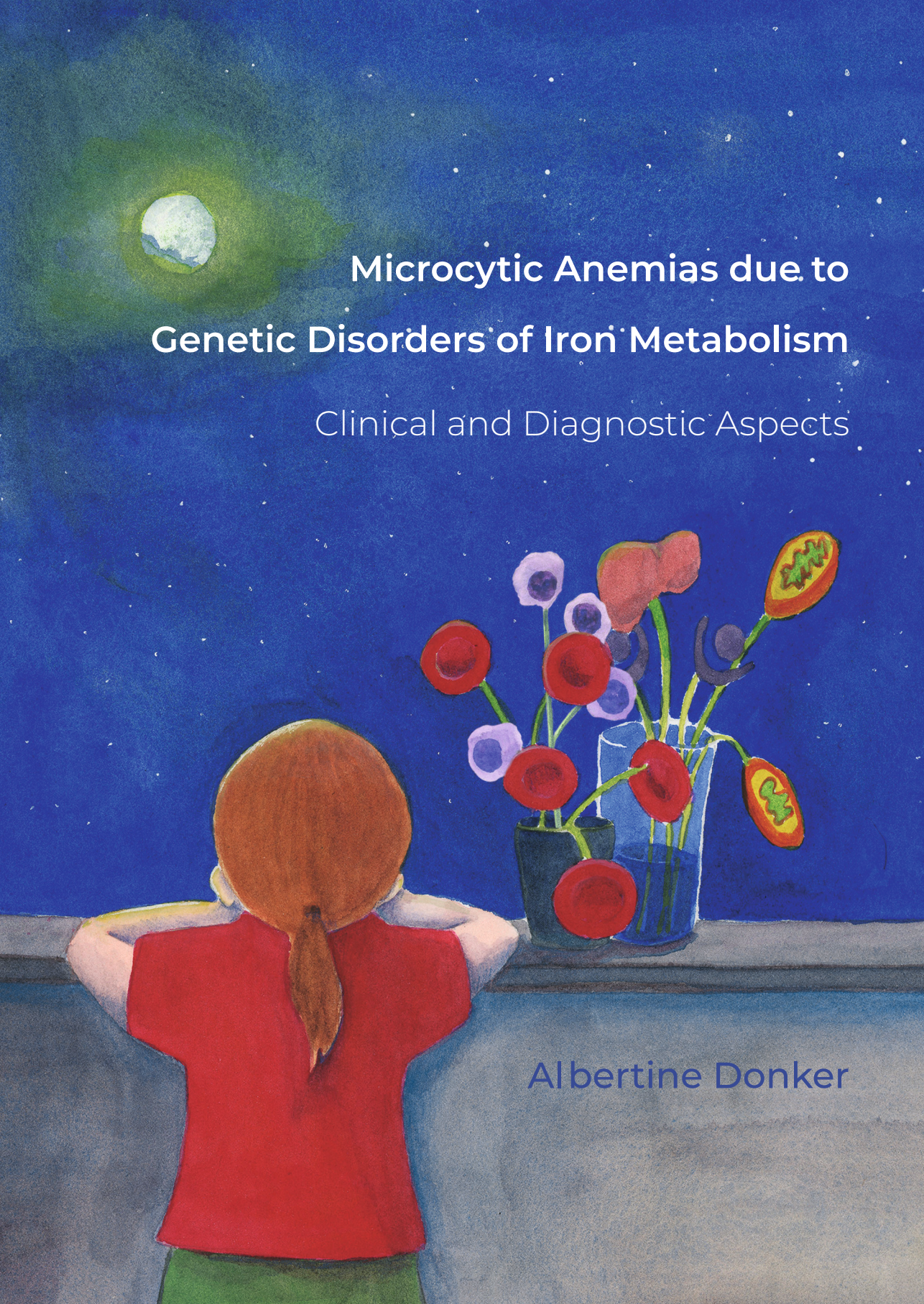
Microcytic Anemias due to Genetic Disorders of Iron Metabolism

Clinical and Diagnostic Aspects



Albertine Donker

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Clinical and Diagnostic Aspects

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The research presented in this thesis was performed at the Translational Metabolic Laboratory, part of the Department of Laboratory Medicine, Radboud Institute for Molecular Life Sciences, Radboud University Medical Center, Nijmegen, the Netherlands and at the Department of Pediatrics, Máxima MC, Veldhoven, the Netherlands. Publication of this thesis was financially supported by the Radboud University Nijmegen, the Netherlands.

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The cover painting represents a paraphrase of a quote by Isaac Newton (1643-1727): “In science we resemble children picking a few flowers from the garden of knowledge, while the wide sky of the unknown unfolds itself above us.”

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General Introduction and Outline of Thesis

GENERAL INTRODUCTION

From childhood to adulthood, anemia is a very common clinical condition. Worldwide, anaemia affects approximately 1.62 billion people, which corresponds to 24.8% of the population.[1] Therefore, both primary care and academic doctors, involved in either pediatric or adult mecidine, are consulted for anemia very often. Despite the multiple causes of anemia, in many patients evaluation is straightforward and reveals a specific diagnosis. A standard diagnostic approach is to classify anemia as microcytic (mean corpuscular volume (MCV) <80 fL), normocytic, or macrocytic (MCV >98 fL). The majority of anemias are microcytic.[1]

Microcytic anemias are primarily caused by iron deficiency (ID). Although the proportion may vary among population groups and in different areas according to the local conditions, it is generally assumed that 50% of the cases of anaemia worldwide are due to ID as a consequence of nutritional deficits, gastro-intestinal or gynecological blood loss or iron malabsorption.[2-5] Furthermore, hemoglobinopathies, especially thalassemia syndromes, need to be considered as a cause of microcytic anemias within specific populations.[1]

In some patients the microcytic anemias cannot be explained by ID or a hemoglobinopathy. In those cases a genetic disorder of iron metabolism or heme synthesis due to defects in genes involved in these processes might be the cause of the microcytic anemia.[6-9] It is a diagnostic challenge for the clinician to distinguish these rare disorders from the far more common afore-mentioned conditions. Missing a microcytic anemia due to a genetic disorder of iron metabolism or heme synthesis may result in an unnecessarily long diagnostic process and ineffective, or even harmful treatments. Moreover, in some genetic anemias, such as the sideroblastic anemias, iron overload (IO) is of greater clinical consequence than the anemia itself since unrecognized tissue iron loading might lead to severe morbidity and even mortality.[8,10]

Apart from diagnostic flowcharts that facilitate clinicians to accurately and timely diagnose these genetic microcytic anemias, treatment protocols are required for the different disorders in order to reduce the disease burden for the individual patient and to avoid lifelong sequelae of ID or IO.

This thesis aims to provide information on several clinical aspects of microcytic anemias due to a genetic disorder of iron metabolism or heme synthesis in order to increase awareness among clinicians for these rare disorders and to facilitate them to establish the right diagnosis and start an appropriate treatment. To achieve these objectives, we studied clinical, genetic and diagnostic aspects of these anemias, with the emphasis on Iron Refractory Iron Deficiency Anemia (IRIDA) due to *TMPRSS6* defects and X-linked Sideroblastic Anemia (XLSA) due to *ALAS2* defects.

PHYSIOLOGY OF SYSTEMIC AND CELLULAR IRON METABOLISM

The vast majority of human body iron is dedicated to hemoglobin synthesis

The human adult body produces 20 mL blood every day, consisting of 200 x 10^9 red blood cells (RBC's). The cytoplasm of RBC's is rich of hemoglobin, an iron-containing bio-molecule that plays a crucial role in oxygen transport. Twenty milligrams of iron every day and 2x 10^{15} iron atoms every second are required for this enormous production, illustrating that the majority of body iron is involved in maintaining adequate erythropoiesis.[11]

In the human body iron alternates between the oxidation states Fe^{2+}(ferrous iron) and Fe^{3+}(ferric iron). This interconversion between Fe^{2+} and Fe^{3+} involves a mechanism whereby iron not only participates in electron transfer, but also has the ability to reversibly bind ligands. These fundamental biochemical characteristics of iron are the basis for its essential roles in many biological processes, e.g. oxygen transport via the heme component of hemoglobin, cellular respiraton as part of heme-containing cytochromes and iron-sulfur (Fe/S) cluster- containing proteins of the electron transport chain, DNA synthesis and cell growth, cell differentiation and regulation of gene expression.[12]

In young children, iron plays an important role in brain growth and development, making them vulnerable to ID.[3,12] On the other hand, iron is a potential toxicant to cells. Unbound iron can catalyze the formation of oxidative radicals that damage proteins, lipids and nucleic acids. Furthermore, many pathogens are dependent of iron for their survival.[13,14] Because both ID and IO may have detrimental effects, a highly sophisticated regulatory system is required to maintain iron homeostasis on both the systemic and cellular level.[11]

Extensive research of the last 20 years revealed that iron metabolism is balanced by two regulatory systems, one that functions systemically and relies on the hormone hepcidin and the cellular iron exporter ferroportin (FPN), and another that predominantly controls cellular iron metabolism through iron-regulatory proteins (IRP's) that bind iron-responsive elements (IRE's) in regulated messenger RNA's of cellular iron importers, exporters and storage genes. Although the machineries of systemic and cellular iron homeostasis are separated, crosstalk exists between the two distinct control systems[11,15,16]

Hepcidin is the key regulator of systemic iron homeostasis

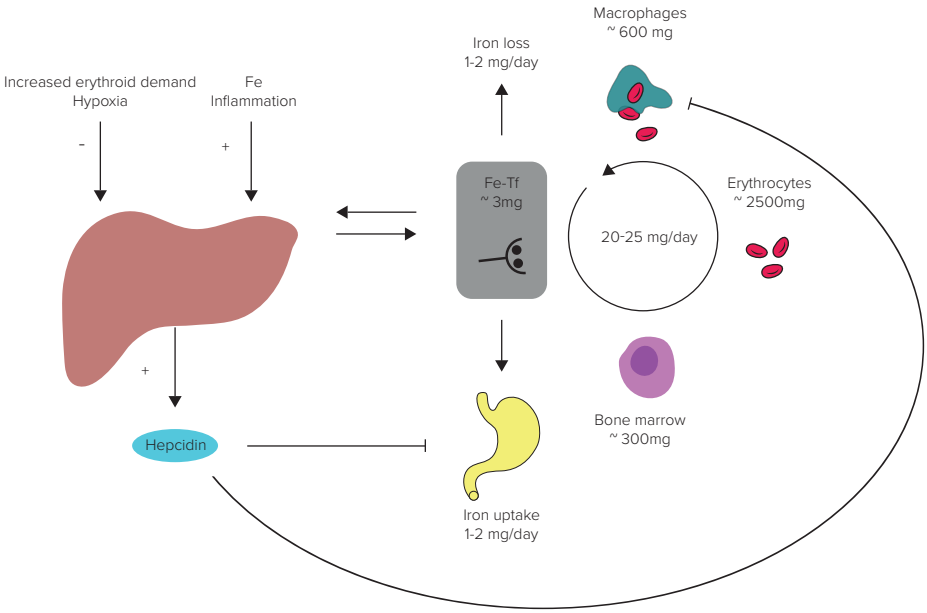
Cells involved in iron homeostasis are duodenal enterocytes, erythroid precursors, hepatocytes and macrophages. The duodenal enterocyte absorbs 1-2 mg per day to balance the small amount of iron that the human body losses via the desquamation of epithelia. Once inside the enterocyte, iron is stored by ferritin or released into the plasma. After oxidation by ferroxidases, iron is loaded to transferrin (Tf) for transport in the plasma. Iron binding to transferrin prevents the formation of unbound iron that is highly toxic because it catalyzes the formation of oxidative radicals. Transferrin binds to specific transferrin receptors, expressed on all iron-containing cells, but mainly on erythroid precursors in the bone marrow, and on hepatocytes. In the bone marrow iron is used for erythropoiesis, in the liver iron is stored in hepatocytes as ferritin or hemosiderin. However, the majority of iron in the circulation is derived from the recycling of senescent erythrocytes (20-25 mg of iron per day). In physiological circumstances, intestinal iron absorption is enhanced in situations of increased iron demands (for example during periods of rapid growth in infancy, childhood and pregnancy and during periods of blood loss e.g. menses).[11] On the other hand, iron absorption is depressed in case of (imminent) IO. Importantly, the human body cannot excrete iron in a regulated way. Therefore, effective communication and fine-tuning between enterocytes, erythroid precursors, hepatocytes and macrophages is crucial in order to prevent both ID and IO. Hepcidin, a 25-amino acid peptide hormone, has emerged as the key regulator of systemic iron homeostasis (**Figure 1**).[11,15,17-19]

Hepcidin, encoded by *HAMP*, is mainly produced and secreted by hepatocytes, circulates in the bloodstream and is excreted by the kidneys. By binding to the (only known) cellular iron exporter FNP and inducing its internalization and degradation, hepcidin regulates the iron dietary iron uptake by the intestines and the release of recycled iron from senescent erythrocytes.[11,15,17,18]

The synthesis of hepcidin is controlled by certain physiologic and pathologic triggers that reflect circulating and stored iron levels, erythropoietic activity, hypoxia and inflammatory signals.[11,15,20,21] Many clinical factors are known to influence circulating hepcidin levels. Impaired renal function, therapy with oral or parenteral iron supplements, systemic infections and inflammatory diseases cause an increase of hepcidin levels while on the opposite anemia (especially iron deficiency anemia (IDA)), treatment with erythropoiesis stimulating agents, chronic

liver disease with impaired synthesis function of the liver and (supplementation of) the sex hormones testosterone and estrogen cause a decrease of hepcidin production.[22,23] Also genetic factors play an important role. Thalassemia syndromes characterized by ineffective erythropoiesis and pathologic variants of the hemochromatosis genes cause inappropriately suppressed serum hepcidin levels.[22,24,25]

Figure 1. Systemic iron homeostasis: key role of hepcidin



The majority of body iron in the circulation is derived from the recycling of senescent erythrocytes by macrophages and its subsequent incorporation in new erythroid precursors in the bone marrow. Only ±1-2 mg of iron is absorbed from the diet and lost from the body every day. Hepcidin regulates the systemic iron concentration by inhibiting iron export from duodenal enterocytes and reticulo-endothelial macrophages. It is predominantly produced by hepatocytes. The synthesis of hepcidin is controlled by certain physiologic and pathologic triggers that reflect circulating and stored iron levels, erythropoietic activity, hypoxia and inflammatory signals. Quantities represent the situation in a male adult with a replete iron status.

