects.

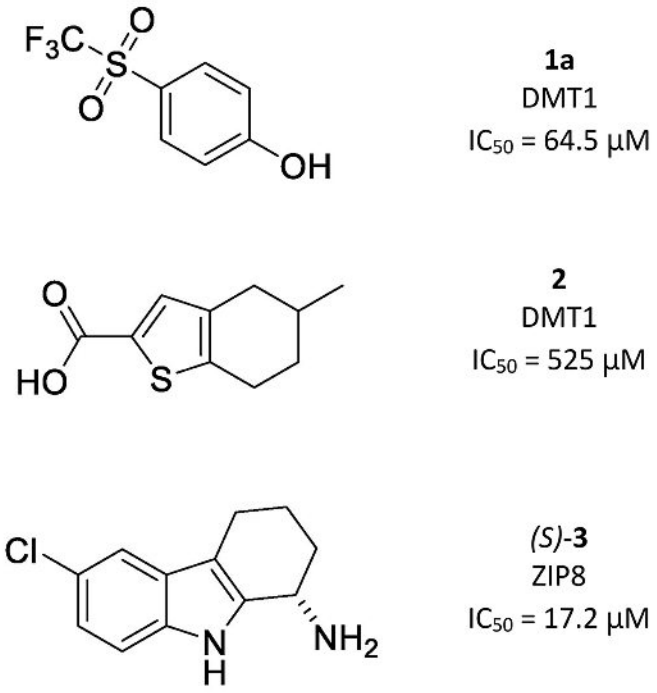

Fig. 4. Novel DMT1 and ZIP8 inhibitors.

Acknowledgements

This work was supported by the Swiss National Science Foundation (SNSF) NCCR TransCure grant # 51NF40-185544, the SNSF grant #310030_197408 to CA, #310030_182272 to MAH and a Gottfried und Julia Bangerter-RhynerFoundation grant to MAH.

Received: July 8, 2022

[1] N. C. Andrews, *Blood* **2008**, *112*, 219, https://doi.org/10.1182/BLOOD-2007-12-077388.
[2] N. Montalbetti, A. Simonin, G. Kovacs, M. A. Hediger, *Mol. Aspects Med.* **2013**, *34*, 270, https://doi.org/10.1016/j.mam.2013.01.002.
[3] G. Papanikolaou, K. Pantopoulos, *IUBMB Life* **2017**, *69*, 399, https://doi.org/10.1002/iub.1629.
[4] T. Ganz, *Physiol. Rev.* **2013**, *93*, 1721, https://doi.org/10.1152/physrev.00008.2013.
[5] A. R. Bogdan, M. Miyazawa, K. Hashimoto, Y. Tsuji, *Trends Biochem. Sci.* **2016**, *41*, 274, https://doi.org/10.1016/j.tibs.2015.11.012.
[6] G. J. Anderson, D. M. Frazer, *Am. J. Clin. Nutr.* **2017**, *106* suppl, p. 1559S, https://doi.org/10.3945/ajcn.
[7] C. Camaschella, A. Nai, L. Silvestri, *Haematologica* **2020**, *105*, 260, https://doi.org/10.3324/haematol.2019.232124.
[8] K. Roemhild, F. von Maltzahn, R. Weiskirchen, R. Knüchel, S. von Stillfried, T. Lammers, *Trends Pharmacol. Sci.* **2021**, *42*, 640, https://doi.org/10.1016/j.tips.2021.05.001.
[9] H. Gunshin, B. Mackenzie, U. V. Berger, Y. Gunshin, M. F. Romero, W. F. Boron, S. Nussberger, J. L. Gollan, M. A. Hediger, *Nature* **1997**, *338*, 482, https://doi.org/10.1038/35001596.
[10] A.-C. S. Vogt, T. Arsiwala, M. Mohsen, M. Vogel, V. Manolova, M. F. Bachmann, *Int. J. Mol. Sci.* **2021**, *22*, 4591, https://doi.org/10.3390/ijms22094591.
[11] S. Recalcati, E. Gammella, P. Buratti, G. Cairo, *IUBMB Life* **2017**, *69*, 389, https://doi.org/10.1002/iub.1628.
[12] M. Nairz, G. Weiss, *Mol. Aspects Med.* **2020**, *75*, 100864, https://doi.org/10.1016/j.mam.2020.100864.