Abbreviations: Fe denotes iron; Fe-Tf, transferrin-bound iron. Figure adapted from Swinkels *et al,* 2006.[19]

On the molecular level, multiple signaling pathways, sensing body iron, inflammation and erythropoietic drive, control hepcidin synthesis. The bone morphogenetic protein (BMP)/ sons of mothers against decapentaplegic (SMAD) is the main signaling pathway to regulate the transcription of the *HAMP* gene encoding hepcidin, in particular inside the hepatocyte (**Figure 2**).[11]

Although several BMP's are expressed in the liver (BMP2, BMP4, BMP5, BMP6. BMP9) only BMP2 and BMP6 have been demonstrated to been involved in hepcidin activation so far.[26] Both BMP2 and BMP6 are produced by liver sinusoidal endothelial cells (LSEC). [27] The iron-induced signaling cascade inside the hepatocyte is initiated following an increase of circulating transferrin-bound iron (TBI) and the endothelial secretion of BMP6. The endothelial production of BMP2 is less iron dependent than the production of BMP6; BMP2 is the prevalent ligand that maintains basal hepcidin transcription while BMP6 is strongly up-regulated in case of (imminent) tissue IO.[27,28] At the hepatocyte membrane level, BMP6 and BMP2 bind to the BMP receptor complex, including the BMP type 1 receptors, activin receptor-like kinase 2 (ALK2) or activin receptor-like kinase 3 (ALK3), and the BMP type 2 receptors, BMP receptor 2 (BMPR2) or activin receptor type 2A (ACVR2A).[29] This binding triggers intra-cellular phosphorylation of SMAD1, SMAD5 and SMAD8 and the formation of heteromeric complexes with SMAD4. Subsequently, the SMAD complex is translocated to the nucleus of the hepatocyte where it activates hepcidin transcription upon binding to BMP-responsive elements (BMP-RE's).[27,28] Efficient iron signaling via the BMP/ SMAD pathway to the *HAMP* gene requires the BMP co-receptor hemojuvelin (HJV), the hemochromatosis protein HFE and the transferrin receptor 2 (TfR2).[27] Increased levels of TBI also positively regulate hepcidin synthesis to prevent IO. High circulating levels of TBI result in a shift of binding of HFE from transferrin receptor 1 (TfR1) to TfR2, which can then activate the BMP-SMAD pathway through interactions with HJV and ALK3.[30,31] Pathologic variants of *HFE* and *TfR2* cause inappropriately decreased serum hepcidin levels resulting in IO.[25,32] In case of inflammation the cytokine interleukin 6 (IL6) and activin B induce hepcidin transcription via the janus kinase (JAK)/ signal transducer and activator of transcription 3 (STAT3) signaling pathway and the BMP-SMAD pathway, respectively. The binding of IL6 triggers dimerization of IL6 receptors (IL6R's) on the membrane of the hepatocyte, with subsequent intracellular phosphorylation of STAT3. This phosphorylated complex is translocated to the nucleus where

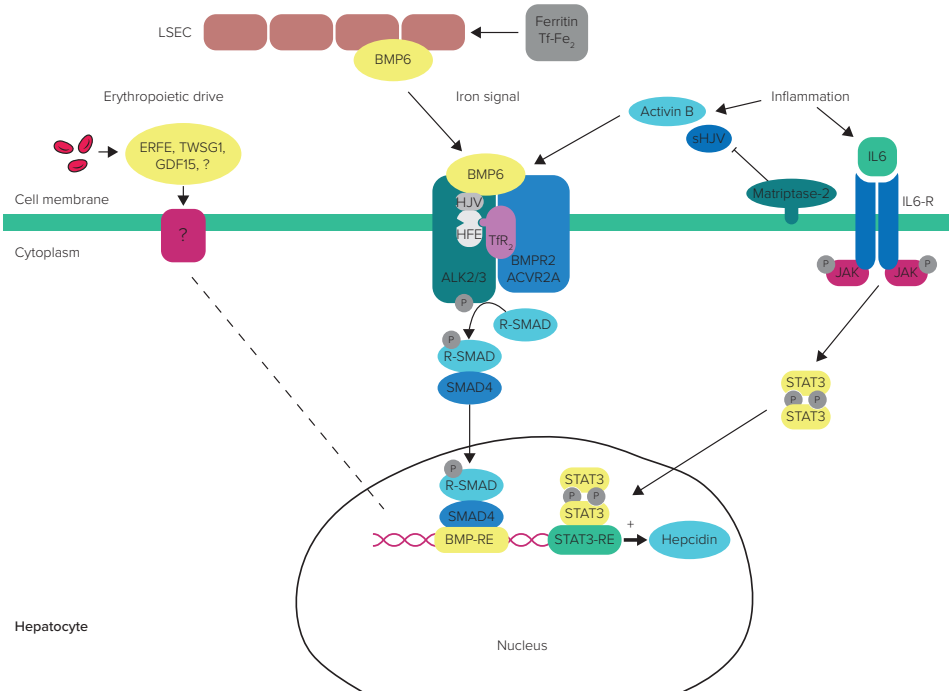
it activates hepcidin transcription upon binding to a STAT-responsive element (STAT-RE) in the *HAMP* promoter.[27,28] On the other hand, suppression of hepcidin transcription is crucial in conditions of ID. The main hepcidin inhibitor responsible for this down-regulation is the transmembrane serine protease 6 (matriptase 2, (MT2), encoded by *TMPRSS6*), mainly expressed in hepatocytes.[27,33-36]

Defects in *TMPRSS6* have provided insights into this mechanism and the crucial role of MT2 in sensing ID and consequent blocking of the hepcidin transcription. Patients with dysfunctional MT2 due to pathologic *TMPRSS6* variants have inappropriately elevated hepcidin levels relative to circulating iron, resulting in IDA that is in general refractory to oral iron supplementation and only partially responsive to parenteral iron administration.[37,38] This condition, called Iron Refractory Iron Deficiency Anemia (IRIDA) is a topic of this thesis and addressed in **Chapter 3, 4, 5** and **8**. Other proteins that have been proposed to mediate hepcidin suppression via the BMP/SMAD signaling pathway are erythroferrone (ERFE), twisted gastrulation (TWSG1) and growth differentiation factor 15 (GDF15).[11,39-41] Mice studies suggest that ERFE suppresses hepcidin transcriptions in both conditions of blood loss with subsequent appropriate increased erythropoiesis and anemias accompanied by iron loading due to ineffective erythropoiesis, whereas GDF15 and TWSG1 seem to play a role only in the last mentioned group. Other erythroid regulators of the hepcidin transcription pathway may exist but still have to be identified. [39]

Iron-regulatory proteins control cellular iron metabolism by binding to Iron-responsive elements in regulated messenger RNA's

Coordination of iron uptake, utilization and storage is also required on the cellular level in order to assure the availability of appropriate supplies and to prevent toxicity due to iron surplus. In contrast to systemic iron metabolism, cellular iron traffic also involves regulated cellular iron excretion.[11,15]

Figure 2. Major pathways of hepcidin regulation



On the molecular level, multiple signaling pathways sensing body iron (middle), inflammation (right) and erythropoietic drive (left), control hepcidin synthesis. The BMP/SMAD is the main signaling pathway to regulate the transcription of the *HAMP* gene encoding hepcidin, in particular inside the hepatocyte. BMP's are produced by LSECs. The iron-induced signaling cascade inside the hepatocyte is initiated following an increase of circulating TBI and stored iron by the endothelial secretion of BMP6. Together with its co-receptor HJV, BMP6 activates type 1 (ALK 2/3) and type 2 (BMPR2, ACVR2A) BMP receptors, leading to phosphorylation of R-SMAD proteins and the formation of active transcriptional complexes with SMAD4. Efficient iron signaling via the BMP/SMAD pathway to the *HAMP* gene also requires the hemochromatosis proteins HFE and the activated TfR2. Subsequently, the SMAD4 complex is translocated to the nucleus of the hepatocyte where it activates hepcidin transcription upon binding to BMP-RE. In case of inflammation the cytokine IL6 and activin B induce hepcidin transcription via the JAK/STAT3 signaling pathway and the BMP-SMAD pathway, respectively. The binding of IL6 triggers dimerization of IL6R and activation of JAK-kinases on the membrane of the hepatocyte, with subsequent intracellular phosphorylation of STAT3. This phosphorylated complex is translocated to the nucleus where it activates hepcidin transcription upon binding to STAT-RE in the *HAMP* promoter. Hepcidin suppression is crucial in conditions of iron deficiency and increased erythropoiesis. The main hepcidin inhibitor responsible for this down-regulation is the transmembrane serine protease 6 (MT2, encoded by *TMPRSS6*) that is able to cleave HJV generating a soluble form of HJV (sHJV) and is mainly expressed in hepatocytes. Other proteins that have been proposed to mediate hepcidin suppression via the BMP/SMAD signaling pathway are ERFE, TWSG1 and GDF15. The signaling pathways of these proteins are unknown. Other erythroid regulators of the hepcidin transcription pathway may exist but still have to be identified.

◂ Abbreviations: ACVR2A denotes Activin receptor type 2A; ALK, Activin receptor-like kinase; BMP, bone morphogenetic protein; BMPR2, BMP receptor 2; BMP-RE, BMP-responsive element; ERFE, erythroferrone; GDF15, growth differentiation factor 15; HJV, hemojuvelin; IL6, interleukin 6; IL6R, IL6 receptor; LSEC, liver sinusoidal endothelial cell; MT2, matriptase 2; R-SMAD, receptor-activated SMAD; sHJV, soluble HJV; SMAD, Sons of mothers against decapentaplegic; STAT3, signal transducer and activator of transcription 3; STAT-RE, STAT-responsive element; TBI, transferrin-bound iron; TfR2, transferrin receptor 2; TWSG1, twisted gastrulation 1. Figure adapted from Muckenthaler *et al*, 2017.[11]

Human cells acquire iron predominantly via TfR1-mediated endocytosis of TBI. In the endosomes, iron is freed from Tf and reduced from Fe^{3+} to Fe^{2+} by a metalloreductase, such as Six-transmembrane epithelial antigen of prostate 3 (STEAP3), prior to its release into the cytosol via Divalent Metal Transporter 1 (DMT1). Of note, DMT is also responsible for the apical resorption of dietary iron after reduction by the ferrireductase duodenal cytochrome B (DCYTB). Apotransferrin and TfR1 return to the plasma membrane to be re-used for next cycles.[15] Biochemical and genetic studies suggests that also transferrin-independent routes of iron uptake exist. Both zinc-regulated transporter (ZRT)/iron-regulated transporter (IRT)-like protein ZIP14 and DMT1 seem to be implicated in non-transferrin-bound iron (NTBI) uptake.[42-45]

Duodenal enterocytes are also able to acquire iron in the form of heme iron. Ingested heme comprises one third of dietary iron but up to two-thirds of absorbed body iron due to the increased bioavailability of heme iron relative to non-heme iron. Although the molecule(s) mediating duodenal enterocyte heme uptake at the brush border has/have not been identified, considerable evidence suggests that uptake occurs via a receptor-mediated endocytosis pathway or by a heme importer, possibly heme carrier protein 1(HCP1).[46]

After the iron has arrived inside the cell, it is stored in the so-called labile iron pool (LIP). The LIP is utilized for direct incorporation into iron proteins or iron transport into mitochondria via mitoferrin (MFRN), where the metal is inserted into heme or Fe/S cluster prosthetic groups. The fraction of the LIP that is not utilized for heme or Fe/S cluster synthesis, can be stored inside the cell as ferritin or exported out of the cell via FPN in the form of Fe^{2+}, after which it is oxidized to Fe^{3+} in order to bind to Tf.

The size of the LIP is determined by the rate of iron uptake, utilization, storage and export. In order to avoid both intracellular detrimental ID and iron toxic iron excess, a tight regulation is required. This regulation occurs post-transcriptionally and involves two cytoplasmic iron regulatory proteins, iron regulatory protein 1 (IRP1) and iron regulatory protein 2 (IRP2) (**Figure 3**).[11,15,16,47,48]

The iron responsive elements (IRE's) constitute of binding sites of the un-translated regions (UTR's) of the mRNA's encoding ferritin and TfR1. Under conditions of iron starvation, IRP1 and IRP2 bind with high affinity to the IRE's of the ferritin mRNA's, thereby inhibiting their translation and preventing intracellular storage or iron. Simultaneously the IRP's stabilize the translation of TfR1 in order to stimulate cellular iron uptake. By contrast, in iron-replete cells IRE-binding activity of both IRP1 and IRP2 is diminished, resulting in the inhibition of further iron uptake and the stimulation of storage of intracellular iron within ferritin.

A characteristic feature of IRP1 is the presence of a Fe/S cluster within its active site. Iron starvation as well as other signals such as hydrogen peroxide and nitric oxide promotes the loss of this Fe/S cluster that triggers a conformational switch, subsequently conferring IRE-binding capacity and conversion from holo-IRP, exhibiting cytosolic aconitase activity, to the apo-IRP1 without cytosolic aconitase activity. On the contrary, IRP2 does not bind a Fe/S cluster, but is regulated at the level of protein stability.[48] The IRE/IRP system also controls the expression of additional IRE-containing mRNA's, including the mRNA's encoding the iron transporters DMT1 and FPN, the enzyme 5-aminolevulinic acid synthase 2 (ALAS2) that catalyzes the first reaction for heme biosynthesis in erythroid progenitor cells,[8] the enzyme of the citric acid cycle mitochondrial aconitase, the cell cycle regulator cell division cycle 14A (CDC14A), and hypoxia inducible factor 2 alpha (HIF2α), a transcription factor that orchestrates molecular responses to hypoxia.[48] The highly complex and sophisticated IRE/IRP system integrates multiple and often opposing signals regarding iron- and oxygen status. As would be expected, the IRE/IRP system impairs translation of HIF2α and FPN in situations of ID since such responses would down-regulate erythropoietin (EPO)-mediated erythropoiesis and would protect enterocytes from intracellular ID. However, such a response might be threatening for iron deficient organisms because iron would be retained in enterocytes instead of being transferred to the plasma. This could be the reason that ID also simulates IRP-mediated stabilization of HIF2α and FPN in combination with HIF2α-mediated transcriptional activation of

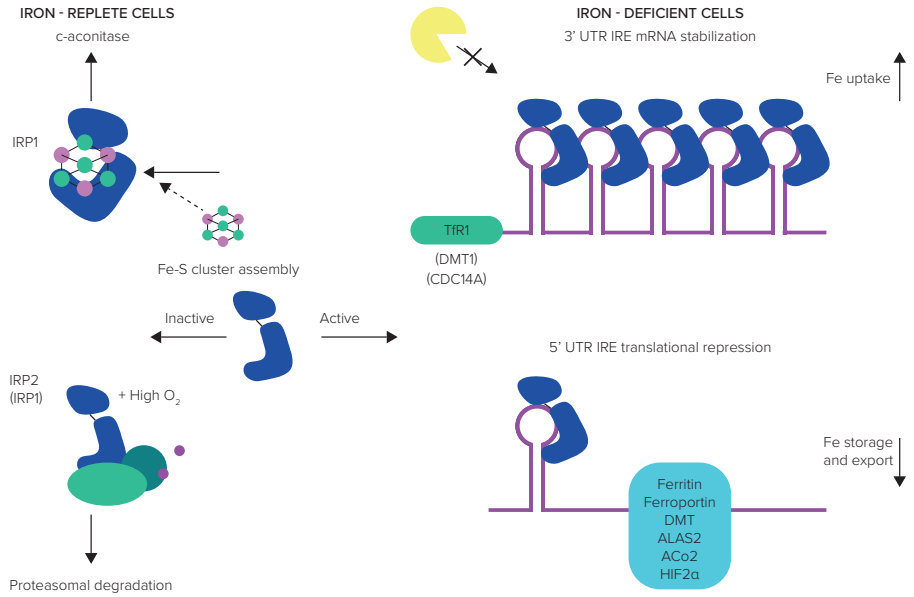
duodenal DMT1 and FPN, resulting in higher transfer of iron from the diet via the plasma to the erythroblasts at the expense of iron depletion of the enterocytes.[48,49] Conversely, in iron replete conditions the IRE/IRP system mediates HIF2α and FPN mRNA translation but also promotes the degradation of HIF2α and FPN via iron- and oxygen dependent signals and hepcidin respectively.[11,48,49]

To summarize, the hepcidin/FPN and IRE/IRP system control the systemic and cellular iron homeostasis respectively via distinct regulatory pathways. However, as described above, connections exist between the two systems on the level of FPN, HIF2α and TfR1.[15,48] First, FPN, that is a critical factor in iron supply to the plasma since it is the only known cellular iron exporter, is subject to both systems. Iron status is sensed on the systemic level and communicated post-translationally via hepcidin, thereby influencing the activity of FPN. On the cellular level, iron levels post-transcriptionally regulate FPN mRNA via the IRE/IRP system.[49] Second, HIF2α is involved in EPO-mediated regulation of hepcidin levels but HIF2α mRNA is also a target of the IRE/IRP system. Third, systemic Tf-Fe_2 displaces TfR1 from HFE inside the hepatocyte, which then subsequently forms a complex with TfR2 and HJV to promote BMP/SMAD signaling leading to *HAMP* transcription, whereas TfR1 expression is modulated by the IRE/IRP system.[15,27] More interconnections between the systemic and cellular iron homeostasis might exist and although the cornerstones of the two systems have been identified, future research is needed to further unravel how the two systems tango and collaborate.