[13] M. D. Fleming, C. C. Trenor, M. A. Su, D. Foernzler, D. R. Beier, W. F. Dietrich, N. C. Andrews, *Nat. Genet.* **1997**, *16*, 383, https://doi.org/10.1038/ng0897-383.
[14] S. Abboud, D. J. Haile, *J. Biol. Chem.* **2000**, *275*, 19906, https://doi.org/10.1074/jbc.M000713200.
[15] A. Donovan, A. Brownlie, Y. Zhou, J. Shepard, S. J. Pratt, J. Moynihan, B. H. Paw, A. Drejer, B. Barut, A. Zapata, T. C. Lawk, C. Brugnarak, S. E. Lux, G. S. Pinkus, J. L. Pinkus, P. D. Kingsley, J. Palis, M. D. Fleming, N. C. Andrews, L. I. Zon, *Nature* **2000**, *403*, 776, https://doi.org/10.1038/35001596.
[16] A. T. McKie, P. Marciani, A. Rolfs, K. Brennan, K. Wehr, D. Barrow, S. Miret, A. Bomford, T. J. Peters, F. Farzaneh, M. A. Hediger, M. W. Hentze, R. J. Simpson, *Mol. Cell* **2000**, *5*, 299, https://doi.org/10.1016/S1097-2765(00)80425-6.
[17] D. M. Frazer, C. D. Vulpe, A. T. McKie, S. J. Wilkins, D. Trinder, G. J. Cleghorn, G. J. Anderson, *Am. J. Physiol. - Gastrointest. Liver Physiol.* **2001**, *281*, 931, https://doi.org/10.1152/ajpgi.2001.281.4.g931.
[18] R. Daher, Z. Karim, *Transfus. Clin. Biol.* **2017**, *24*, 115, https://doi.org/10.1016/j.tracli.2017.06.015.
[19] A. R. West, P. S. Oates, *World J. Gastroenterol.* **2008**, *14*, 4101, https://doi.org/10.3748/wjg.14.4101.
[20] M. Shayeghi, G. O. Latunde-Dada, J. S. Oakhill, A. H. Laftah, K. Takeuchi, N. Halliday, Y. Khan, A. Warley, F. E. McCann, R. C. Hider, D. M. Frazer, G. J. Anderson, C. D. Vulpe, R. J. Simpson, A. T. McKie, *Cell* **2005**, *122*, 789, https://doi.org/10.1016/j.cell.2005.06.025.
[21] A. Qiu, M. Jansen, A. Sakaris, S. H. Min, S. Chattopadhyay, E. Tsai, C. Sandoval, R. Zhao, M. H. Akabas, I. D. Goldman, *Cell* **2006**, *127*, 917, https://doi.org/10.1016/j.cell.2006.09.014.
[22] M. Patel, D V S S Ramavataram, *Indian J. Clin. Biochem.* **2012**, *27*, 322, https://doi.org/10.1007/s12291-012-0250-7.
[23] R. S. Ohgami, D. R. Campagna, E. L. Greer, B. Antiochos, A. Mcdonald, J. Chen, J. J. Sharp, Y. Fujiwara, J. E. Barker, M. D. Fleming, *Nat. Genet.* **2005**, *37*, 1264, https://doi.org/10.1038/ng1658.
[24] M. W. Hentze, M. U. Muckenthaler, B. Galy, C. Camaschella, *Cell* **2010**, *142*, 24, https://doi.org/10.1016/j.cell.2010.06.028.
[25] G. J. Anderson, D. M. Frazer, *Semin. Liver Dis.* **2005**, *25*, 420, https://doi.org/10.1055/s-2005-923314.
[26] G. Rishi, V. N. Subramaniam, *Am. J. Physiol. - Gastrointest. Liver Physiol.* **2017**, *313*, G157, https://doi.org/10.1152/ajpgi.00004.2017.
[27] B. Qiao, P. Sugianto, E. Fung, A. Del-Castillo-Rueda, M. J. Moran-Jimenez, T. Ganz, E. Nemeth, *Cell Metab.* **2012**, *15*, 918, https://doi.org/10.1016/j.cmet.2012.03.018.
[28] C. Camaschella, *N. Engl. J. Med.* **2015**, *373*, 485, https://doi.org/10.1056/NEJMc1507104.