Heme Biosynthesis consists of eight enzymatic reactions in both the mitochondria and cytosol and is regulated by the activity of *ALAS2*

The vast majority of ingested and recycled iron is dedicated to heme synthesis, which plays a crucial role in many fundamental processes because of its oxidation-reduction capacity.[11] Heme serves as the prosthetic group of numerous hemoproteins, a large group of proteins that includes cytochromes (for mitochondrial respiratory chain electron transfer and drug metabolism), oxidases (e.g., nicotinamide adenine dinucleotide phosphate (NADPH) oxidase) and peroxidases, catalases and synthases (e.g., nitric oxide synthase, NOS), as well as the oxygen storage and transport molecules, myoglobin and hemoglobin (Hb).[46,50] Furthermore, heme is essential for the regulation of microRNA processing, protein synthesis and cell differentiation, [51] circadian rhythm [52] and ion-channel functions. [53]

Figure 3. Cellular iron homeostasis: key role of IRE/IRP system



The size of the LIP is determined by the rate of iron uptake, utilization, storage and export. In order to avoid both intracellular detrimental iron deficiency and iron toxic iron excess, a tight regulation is required. This regulation occurs post-transcriptionally and involves two cytoplasmic iron regulatory proteins, IRP1 and IRP2 that are able to bind to IRE's. The IRE's constitute of binding sites of the UTR's of the mRNA's encoding ferritin and TfR1. The binding of IRP's to single IRE' s in the 5′ UTR' s of target mRNA's inhibits their translation, whereas IRP interaction with 3′ UTR IRE's of *TfR1* transcript increases its stability. On the contrary, without IRP binding to the 3′ UTR's of *TfR1*, the transcript is susceptible to endonuclease attack and degradation, leading to down-regulation of translation (yellow figure). Under conditions of iron starvation, IRP1 and IRP2 bind with high affinity to the IRE's of the ferritin mRNAs, thereby inhibiting their translation and preventing intracellular storage or iron. Simultaneously the IRP's stabilize the translation of TfR1 in order to stimulate cellular iron uptake. By contrast, in iron-replete cells IRE-binding activity of both IRP1 and IRP2 is diminished, resulting in the inhibition of further iron uptake and the stimulation of storage of intracellular iron within ferritin. A characteristic feature of IRP1 is the presence of a Fe/S cluster within its active site. Iron starvation promotes the loss of this Fe/S cluster that triggers a conformational switch, subsequently conferring IRE-binding capacity and conversion from holo-IRP, exhibiting cytosolic aconitase activity to the apo-IRP1 without cytosolic aconitase activity; hence the protein is bifunctional. On the contrary, IRP2 does not bind a Fe/S cluster, but is regulated at the level of protein stability. The IRE/IRP system also controls the expression of additional IRE-containing mRNAs, including the mRNA's encoding the iron transporters DMT1 and FPN, the enzyme ALAS2 that catalyzes the first reaction for heme biosynthesis in erythroid progenitor cells, the enzyme AC02, the cell cycle regulator CDC14A, and HIF2α, a transcription factor that orchestrates molecular responses to hypoxia.

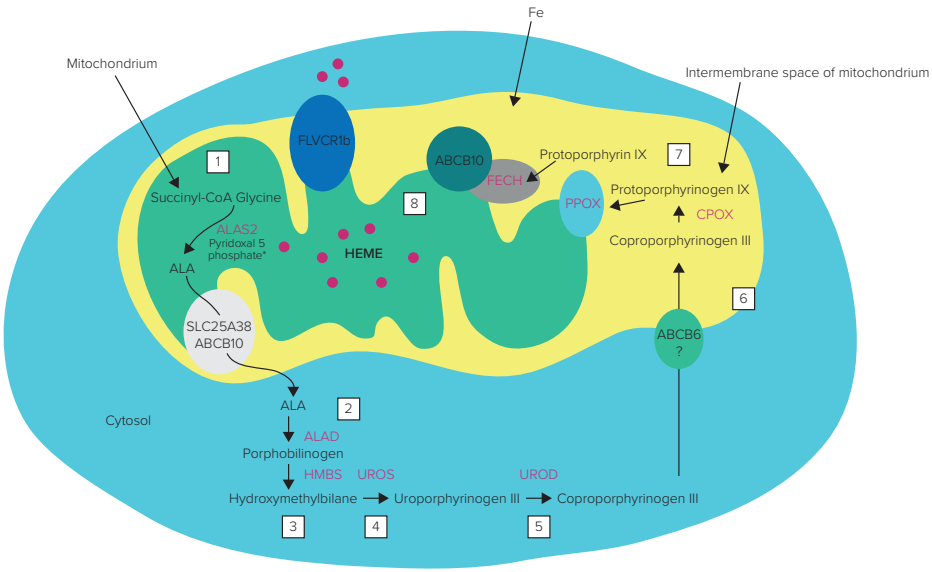
Abbreviations: AC02 denotes citric acid cycle mitochondrial aconitase; ALAS2, 5-aminolevulinic acid synthase 2; CDC14a, Cell division cycle 14A; DMT1, dimetal transporter 1; FNP, ferroportin; HIF2α, hypoxia inducible factor 2 alpha; IRE, iron-responsive elements; IRP, iron-regulatory protein; LIP, labile iron pool; TfR, transferrin receptor; UTR, un-translated regions. Figure adapted from Hentze *et al,* 2010.[15]

Most heme synthesis takes place in developing RBC's in the bone marrow in order to be incorporated in hemoglobin; about 15% of the daily production takes place in the liver for the formation of other heme-containing proteins.[54] the regulatory mechanisms controlling heme synthesis in these two organ systems differ. In the next paragraphs we focus on heme synthesis in the erythropoietin precursors in the bone marrow.

The heme biosynthetic pathway consists of eight enzymatic steps; two reactions take place inside the mitochondria and six inside the cytosol or the intermembrane space of the mitochondria (**Figure 4**).[54,55]

The first and rate-limiting step of heme synthesis occurs in the mitochondrial matrix with the condensation of succinyl-CoA and glycine by ALAS2 to generate aminolevulinic acid (ALA). Pyridoxal 5-Phosphate (vitamin B6) is required as a cofactor in this enzymatic reaction. Both transcriptional and translational mechanisms regulate the synthesis of ALAS2. Transcriptional regulation of *ALAS2* is mediated by erythroid-specific factors (including GATA1), which interact with sequences in the promoter region. Translational regulation involves the IRE/IRP system since the *ALAS2* transcript contains a 5′ IRE that interacts with an IRP. This IRE/IRP complex prevents translation of the ALAS2 mRNA. Addition of a Fe/S abolishes the ability of the IRP to bind to this IRE and permits translation to occur. Fe/S clusters are generated and exported by mitochondria, linking regulation of heme biosynthesis in the red cell to iron availability and mitochondrial function.[56] For further details concerning heme synthesis, see **Figure 4**.[46,54,55]

Figure 4. Heme Biosynthesis consists of eight enzymatic reactions in both the mitochondria and cytosol and is regulated by the activity of *ALAS2*



The heme biosynthetic pathway consists of eight enzymatic steps; two reactions take place inside the mitochondria and six inside the cytosol or the intermembrane space of the mitochondria. The first and rate-limiting step of heme synthesis occurs in the mitochondrial matrix with the condensation of succinyl-CoA and glycine by ALAS2 to generate ALA. Pyridoxal 5-Phosphate (vitamin B6) is required as a cofactor in this enzymatic reaction (**1**). It has been hypothesized that SLC25A38 facilitates ALA production by importing glycine into mitochondria or by exchanging glycine for ALA across the mitochondrial inner membrane. Both SLC25A38 and ABCB10 have been proposed as mitochondrial ALA exporters.

After ALA has been transported out of the mitochondria, it is converted to coproporphyrinogen III by four enzymatic reactions inside the cytosol (**2-5**). ALAD catalyzes the condensation of two molecules of ALA to form one molecule of porphobilinogen (**2**). Next, HMBS catalyzes the synthesis of four porphobilinogen molecules to form the linear hydroxymethylbilane (**3**) that is converted to uroporphyrinogen III by UROS (**4**). The last cytoplasmic step, the synthesis of coproporphyrinogen III (**5**), is catalyzed by UROD. Coproporphyrinogen III is then transported back into the mitochondrial intermembrane space, possibly via ABCB6, where it is converted to protoporphyrinogen IX by CPOX (**6**). The next step is the conversion of protoporphyrinogen IX to protoporphyrin IX by PPOX and its transport into the matrix of the mitochondria (**7**). Inside the mitochondria, Fe+2 that is imported by ABCB10 and MFRN1, is incorporated into protoporphyrin IX by FECH to generate heme, which is the last step of heme biosynthesis (**8**). FLVCR1b may mediate the transfer of heme out of the mitochondria.

Abbreviations: ABCB denotes ATP Binding Cassette Subfamily B; ALA, Aminolevulinic acid; ALAD, aminolevulinate dehydratase; ALAS2, 5-Aminolevulinic acid synthase 2; CPOX, coproporphyrinogen oxidase; FECH, ferrochelatase; FLVCR1, Feline leukemia virus subgroup C receptor 1b; HMBS, hydroxymethylbilane synthase; MFRN, mitoferrin; PPIX, protoporphyrin IX; PPOX, protoporphyrinogen oxidase; SLC25A38, Solute carrier 25A38; UROD, uroporphyrinogen decarboxylase; UROS, uroporphyrinogen synthase. Figure adapted from Chiabrando *et al*, 2014.[55]

DEVELOPMENTAL ASPECTS OF IRON HOMEOSTASIS

Iron homeostasis in early life is fundamentally different from iron homeostasis later in life

As stated above, the key hormone hepcidin and the IRE/IRP system collaborate on the systemic and cellular level, respectively, in order to precisely adjust body iron amounts to the needs of the human individual. However, iron homeostasis during the fetal period and infancy differs from iron homeostasis later in life.[57,58]

Optimal iron status in utero is essential for the development of the fetus and helps establish birth iron stores that are adequate to sustain growth in early infancy. Consistent with the situation in adults, the majority of the iron in the fetus is dedicated to the production of erythrocytes. However, iron *in utero* is also crucial for processes involving brain development as neurotransmitter production, neuronal energy metabolism and myelinisation.[59,60] Therefore, ID in the human fetus and infant is associated with a number of short-term and long-term neurodevelopmental deficits that persist after repletion of iron stores because of critical iron-dependent gestational windows during central nervous system development. Approximately 80% of fetal iron accrues in the last trimester of pregnancy by active placental transport from the mother to the fetus.[61] Under most circumstances, maternal iron status modulates the expression of placental iron transporters in favor of the fetal demands. However, evidence exists that the regulatory system can no longer sustain sufficient transfer of iron from the mother to the fetus in case of severely exhausted maternal iron status, resulting in fetal ID.[62-64] The systemic and local mechanisms that sense and regulate placental iron transport are largely unknown, and the possible role of maternal and/or fetal hepcidin in prenatal iron metabolism still needs clarification.[61,64-68] Also, the molecular pathways of iron trafficking in the placenta on the cellular level are still poorly understood.[65,67,68] Maternal iron status accounts for 6% of the variants in infant iron stores at birth, and the remaining causes of the highly variable size of birth iron endowment are not known, but likely include gestational age, intrauterine growth restriction, time of cord clamping, maternal smoking habits and diabetes.[69]

After the fetus has been born, iron demands remain high to meet the large quantities needed for the rapid growth of the newborn (15-20 mg/kg/day), the accompanying increase in circulating blood volume and the ongoing development of the neonatal

brain. The normal decline in Hb due to the breakdown of fetal Hb significantly increases iron stores in newborns. Moreover, despite the low iron content, the iron-binding protein lactoferrin in breastmilk facilitates iron absorption, resulting in a higher bioavailability than the iron from formula milk and cowmilk.[70,71] Therefore, healthy, term and breastfed infants are initially independent of additional external iron and can double their birth weight before iron stores are depleted.[57,58] Studies in human infants suggest that homeostatic regulation of iron absorption is absent in young infants but matures and is present at nine months of age.[72] Experimental studies in suckling rat pups show similar results; at the age of 10 days when fully nursing, iron absorption is independent of iron status, suggesting a lack of sensing and/or regulation of iron import. At the age of 20 days when pups are partially weaned from breastmilk, ID strongly up-regulates and iron supplementation strongly down-regulates the expression of both DMT1 and FPN suggesting homeostatic regulation of iron absorption that develops and matures during infancy.[73] Furthermore rodent studies have shown that iron absorption is refractory to hepcidin in suckling rats, in spite of intact hepcidin signaling.[74,75] This might be part of an adaptive mechanism to absorb as much iron as possible during the suckling period when milk, which has a low iron content, is the main food. However, this phenomenon might also illustrate the inability to regulate iron absorption by the gut according to physiologic need, which may render the neonate susceptible to iron excess and which can compromise growth and predispose to bacterial infections.[76,77]

Regarding the extracellular transport of iron in the circulation, infants have low levels of ceruloplasmin that is required to oxidize Fe^{2+} to Fe^{3+} in order to enable binding to Tf after cellular iron export. Since also Tf is relatively low in the infant, both the affinity and the capacity to bind free iron is reduced, making the young child vulnerable to damage by NTBI.[78-80] Studies on the intracellular iron metabolism in infants are scarce.[65] Whether further developmental changes in systemic and cellular iron metabolism occur after infancy, during middle and later childhood, is unclear.[81] During adolescence hepcidin levels decrease in response to stimulation of endogenous production of both estrogens and testosterone. Sex hormones result in an increase of growth hormone,[82,83] which in turn also suppresses hepcidin production.[84] This suggests a regulatory mechanism in order to adapt to increased iron demands due to rapid growth and development during puberty in both boys and girls and due to menstrual blood loss in girls.[85,86]

Importantly, hepcidin plays a role as a mediator of antimicrobial activity by causing depletion of extracellular iron, which is thought to be a general defense mechanism against many infections by withholding iron from invading extracellular proliferating pathogens.[87-89] Since (especially young) children are more susceptible to severe life threatening infections, one might argue that relatively high hepcidin levels are favorable in the context of host defense against these extracellular pathogens,[88] outweighing the risk of ID.[14,90] We therefore hypothesize that the set point of systemic iron homeostasis might change during lifetime to adapt to different conditions for which a high amount of iron is relatively favorable or not.

Taken together, it is evident that mechanisms for iron absorption, extracellular iron transport and intracellular iron trafficking change during the journey from early life to adulthood, but many questions remain on both the systemic regulation and cellular pathways that are responsible for adjusting iron supply to the needs of the growing and developing child. Addressing these questions in future research will generate insights in physiological changes of systemic and cellular iron homeostasis during human growth and development and will also facilitate the diagnosis, treatment and monitoring of pediatric genetic disorders of iron metabolism or heme synthesis.

PATHOPHYSIOLOGY OF SYSTEMIC AND CELLULAR IRON METABOLISM

Various genetic and acquired disorders of iron metabolism may result in iron deficiency or iron loading

As described in the previous section, iron homeostasis is tightly regulated on the systemic and cellular level in order to ensure adequate organ supply in a hierarchical manner, with prioritization of the erythroblasts over the brain, heart, skeletal muscle and other organ systems in descending order.[12,91] However, various genetic and acquired conditions may result in a failure of these regulatory systems to maintain adequate systemic and/or cellular iron levels.

On one side of the spectrum, ID occurs when iron demands exceed iron supply. If iron stores are fully depleted, IDA develops. On the systemic level, the mechanisms of adaption to (imminent) ID are centered on the suppression of hepcidin synthesis in order to allow increased enteral iron absorption and iron recycling from the macrophages. MT2, encoded by *TMPRSS6* is essential for this process as described in the previous section. At the cellular level, the IRE/IRP system post-transcriptionally regulates cellular proteins resulting in increased iron import and decreased iron storage and export, and a reduction in heme synthesis in response to (imminent) ID and tissue hypoxia. Furthermore, tissue hypoxia due to anemia induces the kidneys to produce erythropoietin (EPO) in response to enhanced levels of HIF2α. As a consequence of this EPO production, erythropoiesis is increased, resulting in a further decrease of hepcidin production. Mice studies suggest that ERFE, secreted by the erythroblasts, plays a critical role in this pathway.[92] Apart from its effects on the EPO production by the kidneys, HIF2α also increases iron absorption by the intestine via direct transcriptional activation of DMT1, DCYTB and FPN. These mechanisms indicate that hypoxia links erythropoiesis with iron homeostasis.[93] Once stores are exhausted, levels of circulating iron decrease, even if absorption from the enteral lumen is increased. Reduced levels of iron in the liver trigger an increase in the synthesis of the apo-transferrin, further decreasing levels of TBI. Consequently, the uptake of iron from TfRs by all cells and organs is reduced. Owing to the low availability of iron, hypochromic, microcytic erythrocytes are produced, characteristic of IDA.[94,95]

On the other site of the spectrum, both the hepcidin/FPN axis and the IRE/IRP system operate in the opposite way in case of (imminent) iron surplus in order to prevent systemic and cellular overload. Tissue IO may occur when the hepcidin/FPN axis and the IRP system fail to maintain systemic and cellular iron levels below toxic levels.[96] Iron loading occurs in parenchymal tissues or in the reticulo-endothelial system (RES) or both, depending on the underlying pathophysiology. In hereditary hemochromatosis, a group of primary genetic disorders of hepcidin deficiency or resistance, hyper-absorption of dietary iron leads to primarily parenchymal iron accumulation in especially the liver.[97] A similar pattern is seen in iron loading anemias due to ineffective erythropoiesis with subsequently inappropriately decreased hepcidin levels.[98,99] IO of the RES corresponds to iron deposition within Kupffer cells or portal macrophages, and in the spleen, and is usually caused by excessive iron supply (i.e. repeated blood transfusions), infection or inflammation.[97 100] Massive iron surplus eventually results in a mixed type with iron loading in both the parenchyma tissues and the RES. [97]

Iron deficiency anemia is the top-ranking cause of anemia worldwide and has a considerable impact on health

ID affects more than 2 billion people worldwide,[101] and IDA remains the top cause of anemia with a prevalence of over 1.2 billion globally, as confirmed by the analysis of a large number of reports on the burden of disease in 187 countries between 1990 and 2010.[4] Many physiologic and pathologic causes exist that are mostly acquired and commonly occur simultaneously, like increased demands, insufficient dietary intake, malabsorption and/or chronic blood loss.[3,102]

ID and IDA are associated with many adverse effects, depending on the age of the patient, the severity and the progress of the deficiency. ID can cause symptoms of fatigue and paleness, which may worsen to a reduced exercise capacity, cardiac palpitations and dizziness in the case of anemia.[12,103] Furthermore, the development and maturation of the central nervous system in infants and young children is highly dependent on iron-containing enzymes and proteins. ID might therefore have multiple and varied effects on neurocognitive development in children.[59,104] Besides the above-mentioned sequelae and the diverse effects on the central nervous system, ID in children is associated with growth retardation, breath holding spells and febrile convulsions.[2,105] Restless legs, impaired immune response and increased absorption of other metals like aluminum are also associated with decreased iron status in both children and adults.[105,106]

Microcytic anemia not explained by acquired iron deficiency or thalassemia might indicate a genetic disorder of iron metabolism or heme synthesis

Microcytic anemias are primarily caused by ID as a consequece of nutritional deficits, gastro-intestinal or gynecological blood loss or iron malabsorption.[1-3] In specific populations, hemoglobinopathies, espcially thalassemia syndromes are also a common cause of microcytic anemias.[1] However, not all cases of microcytic anemias can be explained by acquired ID or hemoglobinopathy. Assessment of serum iron parameters is paramount in these patients; abnormal transferrin saturation (TSAT) and/or increased ferritin levels might indicate a genetic disorder of iron metabolism or heme synthesis. Especially in case of a positive family history for anemia and/or iron loading, early onset of anemia or anemia that is refractory or incompletely responsive to iron supplementation, clinicians should be aware of a possible genetic cause of microcytic anemia. Additional features as neurologic disease and skin photosensitivity may also be suggestive of these disorders.

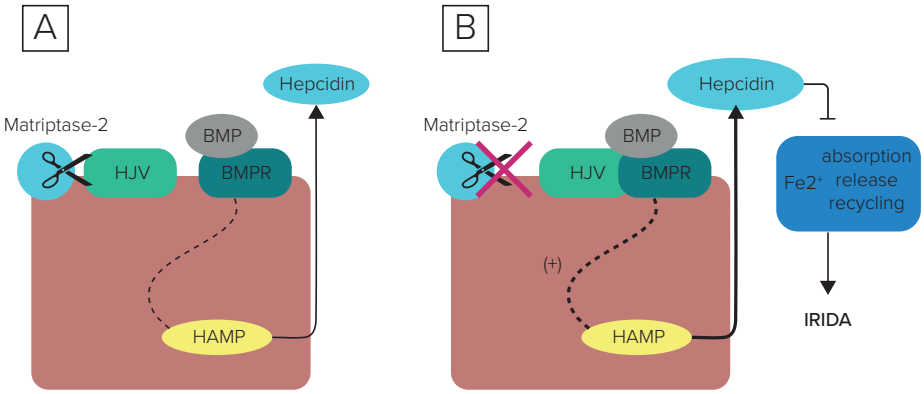
Several defects in genes with roles in systemic and cellular iron metabolism and heme synthesis have been identified as being involved in the pathogenesis of genetic microcytic anemias.[6-9] Pathogenic variants of these genes may result in low iron availability for erythropoiesis, impaired iron acquisition by the erythroid precursors, or defects in heme and/or Fe/S cluster synthesis.

In this thesis we focus on these disorders with the emphasis on Iron Refractory Iron Deficiency Anemia (IRIDA) due to *TMPRSS6* defects, a disorder of low iron availability for erythropoiesis, and on X-linked Sideroblastic anemia (XLSA) due to *ALAS2* defects resulting in impaired heme synthesis.

Iron Refractory Iron Deficiency Anemia: a clinically and genetically a heterogeneous disease

MT2, encoded by *TMPRSS6,* plays an essential role in providing adequate iron supply to the erythroblasts by down-regulating hepcidin (**Figure 2**). Pathogenic *TMPRSS6* defects result in uninhibited hepcidin production despite low body iron levels, causing IRIDA, a disease characterized by a microcytic, hypochromic anemia due to serum hepcidin values that are inappropriately high for circulating iron levels (**Figure 5**). [22,34,37,107-109]

Figure 5. *TMPRSS6* defects cause inappropriately increased hepcidin levels, resulting in Iron Refractory Iron Deficiency Anemia



Matriptase 2, encoded by *TMPRSS6,* plays an essential role in providing adequate iron supply to the erythroblasts and other iron-demanding tissues by down-regulating hepcidin (see also Figure 2). **A**. Physiological situation. MT2 prevents hepcidin overexpression by degrading HJV, which acts as a co-factor for BMP to promote *HAMP* expression, resulting in hepcidin synthesis. **B**. Pathogenic *TMPRSS6* defects leading to matriptase 2 deficiency result in uninhibited hepcidin production despite low body iron levels, due to aberrant cleavage of HJV. This causes IRIDA, a disease characterized by a microcytic, hypochromic anemia due to serum hepcidin values that are inappropriately high for circulating iron levels.

Abbreviations: BMP denotes bone morphogenetic protein; BMPR, BMP receptor; Fe^{2+}, ferrous iron; HJV, hemojuvelin; IRIDA, Iron Refractory Iron Deficiency Anemia. Figure adapted from Cui *et al*, 2009.[109]

IRIDA patients typically present in childhood with microcytic anemia not responding to oral iron, in combination with remarkably low TSAT, which tends to become less severe with increasing age.[110] At the population level, genome wide association studies (GWAS) show that *TMPRSS6* is polymorphic with a relatively high frequency of polymorphisms of which the non-synonymous c.2207C>T (p.Ala736Val) is associated with a significant decrease of the concentrations of iron, Hb, Ht, MCV, MCH and red blood cells.[111,112] These findings are corroborated by functional studies, which show that the 736Ala variant inhibits hepcidin production more efficiently, although GWAS do not support an intermediate role for hepcidin in iron parameters associations. This suggests that other, yet unknown serum hepcidin-independent mechanisms play a role in the association of the p.Ala736Val *TMPRSS6* variant with serum iron parameters.[113-115]

To date, 69 different *TMPRSS6* defects have been identified in 65 IRIDA families with 94 patients of different ethnic origin.[108,116,117] Despite this increasing number of IRIDA cases that are being reported, many questions remain concerning the mode of inheritance, the genotype–phenotype correlation, the diagnostic workup, and the optimal treatment. Until now, IRIDA has been considered as a disorder with an autosomal recessive mode of inheritance but anecdotal data are available of phenotypically affected IRIDA patients in whom only a heterozygous *TMPRSS6* variant was found. [37,38,107,118,119] Concerning the genotype–phenotype relationship, there is a tendency towards lower hemoglobin, MCV and TSAT in patients with two nonsense mutations.[117] The influence of high frequency *TMPRSS6* variants, other still unrecognized genes and environmental factors in the phenotypic expression of the disease still needs clarification. Furthermore, diagnosing IRIDA is challenging because of the highly variable phenotype [108,117] and the unclear genotype–phenotype relation.

In addition, serum hepcidin measurements procedures have not been standardized nor harmonized until now. This hampers comparability of results obtained from different laboratory assays, whereas effective care for IRIDA patients and clinical research on IRIDA and other genetic and acquired disorders of iron metabolism require comparability of laboratory results independent of time, place, and measurement procedure. Failure to recognize that results are standardized nor harmonized may lead to erroneous medical decisions or misinterpretation of research data. As a first step, harmonization ensures traceability to a reference system agreed on by convention. The next step is standardization, ensuring traceability to the International System of Units. [120,121] Standardization but at least harmonization is paramount regarding serum hepcidin measurements procedures.

For children >3-18 years, no reference values of serum hepcidin levels and its concentration relative to TSAT levels are available, hampering the diagnosis of IRIDA in this age group.[122] Also, unsolved questions remain regarding the optimal oral and/or intravenous iron treatment of IRIDA. As the acronym IRIDA implies, patients with *TMPRSS6* defects are usually unresponsive to oral iron. However, anecdotal data suggest that in some IRIDA patients with both mono-allelic and bi-allelic *TMPRSS6* defects hemoglobin levels increase to a clinically acceptable level with prolonged oral iron supplementation, or with a combination of oral iron and vitamin C.[123] Nevertheless, most IRIDA patients require parenteral iron in order

to improve the anemia. To our knowledge, no guidelines are available addressing the route of iron administration, the optimal dose and dosing intervals, taking into account the benefits and possible side effects of oral versus parenteral iron. On one hand, oral iron supplements may result in unabsorbed iron entering the colon causing unwanted side effects on the intestinal host-microbiota interface.[124,125] On the other hand, excess of iron due to parenteral iron treatment will result in iron accumulation in the macrophages of especially the liver and the spleen because of the inappropriately high serum hepcidin levels. This predominantly RES iron storage is also characteristic for patients with loss of function variants in *SLC40A1* encoding FPN, but differs from observations in patients with hereditary hemochromatosis due to variants in *HFE, TfR2, HJV* and *HAMP* and gain of function variants in *SLC40A1* that are associated with iron accumulation in the parenchymal cells, as the hepatocytes, which may result in liver cirrhosis and hepatocellular carcinoma. Therefore, parenchymal iron loading is considered more harmful than RES iron loading.[126-128] However, accumulation in the RES iron has implications for multiple aspects of macrophage antimicrobial activity.

At one hand intracellular iron can be used to support the activity of hemoproteins that exert cytotoxic effects on pathogens. On the other hand, intracellular iron down-regulates the transcription of NOS that is required as a toxic defense against infectious microorganisms.[14,129] Therefore, one might argue that increased levels of RES iron may impair the cytotoxic response of the macrophage against pathogens and promote the survival of intracellular microbes as Salmonellla, Mycobacteria and Legionalla.[88] Moreover, recent data suggest that iron loading of the RES might exacerbate the progression of atherosclerosis by inducing inflammation and enhancing the glycolysis inside the macrophages.[130] Until now, no data exist on these possible side effects of parenteral iron treatment in IRIDA patients but restraint use of intravenous iron supplementation seems justified because of the above-mentioned possible risks of intracellular infections and progression of atherosclerosis.