[29] G. J. Anderson, E. Bardou-Jacquet, *Ann. Transl. Med.* **2021**, *9*, 731, https://doi.org/10.21037/atm-20-5512.
[30] G. Weiss, G. Schett, *Nat. Rev. Rheumatol.* **2013**, *9*, 205, https://doi.org/10.1038/nrrheum.2012.183.
[31] P. G. Mazzara, S. Muggeo, M. Luoni, L. Massimino, M. Zaghi, P. T. T. Valverde, S. Brusco, M. J. Marzi, C. Palma, G. Colasante, A. Iannielli, M. Paulis, C. Cordiglieri, S. G. Giannelli, P. Podini, C. Gellera, F. Taroni, F. Nicassio, M. Rasponi, V. Broccoli, *Nat. Commun.* **2020**, *11*, 4178, https://doi.org/10.1038/s41467-020-17954-3.
[32] L. Weng, L. Laboureur, Q. Wang, L. Guo, P. Xu, L. Gottlieb, D. R. Lynch, C. Mesaros, I. A. Blair, *Sci. Rep.* **2020**, *10*, 15788, https://doi.org/10.1038/s41598-020-72884-w.
[33] M. Morales, X. Xue, *Theranostics* **2021**, *11*, 8412, https://doi.org/10.7150/THNO.59092.
[34] T. A. Rouault, *Nat. Rev. Neurosci.* **2013**, *14*, 551, https://doi.org/10.1038/nrn3453.
[35] B. J. Crielaard, T. Lammers, S. Rivella, *Nat. Rev. Drug Discov.* **2017**, *16*, 400, https://doi.org/10.1038/nrd.2016.248.
[36] T. Veuthey, M. Wessling-Resnick, *Front. Pharmacol.* **2014**, *5*, 1, https://doi.org/10.3389/fphar.2014.00082.
[37] M. Tabuchi, T. Yoshimori, K. Yamaguchi, T. Yoshida, F. Kishi, *J. Biol. Chem.* **2000**, *275*, 22220, https://doi.org/10.1074/jbc.M001478200.
[38] E. A. Farcich, E. H. Morgan, *Am. J. Physiol. - Regul. Integr. Comp. Physiol.* **1992**, *262*, R220, https://doi.org/10.1152/ajpregu.1992.262.2.r220.
[39] H. Gunshin, Y. Fujiwara, A. O. Custodio, C. DiRenzo, S. Robine, N. C. Andrews, *J. Clin. Invest.* **2005**, *115*, 1258, https://doi.org/10.1172/JCI24356.
[40] S. Jenkitkasemwong, Chia-Yu Wang, B. Mackenzie, M. D. Knutson, *BioMetals* **2012**, *25*, 643, https://doi.org/10.1007/s10534-012-9526-x.Physiologic.
[41] J. P. Liuzzi, F. Aydemir, H. Nam, M. D. Knutson, R. J. Cousins, *Proc. Natl. Acad. Sci. U. S. A.* **2006**, *103*, 13612, https://doi.org/10.1073/pnas.0606424103.
[42] N. Zhao, J. Gao, C. A. Enns, M. D. Knutson, *J. Biol. Chem.* **2010**, *285*, 32141, https://doi.org/10.1074/jbc.M110.143248.
[43] S. Hojyo, T. Fukada, S. Shimoda, W. Ohashi, B. H. Bin, H. Koseki, T. Hirano, *PLoS One* **2011**, *6*, e18059, https://doi.org/10.1371/journal.pone.0018059.
[44] B. Wang, L. He, H. Dong, T. P. Dalton, D. W. Nebert, *Biochem. Biophys. Res. Commun.* **2011**, *410*, 289, https://doi.org/10.1016/j.bbrc.2011.05.134.
[45] J. Bastin, H. Drakesmith, M. Rees, I. Sargent, A. Townsend, *Br. J. Haematol.* **2006**, *134*, 532, https://doi.org/10.1111/j.1365-2141.2006.06216.x.
[46] Z. L. Harris, A. P. Durley, T. K. Man, J. D. Gitlin, *Proc. Natl. Acad. Sci. U. S. A.* **1999**, *96*, 10812, https://doi.org/10.1073/pnas.96.19.10812.