X-linked sideroblastic anemia due to *ALAS2* defects might result in severe systemic iron loading even in un-transfused patients

XLSA is the most common inherited form of sideroblastic anemia (SA) and is associated with several mutations in the erythroid specific *ALAS2* gene, which is the first and rate limiting step of heme biosynthesis. [54,131-133] Also defects in an

enhancer element in the intron of *ALAS2,* that contains a GATA-binding site, results in clinical phenotypes of XLSA.[134] The disorder is characterized by hypochromic microcytic anemia with ring sideroblasts in erythroid precursor cells in the bone marrow in combination with systemic IO. The IO in XLSA, which is predominantly of parenchymal nature, is thought to be a result of inappropriately low hepcidin levels as a consequence of elevated ERFE levels due to ineffective erythropoiesis, as seen other iron loading anemias, like β-thalassemias, and also occurs in un-transfused patients.[98,99] However, no data on serum hepcidin levels or ERFE levels in XLSA patients are available. Phenotypic expression of XLSA is highly variable even in patients with identical mutations, but affected males generally present in the first decades of life with symptoms of anemia or later with manifestations of parenchymal IO. As in most X-linked recessive disorders, the majority of female carriers of XLSA are spared from clinical manifestations. Sporadically women with *ALAS2* mutations may be affected due to inactivation of the normal X-chromosome or age-related skewed X-inactivation in hematopoietic cells.[135-137]

Recently a family has been described with multiple females affected by macrocytic anemia whereby whole exome sequencing (WES) revealed an *ALAS2* defect. In the reticulocytes of the affected females a complete skewing towards expression of the wildtype allele was found, in contrast to the above-mentioned possible unfavorable lionization pattern with skewing towards expression of the mutated allele causing a phenotype of XLSA with microcytic anemia and IO. The results of the X inactivation pattern studies in this family illustrate how this X-linked dominant mutation in *ALAS2* can perturb normal erythropoiesis.[138]

Standard treatment of XLSA consists of high dose pyridoxine supplementation and iron reducing strategies like phlebotomies and iron chelation.[10] The effect of high dose vitamin B6 is based on the high prevalence of mutations in the pyridoxine-binding region of the *ALAS2*-gene. The high dose enhances the half-life of ALAS2, however, this is not true for mutations outside this region.[139] Reduction of IO in XLSA improves erythropoiesis and prevents complications of chronic parenchymal iron overload, especially liver cirrhosis and hepatocellular carcinoma.[99,140] A timely diagnosis of XLSA therefore avoids unnecessary investigations for other causes of microcytic anemia and allows early detection of subclinical, progressive IO that may result in severe morbidity of even mortality if it goes unnoticed.

Lack of evidence based clinical guidelines for genetic disorders of iron metabolism or heme synthesis hamper timely diagnosis and adequate treatment of these disorders

Discriminating the rare genetic disorders of iron metabolism or heme synthesis from the highly prevalent microcytic anemias due to acquired ID is a diagnostic challenge for physicians. Timely diagnosis of these diseases is paramount in order to avoid an unnecessarily long diagnostic process or a wrong diagnosis with subsequently a not effective or even harmful treatment. Moreover, in some genetic anemias, such as the sideroblastic anemias, IO is of greater consequence than the anemia itself since unrecognized tissue iron loading might lead to severe morbidity and even mortality.[8,10]

Until now, unawareness among clinicians of theses anemias and lack of evidence-based guidelines delay the diagnostic and therapeutic process of these diseases. Literature largely consists of anecdotal data and narrative reviews. Studies on genotype-phenotype correlation are mostly not available. For the diagnosis of IRIDA, serum hepcidin levels are indispensable since the cornerstone of the diagnosis involves an inappropriately increased serum hepcidin level in relation to body iron status. However, harmonized or standardized reference values of hepcidin for children are not available until now, hampering the diagnosis of IRIDA in children.

OUTLINE AND AIMS OF THIS THESIS

We aim to increase awareness among clinicians and to facilitate them to accurately and timely diagnose these rare disorders in order to optimize management and to prevent not effective or even harmful treatments. To achieve this aim, this thesis explores clinical, genetic and diagnostic aspects of microcytic anemias due to genetic disorders of iron metabolism or heme synthesis.

PART I: LITERATURE AND CLINICAL STUDIES

In **Chapter 2** we review the literature on developmental aspects of systemic iron metabolism, summarize the physiologic changes in iron homeostasis that occur during the journey from fetus to adult and identify research gaps on this topic. Therewith we aim to contribute to a better understanding of pediatric genetic disorders of iron metabolism or heme synthesis.

As a first step to improve the clinical management of microcytic anemias due to iron metabolism and heme synthesis, we explored the literature on clinical, biochemical and genetical studies of these disorders. In **Chapter 3** we present evidence-based multidisciplinary guidelines for the diagnosis and treatment of 12 disorders of microcytic anemia that result from defects in 13 different genes and that lead to genetic disorders of iron metabolism and heme synthesis. We briefly discuss pathogenesis, epidemiology, clinical presentation, diagnosis and treatment, and also provide recommendations on family screening.[141]

In the next chapters we describe case series for a disorder of iron metabolism (IRIDA) and a disorder of heme synthesis (XLSA), respectively. In **Chapter 4** we assess clinical presentation, disease severity, response to (oral) iron supplementation and genotype-phenotype correlation of a case series of Dutch IRIDA patients, in order to get more insight in the clinical and genetic nature of this anemia.[142] In **Chapter 5** we discuss the pathogenicity of the non-synonymous *TMPRSS6* changes c.757A>G (p.Lys253Glu, rs2235324) and c.2207 T>C (p.Val736Ala, rs855791) in response to a paper on a suggested IRIDA case of de Nie *et al.*[143,144] In **Chapter 6** we present clinical and genetic data of Dutch XLSA patients.[145]

PART II: DIAGNOSTIC STUDIES: FOCUS ON IRON REFRACTORY IRON DEFICIENCY ANEMIA

Measurement of serum hepcidin plays an important role in the diagnosis of iron related disorders, especially of IRIDA. In children, diagnosing IRIDA is hampered by the lack of pediatric studies defining reference values of serum hepcidin in healthy children of different age groups, relative to iron status. Therefore, we assessed serum hepcidin levels in healthy children from different age groups, relative to body iron indicators. The results are presented in Chapter 7.[146]

Since the cardinal feature of IRIDA is a discrepantly high serum hepcidin in relation to the low circulating iron levels, we hypothesize that the TSAT/hepcidin ratio could be a useful diagnostic tool in order to discriminate between IRIDA and patients presenting with an iron deficient microcytic anemia because of other reasons, e.g., inadequate intake, blood loss or other forms of refractory IDA, such as celiac disease, autoimmune gastritis, and Helicobacter pylori.[102,147,148] However, before its introduction as a diagnostic test in the work up of iron deficient microcytic anemic in patients suspected for the presence of IRIDA, the ratio needs confirmation. In Chapter 8 we assessed the TSAT/hepcidin ratio in adult non-IRIDA IDA patients.

PART III: DISCUSSION, SUMMARY AND ADDENDA

In Chapter 9 we discuss the results of our studies and the still unsolved issues, after which we suggest future perspectives and a research agenda. In Chapter 10 we summarize our work. Chapter 11 contains the addenda of this thesis.

REFERENCES

1. World Health Organisation. Incidence of Anemia. http://www.who.int/vmnis/anaemia/prevalence/summary/anaemia_data_status_t2/en/. Accessed 28 May 2019
2. World Health Organisation. Asessment of Iron Status. http://www.who.int/nutrition/publications/en/ida_assessment_prevention_control.pdf. Accessed 28 May 2019
3. Camaschella C. Iron-deficiency anemia. *The New England journal of medicine.* 2015;372(19):1832-1843.
4. Kassebaum NJ, Jasrasaria R, Naghavi M, et al. A systematic analysis of global anemia burden from 1990 to 2010. *Blood.* 2014;123(5):615-624.
5. Stevens GA, Finucane MM, De-Regil LM, et al. Global, regional, and national trends in haemoglobin concentration and prevalence of total and severe anaemia in children and pregnant and non-pregnant women for 1995-2011: a systematic analysis of population-representative data. *The Lancet Global health.* 2013;1(1):e16-25.
6. Iolascon A, De Falco L, Beaumont C. Molecular basis of inherited microcytic anemia due to defects in iron acquisition or heme synthesis. *Haematologica.* 2009;94(3):395-408.
7. van Rooijen KL, Raymakers RA, Cuijpers ML, Brons PP, Janssen MC, Swinkels DW. [New causes of microcytic anaemia: hereditary disorders of iron homeostasis]. *Nederlands tijdschrift voor geneeskunde.* 2010;154:A1039.
8. Camaschella C. Recent advances in the understanding of inherited sideroblastic anaemia. *British journal of haematology.* 2008;143(1):27-38.
9. Fleming MD. Congenital sideroblastic anemias: iron and heme lost in mitochondrial translation. *Hematology / the Education Program of the American Society of Hematology American Society of Hematology Education Program.* 2011;2011:525-531.
10. Camaschella C. Hereditary sideroblastic anemias: pathophysiology, diagnosis, and treatment. *Seminars in hematology.* 2009;46(4):371-377.
11. Muckenthaler MU, Rivella S, Hentze MW, Galy B. A Red Carpet for Iron Metabolism. *Cell.* 2017;168(3):344-361.
12. Beard JL. Iron biology in immune function, muscle metabolism and neuronal functioning. *The Journal of nutrition.* 2001;131(2s-2):568S-579S; discussion 580S.
13. Fleming RE, Ponka P. Iron overload in human disease. *The New England journal of medicine.* 2012;366(4):348-359.
14. Armitage AE, Drakesmith H. Genetics. The battle for iron. *Science.* 2014;346(6215):1299-1300.
15. Hentze MW, Muckenthaler MU, Galy B, Camaschella C. Two to tango: regulation of Mammalian iron metabolism. *Cell.* 2010;142(1):24-38.
16. Muckenthaler MU, Galy B, Hentze MW. Systemic iron homeostasis and the iron-responsive element/iron-regulatory protein (IRE/IRP) regulatory network. *Annual review of nutrition.* 2008;28:197-213.
17. Ganz T. Hepcidin and iron regulation, 10 years later. *Blood.* 2011;117(17):4425-4433.
18. Nemeth E, Ganz T. The role of hepcidin in iron metabolism. *Acta haematologica.* 2009;122(2-3):78-86.
19. Swinkels DW, Janssen MC, Bergmans J, Marx JJ. Hereditary hemochromatosis: genetic complexity and new diagnostic approaches. *Clinical chemistry.* 2006;52(6):950-968.
20. Pak M, Lopez MA, Gabayan V, Ganz T, Rivera S. Suppression of hepcidin during anemia requires erythropoietic activity. *Blood.* 2006;108(12):3730-3735.
21. Kroot JJ, Tjalsma H, Fleming RE, Swinkels DW. Hepcidin in human iron disorders: diagnostic implications. *Clinical chemistry.* 2011;57(12):1650-1669.
22. Girelli D, Nemeth E, Swinkels DW. Hepcidin in the diagnosis of iron disorders. *Blood.*

2016;127(23):2809-2813.
23. Nicolas G, Chauvet C, Viatte L, et al. The gene encoding the iron regulatory peptide hepcidin is regulated by anemia, hypoxia, and inflammation. *The Journal of clinical investigation.* 2002;110(7):1037-1044.
24. Nemeth E, Ganz T. Hepcidin and iron-loading anemias. *Haematologica.* 2006;91(6):727-732.
25. Nemeth E, Roetto A, Garozzo G, Ganz T, Camaschella C. Hepcidin is decreased in TFR2 hemochromatosis. *Blood.* 2005;105(4):1803-1806.
26. Zhang AS, Anderson SA, Wang J, et al. Suppression of hepatic hepcidin expression in response to acute iron deprivation is associated with an increase of matriptase-2 protein. *Blood.* 2011;117(5):1687-1699.
27. Silvestri L, Nai A, Dulja A, Pagani A. Hepcidin and the BMP-SMAD pathway: An unexpected liaison. *Vitamins and hormones.* 2019;110:71-99.
28. Katsarou A, Pantopoulos K. Hepcidin Therapeutics. *Pharmaceuticals (Basel).* 2018;11(4).
29. Mayeur C, Leyton PA, Kolodziej SA, Yu B, Bloch KD. BMP type II receptors have redundant roles in the regulation of hepatic hepcidin gene expression and iron metabolism. *Blood.* 2014;124(13):2116-2123.
30. Wu XG, Wang Y, Wu Q, et al. HFE interacts with the BMP type I receptor ALK3 to regulate hepcidin expression. *Blood.* 2014;124(8):1335-1343.
31. Latour C, Besson-Fournier C, Meynard D, et al. Differing impact of the deletion of hemochromatosis-associated molecules HFE and transferrin receptor-2 on the iron phenotype of mice lacking bone morphogenetic protein 6 or hemojuvelin. *Hepatology (Baltimore, Md).* 2016;63(1):126-137.
32. Papanikolaou G, Tzilianos M, Christakis JI, et al. Hepcidin in iron overload disorders. *Blood.* 2005;105(10):4103-4105.
33. Ramsay AJ, Hooper JD, Folgueras AR, Velasco G, Lopez-Otin C. Matriptase-2 (TMPRSS6): a proteolytic regulator of iron homeostasis. *Haematologica.* 2009;94(6):840-849.
34. Du X, She E, Gelbart T, et al. The serine protease TMPRSS6 is required to sense iron deficiency. *Science.* 2008;320(5879):1088-1092.
35. Knutson MD. Into the matrix: regulation of the iron regulatory hormone hepcidin by matriptase-2. *Nutrition reviews.* 2009;67(5):284-288.
36. Muckenthaler MU. Fine tuning of hepcidin expression by positive and negative regulators. *Cell metabolism.* 2008;8(1):1-3.
37. Finberg KE, Heeney MM, Campagna DR, et al. Mutations in TMPRSS6 cause iron-refractory iron deficiency anemia (IRIDA). *Nature genetics.* 2008;40(5):569-571.
38. Beutler E, Van Geet C, te Loo DM, et al. Polymorphisms and mutations of human TMPRSS6 in iron deficiency anemia. *Blood cells, molecules & diseases.* 2010;44(1):16-21.
39. Kautz L, Nemeth E. Molecular liaisons between erythropoiesis and iron metabolism. *Blood.* 2014;124(4):479-482.
40. Casanovas G, Vujic Spasic M, Casu C, et al. The murine growth differentiation factor 15 is not essential for systemic iron homeostasis in phlebotomized mice. *Haematologica.* 2013;98(3):444-447.
41. Tanno T, Bhanu NV, Oneal PA, et al. High levels of GDF15 in thalassemia suppress expression of the iron regulatory protein hepcidin. *Nature medicine.* 2007;13(9):1096-1101.
42. Nam H, Wang CY, Zhang L, et al. ZIP14 and DMT1 in the liver, pancreas, and heart are differentially regulated by iron deficiency and overload: implications for tissue iron uptake in iron-related disorders. *Haematologica.* 2013;98(7):1049-1057.
43. Coffey R, Knutson MD. The plasma membrane metal-ion transporter ZIP14 contributes to nontransferrin-bound iron uptake by human beta-cells. *American journal of physiology Cell physiology.* 2017;312(2):C169-c175.
44. Jenkitkasemwong S, Wang CY, Coffey R, et al. SLC39A14 Is Required for the Development of Hepatocellular Iron Overload in Murine

Models of Hereditary Hemochromatosis. *Cell metabolism.* 2015;22(1):138-150.
45. Knutson MD. Non-transferrin-bound iron transporters. *Free radical biology & medicine.* 2019;133:101-111.
46. Khan AA, Quigley JG. Control of intracellular heme levels: heme transporters and heme oxygenases. *Biochimica et biophysica acta.* 2011;1813(5):668-682.
47. Hentze MW, Caughman SW, Rouault TA, et al. Identification of the iron-responsive element for the translational regulation of human ferritin mRNA. *Science.* 1987;238(4833):1570-1573.
48. Wilkinson N, Pantopoulos K. The IRP/IRE system in vivo: insights from mouse models. *Frontiers in pharmacology.* 2014;5:176.
49. Drakesmith H, Nemeth E, Ganz T. Ironing out Ferroportin. *Cell metabolism.* 2015;22(5):777-787.
50. Ponka P. Cell biology of heme. *Am J Med Sci.* 1999;318(4):241-256.
51. Faller M, Matsunaga M, Yin S, Loo JA, Guo F. Heme is involved in microRNA processing. *Nature structural & molecular biology.* 2007;14(1):23-29.
52. Imaizumi T, Kay SA, Schroeder JI. Circadian rhythms. Daily watch on metabolism. *Science.* 2007;318(5857):1730-1731.
53. Wang S, Publicover S, Gu Y. An oxygen-sensitive mechanism in regulation of epithelial sodium channel. *Proceedings of the National Academy of Sciences of the United States of America.* 2009;106(8):2957-2962.
54. Ajioka RS, Phillips JD, Kushner JP. Biosynthesis of heme in mammals. *Biochimica et biophysica acta.* 2006;1763(7):723-736.
55. Chiabrando D, Mercurio S, Tolosano E. Heme and erythropoieis: more than a structural role. *Haematologica.* 2014;99(6):973-983.
56. Ye H, Rouault TA. Erythropoiesis and iron sulfur cluster biogenesis. *Advances in hematology.* 2010;2010.
57. Lonnerdal B, Georgieff MK, Hernell O. Developmental Physiology of Iron Absorption, Homeostasis, and Metabolism in the Healthy Term Infant. *The Journal of pediatrics.* 2015;167(4 Suppl):S8-14.
58. Lonnerdal B. Development of iron homeostasis in infants and young children. *The American journal of clinical nutrition.* 2017;106(Suppl 6):1575s-1580s.
59. Georgieff MK. Long-term brain and behavioral consequences of early iron deficiency. *Nutrition reviews.* 2011;69 Suppl 1:S43-48.
60. Prado EL, Dewey KG. Nutrition and brain development in early life. *Nutrition reviews.* 2014;72(4):267-284.
61. Cao C, Fleming MD. The placenta: the forgotten essential organ of iron transport. *Nutrition reviews.* 2016;74(7):421-431.
62. Kumar A, Rai AK, Basu S, Dash D, Singh JS. Cord blood and breast milk iron status in maternal anemia. *Pediatrics.* 2008;121(3):e673-677.
63. Singla PN, Tyagi M, Shankar R, Dash D, Kumar A. Fetal iron status in maternal anemia. *Acta paediatrica (Oslo, Norway : 1992).* 1996;85(11):1327-1330.
64. Basu S, Kumar N, Srivastava R, Kumar A. Maternal and Cord Blood Hepcidin Concentrations in Severe Iron Deficiency Anemia. *Pediatrics and neonatology.* 2016;57(5):413-419.
65. Lipinski P, Stys A, Starzynski RR. Molecular insights into the regulation of iron metabolism during the prenatal and early postnatal periods. *Cellular and molecular life sciences : CMLS.* 2013;70(1):23-38.
66. Koenig MD, Tussing-Humphreys L, Day J, Cadwell B, Nemeth E. Hepcidin and iron homeostasis during pregnancy. *Nutrients.* 2014;6(8):3062-3083.
67. Fisher AL, Nemeth E. Iron homeostasis during pregnancy. *The American journal of clinical nutrition.* 2017;106(Suppl 6):1567s-1574s.
68. Sangkhae V, Nemeth E. Placental iron transport: The mechanism and regulatory circuits. *Free radical biology & medicine.* 2019;133:254-261.
69. Siddappa AM, Rao R, Long JD, Widness JA, Georgieff MK. The assessment of newborn iron stores at birth: a review of the literature

and standards for ferritin concentrations. *Neonatology.* 2007;92(2):73-82.
70. Suzuki YA, Shin K, Lonnerdal B. Molecular cloning and functional expression of a human intestinal lactoferrin receptor. *Biochemistry.* 2001;40(51):15771-15779.
71. Yalcin SS, Baykan A, Yurdakok K, Yalcin S, Gucus AI. The factors that affect milk-to-serum ratio for iron during early lactation. *Journal of pediatric hematology/oncology.* 2009;31(2):85-90.
72. Domellof M, Lonnerdal B, Abrams SA, Hernell O. Iron absorption in breast-fed infants: effects of age, iron status, iron supplements, and complementary foods. *The American journal of clinical nutrition.* 2002;76(1):198-204.
73. Leong WI, Bowlus CL, Tallkvist J, Lonnerdal B. DMT1 and FPN1 expression during infancy: developmental regulation of iron absorption. *American journal of physiology Gastrointestinal and liver physiology.* 2003;285(6):G1153-1161.
74. Frazer DM, Wilkins SJ, Darshan D, Mirciov CSG, Dunn LA, Anderson GJ. Ferroportin Is Essential for Iron Absorption During Suckling, But Is Hyporesponsive to the Regulatory Hormone Hepcidin. *Cellular and molecular gastroenterology and hepatology.* 2017;3(3):410-421.
75. Darshan D, Wilkins SJ, Frazer DM, Anderson GJ. Reduced expression of ferroportin-1 mediates hyporesponsiveness of suckling rats to stimuli that reduce iron absorption. *Gastroenterology.* 2011;141(1):300-309.
76. Lind T, Seswandhana R, Persson LA, Lonnerdal B. Iron supplementation of iron-replete Indonesian infants is associated with reduced weight-for-age. *Acta paediatrica (Oslo, Norway : 1992).* 2008;97(6):770-775.
77. Lonnerdal B. Excess iron intake as a factor in growth, infections, and development of infants and young children. *The American journal of clinical nutrition.* 2017;106(Suppl 6):1681s-1687s.
78. Scott PH, Berger HM, Kenward C, Scott P, Wharton BA. Effect of gestational age and intrauterine nutrition on plasma transferrin and iron in the newborn. *Arch Dis Child.* 1975;50(10):796-798.
79. Fryer AA, Jones P, Strange R, Hume R, Bell JE. Plasma protein levels in normal human fetuses: 13 to 41 weeks' gestation. *British journal of obstetrics and gynaecology.* 1993;100(9):850-855.
80. Collard KJ. Iron homeostasis in the neonate. *Pediatrics.* 2009;123(4):1208-1216.
81. Domellof M. Iron requirements, absorption and metabolism in infancy and childhood. *Curr Opin Clin Nutr Metab Care.* 2007;10(3):329-335.
82. Melmed. *Williams Textbook of Endocrinology.* 13 ed2016.
83. Veldhuis JD, Roemmich JN, Richmond EJ, et al. Endocrine control of body composition in infancy, childhood, and puberty. *Endocrine reviews.* 2005;26(1):114-146.
84. Troutt JS, Rudling M, Persson L, et al. Circulating human hepcidin-25 concentrations display a diurnal rhythm, increase with prolonged fasting, and are reduced by growth hormone administration. *Clinical chemistry.* 2012;58(8):1225-1232.
85. Lehtihet M, Bonde Y, Beckman L, et al. Circulating Hepcidin-25 Is Reduced by Endogenous Estrogen in Humans. *PloS one.* 2016;11(2):e0148802.
86. Bachman E, Feng R, Travison T, et al. Testosterone suppresses hepcidin in men: a potential mechanism for testosterone-induced erythrocytosis. *J Clin Endocrinol Metab.* 2010;95(10):4743-4747.
87. Michels K, Nemeth E, Ganz T, Mehrad B. Hepcidin and Host Defense against Infectious Diseases. *PLoS pathogens.* 2015;11(8):e1004998.
88. Ganz T. Iron and infection. *International journal of hematology.* 2017.
89. Burte F, Brown BJ, Orimadegun AE, et al. Circulatory hepcidin is associated with the anti-inflammatory response but not with iron or anemic status in childhood malaria. *Blood.* 2013;121(15):3016-3022.

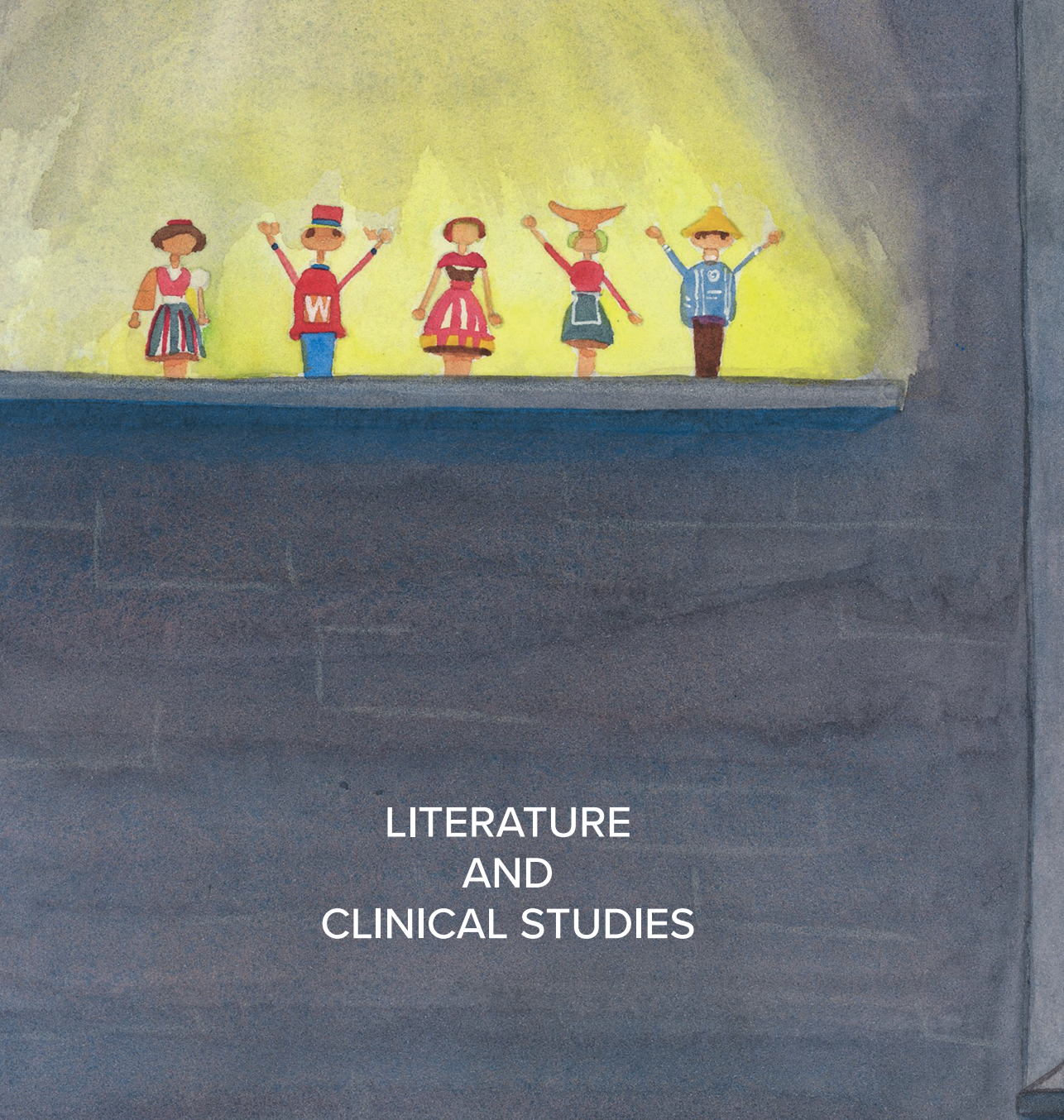
90. Pietrangelo A. Pathogens, Metabolic Adaptation, and Human Diseases--An Iron-Thrifty Genetic Model. *Gastroenterology.* 2015;149(4):834-838.
91. Georgieff MK. Iron assessment to protect the developing brain. *The American journal of clinical nutrition.* 2017;106(Suppl 6):1588s-1593s.
92. Kautz L, Jung G, Valore EV, Rivella S, Nemeth E, Ganz T. Identification of erythroferrone as an erythroid regulator of iron metabolism. *Nature genetics.* 2014;46(7):678-684.
93. Renassia C, Peyssonnaux C. New insights into the links between hypoxia and iron homeostasis. *Current opinion in hematology.* 2019.
94. Camaschella C. Iron and hepcidin: a story of recycling and balance. *Hematology / the Education Program of the American Society of Hematology American Society of Hematology Education Program.* 2013;2013:1-8.
95. Camaschella C. Iron-Deficiency Anemia. *The New England journal of medicine.* 2015;373(5):485-486.
96. Ganz T, Nemeth E. Hepcidin and iron homeostasis. *Biochimica et biophysica acta.* 2012;1823(9):1434-1443.
97. Deugnier Y, Turlin B. Pathology of hepatic iron overload. *Seminars in liver disease.* 2011;31(3):260-271.
98. Origa R, Cazzola M, Mereu E, et al. Differences in the erythropoiesis-hepcidin-iron store axis between hemoglobin H disease and beta-thalassemia intermedia. *Haematologica.* 2015;100(5):e169-171.
99. Pippard MJ, Weatherall DJ. Iron absorption in non-transfused iron loading anaemias: prediction of risk for iron loading, and response to iron chelation treatment, in beta thalassaemia intermedia and congenital sideroblastic anaemias. *Haematologia.* 1984;17(1):17-24.
100. Weiss G, Ganz T, Goodnough LT. Anemia of inflammation. *Blood.* 2019;133(1):40-50.
101. McLean E, Cogswell M, Egli I, Wojdyla D, de Benoist B. Worldwide prevalence of anaemia, WHO Vitamin and Mineral Nutrition Information System, 1993-2005. *Public health nutrition.* 2009;12(4):444-454.
102. Camaschella C. New insights into iron deficiency and iron deficiency anemia. *Blood reviews.* 2017.
103. Lopez A, Cacoub P, Macdougall IC, Peyrin-Biroulet L. Iron deficiency anaemia. *Lancet.* 2015.
104. Lozoff B, Jimenez E, Wolf AW. Long-term developmental outcome of infants with iron deficiency. *New England journal of medicine.* 1991;325(10):687-694.
105. Yadav D, Chandra J. Iron deficiency: beyond anemia. *Indian journal of pediatrics.* 2011;78(1):65-72.
106. Agarwal R. Nonhematological benefits of iron. *American journal of nephrology.* 2007;27(6):565-571.
107. Silvestri L, Guillem F, Pagani A, et al. Molecular mechanisms of the defective hepcidin inhibition in TMPRSS6 mutations associated with iron-refractory iron deficiency anemia. *Blood.* 2009;113(22):5605-5608.
108. De Falco L, Sanchez M, Silvestri L, et al. Iron refractory iron deficiency anemia. *Haematologica.* 2013;98(6):845-853.
109. Cui Y, Wu Q, Zhou Y. Iron-refractory iron deficiency anemia: new molecular mechanisms. *Kidney international.* 2009;76(11):1137-1141.
110. Melis MA, Cau M, Congiu R, et al. A mutation in the TMPRSS6 gene, encoding a transmembrane serine protease that suppresses hepcidin production, in familial iron deficiency anemia refractory to oral iron. *Haematologica.* 2008;93(10):1473-1479.
111. van der Harst P, Zhang W, Mateo Leach I, et al. Seventy-five genetic loci influencing the human red blood cell. *Nature.* 2012;492(7429):369-375.
112. Benyamin B, Esko T, Ried JS, et al. Novel loci affecting iron homeostasis and their effects in individuals at risk for hemochromatosis. *Nature communications.* 2014;5:4926.
113. Nai A, Pagani A, Silvestri L, et al. TMPRSS6

rs855791 modulates hepcidin transcription in vitro and serum hepcidin levels in normal individuals. *Blood.* 2011;118(16):4459-4462.