[47 H. Chen, Z. K. Attieh, B. A. Syed, Y. Kuo, V. Stevens, B. K. Fuqua, H. S. Andersen, C. E. Naylor, R. W. Evans, L. Gambling, R. Danzeisen, M.

Bacouri-haidar, J. Usta, C. D. Vulpe, H. J. Mcardle, *J. Nutr*. **2010**, *140*, 1728, https://doi.org/10.3945/jn.109.117531.
[48] I. Yanatori, D. R. Richardson, K. Imada, F. Kishi, *J. Biol. Chem*. **2016**, *291*, 17303, https://doi.org/10.1074/jbc.M116.721936.
[49] C. Cao, M. D. Fleming, *Nutr. Rev*. **2016**, *74*, 421, https://doi.org/10.1093/nutrit/nuw009.
[50] K. O. O'Brien, N. Zavaleta, S. A. Abrams, L. E. Caulfield, *Am. J. Clin. Nutr*. **2003**, *77*, 924, https://doi.org/10.1093/ajcn/77.4.924.
[51] M. F. Young, E. Pressman, M. L. Foehr, T. McNanley, E. Cooper, R. Guillet, M. Orlando, A. W. McIntyre, J. Lafond, K. O. O'Brien, *Placenta* **2010**, *31*, 1010, https://doi.org/10.1016/j.placenta.2010.08.009.
[52] L. Gambling, R. Danzeisen, S. Gair, R. G. Lea, Z. Charania, N. Solanky, K. D. Joory, S. Kaila, S. Srai, H. J. McArdle, *Biochem. J.* **2001**, *356*, 883, https://doi.org/10.1042/0264-6021:3560883.
[53] G. Nicolas, M. Bennoun, A. Porteu, S. Mativet, C. Beaumont, B. Grandchamp, M. Sirito, M. Sawadogo, A. Kahn, S. Vaulont, *Proc. Natl. Acad. Sci. U. S. A*. **2002**, *99*, 4596, https://doi.org/10.1073/pnas.072632499.
[54] R. Mazgaj, P. Lipiński, E. S. Edison, A. Bednarz, R. Staroń, O. Haberkiewicz, M. Lenartowicz, E. Smuda, A. Jończy, R. R. Starzyński, *Am. J. Hematol*. **2021**, *96*, 659, https://doi.org/10.1002/ajh.26152.
[55] M. D. Koenig, L. Tussing-Humphreys, J. Day, B. Cadwell, E. Nemeth, *Nutrients* **2014**, *6*, 3062, https://doi.org/10.3390/nu6083062.
[56] V. Sangkhae, E. Nemeth, *Free Radic. Biol. Med.* **2019**, *133*, 254, https://doi.org/10.1016/j.freeradbiomed.2018.07.001.Placental.
[57] V. Sangkhae, A. L. Fisher, S. Wong, M. D. Koenig, L. Tussing-Humphreys, A. Chu, M. Lelić, T. Ganz, E. Nemeth, *J. Clin. Invest*. **2020**, *130*, 625, https://doi.org/10.1172/JCI127341.
[58] S. Parkkila, A. Waheed, R. S. Britton, B. R. Bacon, X. Y. Zhou, S. Tomatsu, R. E. Fleming, W. S. Sly, *Proc. Natl. Acad. Sci. U. S. A*. **1997**, *94*, 13198, https://doi.org/10.1073/pnas.94.24.13198.
[59] J. N. Feder, A. Gnirke, W. Thomas, Z. Tsuchihashi, D. A. Ruddy, A. Basava, F. Dormishian, R. Domingo, M. C. Ellis, A. Fullan, L. M. Hinton, N. L. Jones, B. E. Kimmel, G. S. Kronmal, P. Lauer, V. K. Lee, D. B. Loeb, F. A. Mapa, E. McClelland, N. C. Meyer, G. A. Mintier, N. Moeller, T. Moore, E. Morikang, C. E. Prass, L. Quintana, S. M. Starnes, R. C. Schatzman, K. J. Brunke, D. T. Drayna, N. J. Risch, B. R. Bacon, R. K. Wolff, *Nat. Genet*. **1996**, *13*, 399, https://doi.org/10.1038/ng0896-399.
[60] C. N. Gross, A. Irrinki, J. N. Feder, C. A. Enns, *J. Biol. Chem.* **1998**, *273*, 22068, https://doi.org/10.1074/jbc.273.34.22068.