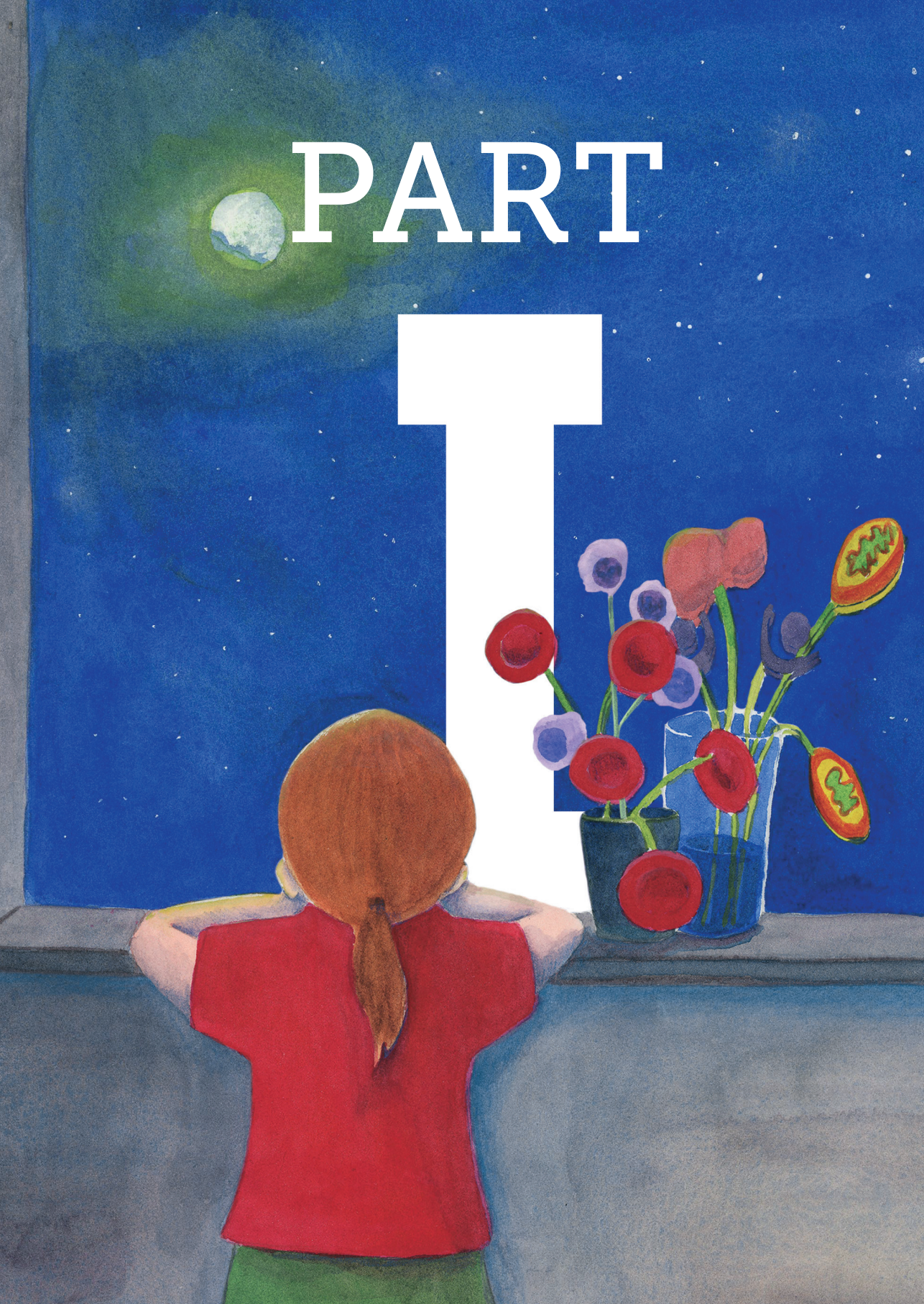
114. Traglia M, Girelli D, Biino G, et al. Association of HFE and TMPRSS6 genetic variants with iron and erythrocyte parameters is only in part dependent on serum hepcidin concentrations. *J Med Genet.* 2011;48(9):629-634.
115. Galesloot TE, Geurts-Moespot AJ, den Heijer M, et al. Associations of common variants in HFE and TMPRSS6 with iron parameters are independent of serum hepcidin in a general population: a replication study. *J Med Genet.* 2013;50(9):593-598.
116. Wang CY, Meynard D, Lin HY. The role of TMPRSS6/matriptase-2 in iron regulation and anemia. *Frontiers in pharmacology.* 2014;5:114.
117. De Falco L, Silvestri L, Kannengiesser C, et al. Functional and clinical impact of novel TMPRSS6 variants in iron-refractory iron-deficiency anemia patients and genotype-phenotype studies. *Human mutation.* 2014;35(11):1321-1329.
118. Parlare M, Ferrao A, Relvast L, C B. TMPRSS6 Gene - Two new nonsense mutations associated with IRIDA. *Haematologica.* 2010;95(supplement 2):1-773.
119. Pellegrino RM, Coutinho M, D'Ascola D, et al. Two novel mutations in the tmprss6 gene associated with iron-refractory iron-deficiency anaemia (irida) and partial expression in the heterozygous form. *British journal of haematology.* 2012;158(5):668-672.
120. Greg Miller W, Myers GL, Lou Gantzer M, et al. Roadmap for Harmonization of Clinical Laboratory Measurement Procedures. *Clinical chemistry.* 2011;57(8):1108-1117.
121. Vesper HW, Myers GL, Miller WG. Current practices and challenges in the standardization and harmonization of clinical laboratory tests. *The American journal of clinical nutrition.* 2016;104 Suppl 3:907s-912s.
122. http://www.hepcidinanalysis.com. Accessed 28 May 2019.
123. Cau M, Galanello R, Giagu N, Melis MA. Responsiveness to oral iron and ascorbic acid in a patient with IRIDA. *Blood cells, molecules & diseases.* 2012;48(2):121-123.
124. Kortman G. *Responses of the gut microbiota to supplementary iron: a survey at the host-microbial interface* 2014.
125. Kortman GA, Raffatellu M, Swinkels DW, Tjalsma H. Nutritional iron turned inside out: intestinal stress from a gut microbial perspective. *FEMS microbiology reviews.* 2014;38(6):1202-1234.
126. Gualdi R, Casalgrandi G, Montosi G, Ventura E, Pietrangelo A. Excess iron into hepatocytes is required for activation of collagen type I gene during experimental siderosis. *Gastroenterology.* 1994;107(4):1118-1124.
127. Pietrangelo A, Montosi G, Totaro A, et al. Hereditary hemochromatosis in adults without pathogenic mutations in the hemochromatosis gene. *The New England journal of medicine.* 1999;341(10):725-732.
128. Pietrangelo A, Caleffi A, Corradini E. Non-HFE hepatic iron overload. *Seminars in liver disease.* 2011;31(3):302-318.
129. Soares MP, Hamza I. Macrophages and Iron Metabolism. *Immunity.* 2016;44(3):492-504.
130. Hu X, Cai X, Ma R, Fu W, Zhang C, Du X. Iron-load exacerbates the severity of atherosclerosis via inducing inflammation and enhancing the glycolysis in macrophages. *Journal of cellular physiology.* 2019.
131. Cotter PD, Rucknagel DL, Bishop DF. X-linked sideroblastic anemia: identification of the mutation in the erythroid-specific delta-aminolevulinate synthase gene (ALAS2) in the original family described by Cooley. *Blood.* 1994;84(11):3915-3924.
132. Bottomley SS, Healy HM, Brandenburg MA, May BK. 5-Aminolevulinate synthase in sideroblastic anemias: mRNA and enzyme activity levels in bone marrow cells. *American journal of hematology.* 1992;41(2):76-83.
133. Cox TC, Bottomley SS, Wiley JS, Bawden MJ, Matthews CS, May BK. X-linked pyridoxine-responsive sideroblastic anemia due to a Thr388-to-Ser substitution in erythroid 5-aminolevulinate synthase. *The New England*

journal of medicine. 1994;330(10):675-679.

134. Campagna DR, de Bie CI, Schmitz-Abe K, et al. X-linked sideroblastic anemia due to ALAS2 intron 1 enhancer element GATA-binding site mutations. *American journal of hematology.* 2014;89(3):315-319.
135. Aivado M, Gattermann N, Rong A, et al. X-linked sideroblastic anemia associated with a novel ALAS2 mutation and unfortunate skewed X-chromosome inactivation patterns. *Blood cells, molecules & diseases.* 2006;37(1):40-45.
136. Cazzola M, May A, Bergamaschi G, Cerani P, Rosti V, Bishop DF. Familial-skewed X-chromosome inactivation as a predisposing factor for late-onset X-linked sideroblastic anemia in carrier females. *Blood.* 2000;96(13):4363-4365.
137. Cortesao E, Vidan J, Pereira J, Goncalves P, Ribeiro ML, Tamagnini G. Onset of X-linked sideroblastic anemia in the fourth decade. *Haematologica.* 2004;89(10):1261-1263.
138. Sankaran VG, Ulirsch JC, Tchaikovskii V, et al. X-linked macrocytic dyserythropoietic anemia in females with an ALAS2 mutation. *The Journal of clinical investigation.* 2015;125(4):1665-1669.
139. May A, Bishop DF. The molecular biology and pyridoxine responsiveness of X-linked sideroblastic anaemia. *Haematologica.* 1998;83(1):56-70.
140. Cotter PD, May A, Li L, et al. Four new mutations in the erythroid-specific 5-aminolevulinate synthase (ALAS2) gene causing X-linked sideroblastic anemia: increased pyridoxine responsiveness after removal of iron overload by phlebotomy and coinheritance of hereditary hemochromatosis. *Blood.* 1999;93(5):1757-1769.
141. Donker AE, Raymakers RA, Vlasveld LT, et al. Practice guidelines for the diagnosis and management of microcytic anemias due to genetic disorders of iron metabolism or heme synthesis. *Blood.* 2014;123(25):3873-3886; quiz 4005.
142. Donker AE, Schaap Schaap CC, Novotny VM, et al. Iron Refractory Iron Deficiency Anemia (IRIDA): A heterogeneous disease that is not always iron refractory. *American journal of hematology.* 2016.;*91(12):E482-E490.*
143. Nie N, Shi J, Shao Y, et al. A novel tri-allelic mutation of TMPRSS6 in iron-refractory iron deficiency anaemia with response to glucocorticoid. *British journal of haematology.* 2014;166(2):300-303.
144. Donker AE, Brons PP, Swinkels DW. Microcytic anaemia with low transferrin saturation, increased serum hepcidin and non-synonymous TMPRSS6 variants: not always iron-refractory iron deficiency anaemia. *British journal of haematology.* 2015;169(1):150-151.
145. Donker AE, Raymakers RA, Nieuwenhuis HK, et al. X-linked sideroblastic anaemia due to ALAS(2) mutations in the Netherlands: a disease in disguise. *The Netherlands journal of medicine.* 2014;72(4):210-217.
146. Donker AE, Galesloot TE, Laarakkers CM et al. Standardized serum hepcidin values in Dutch children: set point relative to body iron changes during childhood. *Pediatric blood and cancer.* 2020;67(3):e28038.
147. Heeney MM, Campagna DR, Westerman M, Fleming MD. The clinical and genetic spectrum of TMPRSS6 mutations leading to inappropriate hepcidin expression and iron refractory iron deficiency anemia (IRIDA). Paper presented at: Blood2009.
148. Heeney MM, Guo D, De Falco L, et al. Normalizing hepcidin predicts TMPRSS6 mutation status in patients with chronic iron deficiency. *Blood.* 2018;132(4):448-452.



LITERATURE AND CLINICAL STUDIES


PART
I

Chapter 2

The Critical Roles of Iron during the Journey from Fetus to Adult: a Narrative Review

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Submitted

ABSTRACT

Iron is indispensable for human life. However, iron is also a potential toxicant to cells since unbound iron can catalyze the formation of harmful oxidative radicals. Moreover, iron makes the human body an attractive host for iron-dependent pathogens. Therefore, iron homeostasis is tightly regulated both on the systemic and cellular level in order to prevent both iron deficiency and overload.

The opposing forces regarding iron metabolism are even more critical for children than for adults. Rapid growth and development during childhood requires large amounts of iron, while (especially young) children are vulnerable to infections with iron-dependent pathogens because of a still immature immune system. Moreover, both iron deficiency and iron excess early in life may have longlasting effects on the central nervous system, the immune system and the gut microbiota that persist into adulthood.

In this narrative review we assess the critical roles of iron for growth and development and how the human body adapts to the physiological high iron demands during the journey from fetus to adult, with a focus on the systemic level. We finally summarize unmet needs and research ideas exploring the developmental aspects of iron homeostasis, as a first step on the road to the development of clinical guidelines on a balanced iron supply and on the accurate diagnosis and treatment of iron disorders of the growing and developing child.