[61] L. Salter-Cid, A. Brunmark, Y. Li, D. Leturcq, P. A. Peterson, M. R. Jackson, Y. Yang, *Proc. Natl. Acad. Sci. U. S. A*. **1999**, *96*, 5434, https://doi.org/10.1073/pnas.96.10.5434.
[62] Y. Gruper, J. Bar, E. Bacharach, R. Ehrlich, *J. Cell. Physiol*. **2005**, *204*, 901, https://doi.org/10.1002/jcp.20349.
[63] H.G.Quezada-pinedo,F.Cassel,L.Duijts,M.U.Muckenthaler,M.Gassmann, V. W. V. Jaddoe, I. K. M. Reiss, M. J. Vermeulen, *Nutrients* **2021**, *13*, 1, https://doi.org/10.3390/nu13072221.
[64] S. Hanieh, T. T. Ha, J. A. Simpson, G. J. Casey, N. C. Khuong, D. D. Thoang, T. T. Thuy, S. R. Pasricha, T. D. Tran, T. Tuan, T. Dwyer, J. Fisher, B. A. Biggs, *PLoS Med*. **2013**, *10*, https://doi.org/10.1371/journal.pmed.1001470.
[65] S. Hanieh, T. T. Ha, A. M. De Livera, J. A. Simpson, T. T. Thuy, N. C. Khuong, D. D. Thoang, T. D. Tran, T. Tuan, J. Fisher, B. A. Biggs, *Arch. Dis. Child*. **2015**, *100*, 165, https://doi.org/10.1136/archdischild-2014-306328.
[66] R. L. Goldenberg, B. M. Mercer, M. Miodovnik, G. R. Thurnau, P. J. Meis, A. Moawad, R. H. Paul, S. F. Bottoms, A. Das, J. M. Roberts, D. McNeilis, T. Tamura, *Am. J. Obstet. Gynecol*. **1998**, *179*, 1599, https://doi.org/10.1016/S0002-9378(98)70032-8.
[67] K. G. Dewey, B. M. Oaks, *Am. J. Clin. Nutr*. **2017**, *106*, 1694S, https://doi.org/10.3945/ajcn.117.156075.
[68] K. Størdal, H. J. McArdle, H. Hayes, G. Tapia, M. K. Viken, N. A. Lund-Blix, M. Haugen, G. Joner, T. Skrivarhaug, K. Mårild, P. R. Njølstad, M. Eggesbø, S. Mandal, C. M. Page, S. J. London, B. A. Lie, L. C. Stene, *Sci. Rep*. **2018**, *8*, 1, https://doi.org/10.1038/s41598-018-27391-4.
[69] A. Agarwal, A. Aponte-Mellado, B. J. Premkumar, A. Shaman, S. Gupta, *Reprod. Biol. Endocrinol*. **2012**, *10*, 49, https://doi.org/10.1186/1477-7827-10-49.
[70] P. Guerby, O. Tasta, A. Swiader, F. Pont, E. Bujold, O. Parant, C. Vayssiere, R. Salvayre, A. Negre-Salvayre, *Redox Biol*. **2021**, *40*, https://doi.org/10.1016/j.redox.2021.101861.
[71] M. Aires Rodrigues De Freitas, A. Vieira Da Costa, L. Alves De Medeiros, M. Da Silva Garrote Filho, A. Lemos Debs Diniz, N. Penha-Silva, *Obstet. Gynecol. Int.* **2018**, https://doi.org/10.1155/2018/9628726.
[72] Y. P. Orlov, N. V Govorova, V. N. Lukach, A. V Mitrofnov, O. D. Dmitriva, *Anesteziol. Reanimatol*. **2016**, *61*, 442.
[73] H. Zhang, Y. He, J. Wang, M. Chen, J. Xu, M. Jiang, Y. Feng, Y. Gu, *Redox Biol.* **2020**, *29*, 101402, https://doi.org/10.1016/j.redox.2019.101402.
[74] J. Zaugg, F. Solenthaler, C. Albrecht, *Biochem. Pharmacol.* **2022**, *202*, 115141, https://doi.org/10.1016/j.bcp.2022.115141.
[75] A. Orecchio, D. Periard, A. Kashef, J. L. Magnin, D. Hayoz, E. Fontana, *Gynecol. Endocrinol.* **2014**, *30*, 561, https://doi.org/10.3109/09513590.2013.879853.
[76] C. L. DeSisto, S. Y. Kim, A. J. Sharma, *Prev. Chronic Dis*. **2014**, *11*, 1, https://doi.org/10.5888/pcd11.130415.
[77] S. Bo, G. Menato, P. Villois, R. Gambino, M. Cassader, I. Cotrino, P. Cavallo-Perin, *Am. J. Obstet. Gynecol.* **2009**, *201*, 158.e1, https://doi.org/10.1016/j.ajog.2009.04.049.
[78] S. Zein, S. Rachidi, S. Awada, M. Osman, A. Al-Hajje, N. Shami, I. Sharara, K. Cheikh-Ali, P. Salameh, I. Hininger-Favier, *J. Trace Elem. Med. Biol.* **2015**, *30*, 220, https://doi.org/10.1016/j.jtemb.2014.09.004.
[79] S. Rawal, S. N. Hinkle, W. Bao, Y. Zhu, J. Grewal, S. Paul, N. L. Weir, M. Y. Tsai, C. Zhang, E. Kennedy, I. City, E. Kennedy, B. Branch, *Diabetologia* **2017**, *60*, 249, https://doi.org/10.1007/s00125-016-4149-3.A.
[80] S. Fu, F. Li, J. Zhou, Z. Liu, *Med. (United States)* **2016**, *95*, e2383, https://doi.org/10.1097/MD.0000000000002383.
[81] M. Afkhami-Ardekani, M. Rashidi, *J. Diabetes Complications* **2009**, *23*, 194, https://doi.org/10.1016/j.jdiacomp.2007.11.006.
[82] F. N. Amiri, Z. Basirat, S. Omidvar, M. Sharbatdaran, K. H. Tilaki, M. Pouramir, *J. Nat. Sci. Biol. Med*. **2013**, *4*, 302, https://doi.org/10.4103/0976-9668.116977.
[83] S. Zein, S. Rachidi, I. Hininger-Favier, *J. Trace Elem. Med. Biol.* **2014**, *28*, 65, https://doi.org/10.1016/j.jtemb.2013.09.009.
[84] B. Zhu, C. Liang, X. Xia, K. Huang, S. Yan, J. Hao, P. Zhu, H. Gao, F. Tao, *Biol. Trace Elem. Res*. **2019**, *191*, 45, https://doi.org/10.1007/s12011-018-1595-4.
[85] K. Bowers, E. Yeung, M. A. Williams, L. Qi, D. K. Tobias, F. B. Hu, C. Zhang, *Diabetes Care* **2011**, *34*, 1557, https://doi.org/10.2337/dc11-0134.
[86] C. Qiu, C. Zhang, B. Gelaye, D. A. Enquobahrie, I. O. Frederick, M. A. Williams, *Diabetes Care* **2011**, *34*, 1564, https://doi.org/10.2337/dc11-0135.
[87] J. Zaugg, H. Melhem, X. Huang, M. Wegner, M. Baumann, D. Surbek, M. Körner, C. Albrecht, *FASEB J.* **2020**, *34*, 7311, https://doi.org/10.1096/fj.201903054R.
[88] G. Papanikolaou, K. Pantopoulos, *Toxicol. Appl. Pharmacol*. **2005**, *202*, 199, https://doi.org/10.1016/j.taap.2004.06.021.
[89] S. Ziaei, M. Norrozi, S. Faghihzadeh, E. Jafarbegloo, *BJOG An Int. J. Obstet. Gynaecol.* **2007**, *114*, 684, https://doi.org/10.1111/j.1471-0528.2007.01325.x.
[90] A. N. Sferruzzi-Perri, O. R. Vaughan, M. Haro, W. N. Cooper, B. Musial, M. Charalambous, D. Pestana, S. Ayyar, A. C. Ferguson-Smith, G. J. Burton, M. Constancia, A. L. Fowden, *FASEB J.* **2013**, *27*, 3928, https://doi.org/10.1096/fj.13-234823.
[91] B. Musial, O. R. Vaughan, D. S. Fernandez-Twinn, P. Voshol, S. E. Ozanne, A. L. Fowden, A. N. Sferruzzi-Perri, *J. Physiol*. **2017**, *595*, 4875, https://doi.org/10.1113/JP273684.
[92] J. Zaugg, L. Zurkinden, B. Musial, O. R. Vaughan, A. L. Fowden, A. N. Sferruzzi-Perri, C. Albrecht, *Placenta* **2021**, *112*, e35, https://doi.org/10.1016/j.placenta.2021.07.115.
[93] W. Xie, S. Lorenz, S. Dolder, W. Hofstetter, *Calcif. Tissue Int*. **2016**, *98*, 275, https://doi.org/10.1007/s00223-015-0087-1.
[94] L. F. D. Van Vulpen, G. Roosendaal, B. S. Van Asbeck, S. C. Mastbergen, F. P. J. G. Lafeber, R. E. G. Schutgens, *J. Clin. Pathol*. **2015**, *68*, 592, https://doi.org/10.1136/jclinpath-2015-202967.
[95] M. G. Ledesma-Colunga, H. Weidner, M. Vujic Spasic, L. C. Hofbauer, U. Baschant, M. Rauner, *Semin. Hematol*. **2021**, *58*, 188, https://doi.org/10.1053/j.seminhematol.2021.06.002.
[96] E. F. Eriksen, *Endocr. Rev*. **1986**, *7*, 379, https://doi.org/10.1038/ng0897-383.
[97] A. M. Parfitt, *Metab. Bone Dis. Relat. Res.* **1982**, *4*, 1, https://doi.org/10.1016/0221-8747(82)90002-9.
[98] R. G. Erben, *Anat. Rec.* **1996**, *246*, 39, https://doi.org/10.1002/(SICI)1097-0185(199609)246:1<39::AID-AR5>3.0.CO;2-A.
[99] D. M. Black, C. J. Rosen, *N. Engl. J. Med*. **2016**, *374*, 254, https://doi.org/10.1056/NEJMcp1513724.
[100] C. Sobacchi, A. Schulz, F. P. Coxon, A. Villa, M. H. Helfrich, *Nat. Rev. Endocrinol*. **2013**, *9*, 522, https://doi.org/10.1038/nrendo.2013.137.
[101] J. Tsay, Z. Yang, F. P. Ross, S. Cunningham-Rundles, H. Lin, R. Coleman, P. Mayer-Kuckuk, S. B. Doty, R. W. Grady, P. J. Giardina, A. L. Boskey, M. G. Vogiatzi, *Blood* **2010**, *116*, 2582, https://doi.org/10.1182/blood-2009-12-260083.
[102] E. D. Weinberg, *Pediatr. Endocrinol. Rev.* **2008**, Suppl 1, 82.
[103] V. Jeney, *Front. Pharmacol*. **2017**, *8*, 1, https://doi.org/10.3389/fphar.2017.00077.
[104] E. Balogh, G. Paragh, V. Jeney, *Pharmaceuticals* **2018**, *11*, 107, https://doi.org/10.3390/ph11040107.
[105] B. J. Kim, S. H. Lee, J. M. Koh, G. S. Kim, *Osteoporos. Int.* **2013**, *24*, 2627, https://doi.org/10.1007/s00198-013-2363-0.

[106] J. E. Aubin, *Rev. Endocr. Metab. Disord.* **2001**, *2*, 81, https://doi.org/10.1023/A:1010011209064.
[107] S. L. Teitelbaum, *Science* **2000**, *289*, 1504, https://doi.org/10.1126/science.289.5484.1504.
[108] R. Felix, W. Hofstetter, A. Wetterwald, M. G. Cecchini, H. Fleisch, *J. Cell. Biochem.* **1994**, *55*, 340, https://doi.org/10.1002/jcb.240550311.
[109] Y. Lu, G. Basatemur, I. C. Scott, D. Chiarugi, M. Clement, J. Harrison, R. Jugdaohsingh, X. Yu, S. A. Newland, H. E. Jolin, X. Li, X. Chen, M. Szymanska, G. Haraldsen, G. Palmer, P. G. Fallon, E. S. Cohen, A. N. J. McKenzie, Z. Mallat, *Immunity* **2020**, *52*, 782, https://doi.org/10.1016/j.immuni.2020.03.006.
[110] T. Ganz, *Microbiol. Spectr.* **2016**, *4*, https://doi.org/10.1128/9781555819194.ch46.
[111] E. Nemeth, T. Ganz, *Int. J. Mol. Sci.* **2021**, *22*, 6493, https://doi.org/10.3390/ijms22126493.
[112] R. Baron, L. Neff, C. Roy, A. Boisvert, M. Caplan, *Cell* **1986**, *46*, 311, https://doi.org/10.1016/0092-8674(86)90748-8.
[113] J. Wu, L. H. Glimcher, A. O. Aliprantis, *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105*, 16934, https://doi.org/10.1073/pnas.0808763105.
[114] A. V. Neutzsky-Wulff, M. A. Karsdal, K. Henriksen, *Calcif. Tissue Int.* **2008**, *83*, 425, https://doi.org/10.1007/s00223-008-9185-7.
[115] D. Brown, S. Breton, *J. Exp.* **1996**, *199*, 2345, https://doi.org/10.1242/jeb.199.11.2345.
[116] K. A. Ishii, T. Fumoto, K. Iwai, S. Takeshita, M. Ito, N. Shimohata, H. Aburatani, S. Taketani, C. J. Lelliott, A. Vidal-Puig, K. Ikeda, *Nat. Med.* **2009**, *15*, 259, https://doi.org/10.1038/nm.1910.
[117] W. Xie, S. Dolder, M. Siegrist, A. Wetterwald, W. Hofstetter, *Calcif. Tissue Int.* **2016**, *99*, 142, https://doi.org/10.1007/s00223-016-0129-3.
[118] R. Balga, A. Wetterwald, J. Portenier, S. Dolder, C. Mueller, W. Hofstetter, *Bone* **2006**, *39*, 325, https://doi.org/10.1016/j.bone.2006.02.056.
[119] D. Yang, Y. wan, *Semin. Immunopathol.* **2019**, *41*, 551, https://doi.org/doi:10.1007/s00281-019-00754-3.
[120] Z. Zhang, V. Kodumuru, S. Sviridov, S. Liu, M. Chafeev, S. Chowdhury, N. Chakka, J. Sun, S. J. Gauthier, M. Mattice, L. G. Ratkay, R. Kwan, J. Thompson, A. B. Cutts, J. Fu, R. Kamboj, Y. P. Goldberg, J. A. Cadieux, *Bioorg. Med. Chem. Lett.* **2012**, *22*, 5108, https://doi.org/10.1016/j.bmcl.2012.05.129.
[1211] J. A. Cadieux, Z. Zhang, M. Mattice, A. Brownlie-Cutts, J. Fu, L. G. Ratkay, R. Kwan, J. Thompson, J. Sanghara, J. Zhong, Y. P. Goldberg, *Bioorg. Med. Chem. Lett.* **2012**, *22*, 90, https://doi.org/10.1016/j.bmcl.2011.11.069.
[122] N. Montalbetti, A. Simonin, C. Simonin, M. Awale, J. L. Reymond, M. A. Hediger, *Biochem. Pharmacol.* **2015**, *96*, 216, https://doi.org/10.1016/j.bcp.2015.05.002.
[123] C. Manatschal, J. Pujol-Giménez, M. Poirier, J. L. Reymond, M. A. Hediger, R. Dutzler, *Elife* **2019**, *8*, e51913, https://doi.org/10.7554/eLife.51913.
[124] M. Poirier, J. Pujol-Giménez, C. Manatschal, S. Bühlmann, A. Embaby, S. Javor, M. A. Hediger, J. L. Reymond, *RSC Med. Chem.* **2020**, *11*, 1023, https://doi.org/10.1039/d0md00085j.
[125] N. Montalbetti, A. Simonin, M. G. Dalghi, G. Kovacs, M. A. Hediger, *J. Biomol. Screen.* **2014**, *19*, 900, https://doi.org/10.1177/1087057114521663.
[126] J. Pujol-Giménez, M. Poirier, S. Bühlmann, C. Schuppisser, R. Bhardwaj, M. Awale, R. Visini, S. Javor, M. A. Hediger, J. L. Reymond, *ChemMedChem* **2021**, *16*, 3306, https://doi.org/10.1002/cmdc.202100467.
[127] C. Manatschal, R. Dutzler, *CHIMIA* **2022**, *76* 1005, https://doi.org/10.2533/chimia.2022.1005.