Key words
iron metabolism, fetus, neonate, adolescent, developmental, hepcidin

INTRODUCTION

Iron is indispensable for the normal development and function of all tissues in the human body. However, iron is also a potential toxicant to cells; unbound iron can catalyze the formation of oxidative radicals that damage proteins, lipids and nucleic acids.[1]

Furthermore, although the immune system needs iron in order to defend the host against pathogens, the virulence of many infectious organisms conversely depends on their ability to assimilate iron from their host.[2,3] Therefore, the human body has to deal with two opposite forces regarding iron metabolism; the need to obtain iron from food for the synthesis of heme, hemoglobin and other iron-containing vital proteins and the opposite need to withhold iron from iron- stealing pathogens.[4]

Because both iron deficiency (ID) and iron overload (IO) may have detrimental effects, a highly sophisticated regulatory system is required to maintain iron homeostasis on both the systemic and cellular level. This is particularly critical during childhood. Rapid growth and development requires greater amounts of micronutrients than in adults while (especially young) children are vulnerable to infections with iron-dependent pathogens because of a still immature immune system.

Growth and development in children occurs in transition phases from fetus to adult.[5,6] Understanding the specific contribution of iron and the mechanisms of iron handling during these different stages of fetal life and childhood is crucial. This forms the basis to define measures for adequate iron supply to the growing and developing child and enable the diagnosis and tailored treatment of iron disorders associated with a defective iron homeostasis both on the cellular and systemic level.[7]

In this narrative review we focus on the critical roles of iron for human growth and development during the specific transition phases from fetus to adult. Different from earlier reviews that predominantly focus on infants and young children,[8-10] we aim to cover the whole period of childhood, from conception to adulthood for both low and high income parts of the world, with a focus on the systemic iron homeostasis.

In the first and second section we briefly review the main functions of iron in the human body and the current insights regarding systemic iron homeostasis - with a focus on the key role of the iron regulating hormone hepcidin-, respectively. In the third section we consider the various organs and physiologic systems that require a balanced iron status in order to achieve growing and developmental tasks that are typical for childhood. In the fourth section we discuss the specific challenges regarding iron and iron handling for the different developmental phases of childhood, from fetus to adult. In the final section we summarize the identified research gaps and explore future research to address these gaps in order to obtain a better insight in the critical roles of iron and iron handling in children, with the ultimate goal of improving diagnosis and management of ID and other iron disorders in childhood worldwide.

IRON IS CRUCIAL FOR HUMAN LIFE

The ability of iron to act as an electron receptor or electron donor forms the fundamental basis for its essential role in many biological processes; oxygen transport via the iron-containing heme component of hemoglobin, cellular respiration as part of heme-containing cytochromes and Fe/S cluster- containing proteins of the electron transport chain, DNA synthesis and cell growth, cell differentiation and regulation of gene expression.[1] In the human body, the great majority of body iron -approximately two-thirds- is dedicated to hemoglobin synthesis.[11] Body iron distribution and its function in the various compartments of the human body are summarized in Table 1. For children, iron is especially essential for brain development.[1,12]

Table 1. Distribution and function of iron in the human adult male body

Compartment	Iron content (mg)	Key iron-containing protein(s)	Function of iron-containing protein(s)
Circulation[1,200-202]	4	Transferrin	Iron transport
Erythropoietic system			
Erythroblasts in bone marrow[200-203]	300	Hemoglobin	Critical ligand for the binding and transport of O_2
Red blood cells in circulation[1,200-204]	2500	Hemoglobin	Critical ligand for the binding and transport of O_2
Circulating macrophages[200,202,205]	600	Ferritin, hemosiderin	Iron storage
Muscular system[1,201]	130	Myoglobin	Critical ligand for the binding and transport of O_2
Central nervous system[1,12,206,207,]	?	Central nervous system oligodendrocytes	Myelin synthesis
		Tryptophan hydroxylase	Serotonin synthesis
		Tyrosine hydroxylase	Norepinephrine and dopamine synthesis
		Monoamine oxydase	Degradation of neurotransmitters
Bone[62]	?	Hydroxylases	Production of collagen type I
		Cytochrome P450	Activation of vitamin D

Table 1. Continued

Compartment	Iron content (mg)	Key iron-containing protein(s)	Function of iron-containing protein(s)
Bone[62] (Continued)		?	Differentiation from mesenchymal stem cells to osteoblasts
		Proteins involved in mitochondrial activity (see below: cellular level)	Osteoclast activity (rich of mitochondria)
Thyroid gland[190-192,208]	?	Heme-containing enzyme thyroid peroxidase (TPO)	Catalyzes iodide oxidation, thyroglobulin iodination and iodothyronine coupling
		Deiodinases	Tissue conversion of T4 to T3
		?	TSH secretion
Cellular level[1,201*]	150		
Heme-containing enzymes[1,107,109]		Cytochrome P450 monooxygenase, cytochrome C	Mitochondrial electron transport chain, drug metabolism
		NADPH oxygenase, myeloperoxidase	Antimicrobial oxidative burst in neutrophils
Iron-sulfur enzymes[209]		Iron Responsive Protein-1 (IRP-1)	Cellular iron homeostasis
		Ferrochelatase	Last step of heme biosynthesis
		Complex I-III, tricarboxylic acid (TCA) cycle	Mitochondrial electron transport chain
		DNA polymerase, DNA helicase	DNA replication and repair
Other iron containing proteins, non-heme, non iron-sulfur [210]		Lipoxygenase	Metabolisation of polyunsaturated fatty acids
Parenchymal iron (liver) and reticuloendothelial iron (liver, spleen)[1,201,202,204]	1000	Ferritin, hemosiderin	Iron storage
Total	4000-5000		

HEPCIDIN PLAYS A KEY ROLE IN SYSTEMIC IRON HOMEOSTASIS

The systemic and cellular iron homeostasis cooperate in the human body

Cells predominantly involved in the systemic iron homeostasis are duodenal enterocytes, erythroid precursors, hepatocytes and macrophages. The duodenal enterocyte absorbs 1-2 mg per day to compensate for the small amount of iron loss that occurs through epidermal and enteral desquamation. Once inside the enterocyte, iron is stored by ferritin or released into the plasma. After oxidation by ferroxidases, iron is loaded to transferrin for transport in the plasma. Iron binding to transferrin prevents the formation of unbound iron (non-transferrin-bound iron; NTBI) that is highly toxic because it catalyzes the formation of oxidative radicals and allows the unrestricted influx of iron in parenchymal organs.[13] Ferric-transferrin binds to the transferrin receptor, expressed on all iron-containing cells, but mainly on erythroid precursors in the bone marrow and on hepatocytes. In the bone marrow, iron is used for erythropoiesis, in the liver iron is stored in hepatocytes as ferritin or hemosiderin. However, the majority of iron in the circulation is derived from the recycling of senescent erythrocytes (20-25 mg of iron per day).

Because both ID and IO may have detrimental effects, a highly sophisticated regulatory system is required to maintain iron homeostasis on both the systemic and cellular level. Systemic iron homeostasis relies on the regulatory hormone hepcidin and the cellular iron exporter ferroportin.[11,14] Cellular iron homeostasis is predominantly controlled in a post-transcriptional way by iron-regulatory proteins (IRP) that bind iron-responsive elements (IRE) in regulated messenger RNA's of cellular iron importers, exporters and storage genes.[11,14] This complex system is beyond the scope of this review; the interested reader is referred to the literature.[11,14-16]

Intracellular iron is also sensed and regulated by the hypoxia-inducible factor (HIF) system.[17-19] Under low iron and oxygen (O2) conditions, HIFα induces the expression of various genes involved in iron homeostasis, encoding the transferrin receptor 1(TfR1), ferroportin, ceruloplasmin and erythropoietin (EPO). On the contrary, under iron-replete, normoxic conditions, degradation of HIFα occurs by iron- and oxygen independent hydoxylases.[17-19]

Although the machinery of systemic and cellular iron homeostasis is separated, crosstalk exists between distinct control systems,[11,14,15] on the level of ferroportin, HIF2α and TfR1.[14,16] First, ferroportin, that as iron exporter is a critical factor for plasma iron supply, is subject to both systems. Iron status is sensed on the systemic level and communicated post-translationally via hepcidin, thereby influencing the activity of ferroportin. On the cellular level, iron levels post-transcriptionally regulate ferroportin mRNA via the IRE/IRP system.[20] Second, HIF2α is involved in EPO-mediated regulation of hepcidin levels, but HIF2α mRNA is also a target of the IRE/IRP system. Third, systemic Tf-Fe_2 displaces TfR1 from HFE inside the hepatocyte, which then subsequently forms a complex with TfR2 and HJV to promote BMP/SMAD signaling leading to *HAMP* and subsequent hepcidin production. On the cellular level, TfR1 expression is modulated by the IRE/IRP system.[14,21] More interconnections between the systemic and cellular iron homeostasis might exist and although the cornerstones of the two systems have been identified, future research is needed to further unravel how the two systems tango and collaborate.

Systemic iron homeostasis and the central role of hepcidin

In physiological circumstances, intestinal iron absorption is enhanced in situations of increased iron demands (for example during periods of rapid growth in infancy, childhood and pregnancy and during periods of blood loss e.g. menses).[11] On the other hand, iron absorption is depressed in case of (imminent) IO Importantly, the human body cannot excrete iron in a regulated way. Therefore, effective communication and fine-tuning between enterocytes, erythroid precursors, hepatocytes and macrophages is crucial in order to optimally distribute iron between the different body compartments and to prevent both ID and IO. Hepcidin, a 25-amino acid peptide hormone that is predominantly synthesized by hepatocytes and excreted by the kidneys, has emerged as the key regulator of systemic iron homeostasis.[7] Hepcidin acts by binding to ferroportin, thereby inducing its degradation and internalization and subsequently blocking iron export out of the enterocytes, hepatocytes, and macrophages into the plasma. Because of this characteristic hepcidin is able to orchestrate iron flows in the body and to regulate iron uptake and iron utilization in favor of storage or vice versa.[11,14] The synthesis of hepcidin is controlled by certain physiologic and pathologic triggers that reflect circulating and stored iron levels, erythropoietic activity, hypoxia (see above) and inflammatory signals.[7,11,14,22-24]

In case of ID enhanced erythropoiesis and tissue hypoxia due to (especially iron deficiency) anemia, hepcidin production drops in order to ensure sufficient iron supply to the bone marrow for the production of erythrocytes. Treatment with erythropoiesis stimulating agents, chronic liver disease with impaired synthesis function of the liver and (supplementation of) the sex hormones testosterone and estrogen also cause a decrease of hepcidin production.[7,25]

Conversely, iron excess and inflammation induce hepcidin production while impaired renal function results in decreased excretion, both leading to impaired iron uptake by the enterocytes and also to impaired iron release from the macrophages.[7,23,25] Restriction of iron availability in the serum by inducing hepcidin synthesis is part of the host defense because this mechanism limits the iron supply to extracellular iron-dependent pathogens.[24,26-28]

The set point of serum hepcidin relative to indicators of body iron changes during childhood

Measurement of hepcidin is a promising clinical tool in the diagnosis and management of both genetic (e.g. Iron Refractory Iron Deficiency Anemia (IRIDA)[29]) and acquired iron disorders (iron deficiency, iron deficiency anemia, iron loading, iron maldistribution).[7]

Determination of serum hepcidin levels might also help in predicting the response to oral iron therapy and in guiding iron treatment under conditions of competing signals (anemia, iron deficiency, inflammation), in combination with other iron and inflammation parameters.[30]

Data on this application in childhood are limited.[31-33] Moreover, implementation of serum hepcidin levels in clinical pediatrics is hampered by the lack of standardized reference values of healthy children from different age groups, relative to iron status.[34-36] Available studies concern either small series with limited age groups or series including children with (anemia of) inflammation.[31,37-41] We therefore recently determined serum hepcidin values, hepcidin/ferritin rations and TSAT/hepcidin ratios in 266 Dutch children aged 0.3-17 year (www.hepcidinanalysis.com[42]),[43] using a standardized assay.[44] Interestingly, we found that hepcidin levels are relatively high corrected for indicators of stored and circulating iron in children < 12 years compared to children > 12 years. Since especially young children are more susceptible to severe life threatening infections one might argue that relatively high hepcidin

levels are favorable in the context of host defense against extracellular pathogens,[27] outweighing the risk of ID.[3,4] We found the opposite in adolescents, i.e. relatively low hepcidin levels relative to body iron, possibly due to the effect of sex hormones,[7] and as part of an adaptive response in order to obtain sufficient iron to meet the high requirements for the growth spurt typical of puberty. We therefore hypothesized that the set point of systemic iron homeostasis might change during lifetime to adapt to different conditions for which a high amount of iron is relatively favorable or not.[43] This point needs further confirmation in larger studies since in our study young children and children from a non-Western European descent were underrepresented. We therefore advocate calibration of worldwide hepcidin assays using secondary (matrix) reference material that has been value assigned by primary reference material.

Iron availability versus iron restriction in inflammation

Since many of both stimulating and suppressing stimuli might be present simultaneously, the hepcidin level in the human body reflects the net effect of these multiple key signals, depending on the relative strength of each stimulus.[45] In adult ICU patients, serum hepcidin levels decline with the onset of ID.[46]In a recent study in women diagnosed with mild iron deficiency anemia (IDA), mild inflammation did not increase serum hepcidin levels. [47,48]

Also in severely anemic children, hepcidin tends to be low even in de presence of inflammation.[46,49] However, other data suggest that even low-grade inflammation induce and increase of serum hepcidin levels, resulting in hepcidin-mediated blockade of iron absorption.[50]

Whether iron acquisition will get priority over iron restriction in case of a combination of ID and inflammation, is still unclear, and might depend on the degree of these opposing forces.

We hypothesize that inflammation initially induces hepcidin synthesis resulting in iron-restricted erythropoiesis,[51] until body iron decreases below a critical threshold (and anemia occurs). Below this threshold, hepcidin production will drop in order to guarantee sufficient iron supply to the erythroblasts for the productions of erythrocytes, preventing severe anemia.

IRON IS PARAMOUNT FROM FETUS TO ADULT

Iron and Growth

Human growth occurs in phases; the infancy-childhood-puberty (ICP) model

Children are not small adults; they differ in many aspects. Opposite to the steady state of adulthood, childhood is characterized by growth and development. Children going through childhood and adolescence grow tremendously from and average weight of 3.5 kg and 50 cm at birth [52] to an average length of 72 kg and 184 cm for boys and 60 kg and 171 cm for girls at the age of 18 years (children of Dutch origin).[53]

Growth and development are dynamic processes, driven by age- and gender-dependent genetic, environmental, dietary, socioeconomic, developmental, behavioral, nutritional, metabolic, biochemical factors, and hormonal factors.[54] Starting at the time of conception, the child transits through different stages and achieves multiple milestones regarding growth and development on the road from fetus to adult.[5,6,55,56] The infancy-childhood-puberty (ICP) model describes this process as three additive and partly superimposed stages that strongly reflect the different hormonal phases involved in human growth.[5,6,54-57] While growth during infancy (0 to 2-3 years) is largely nutrition dependent and closely linked to insulin-growth-factor 1 (IGF1), growth in childhood (2-3 to 11-12 years) is associated with the setting of the growth hormone (GH) – IGF1 axis. During this phase, growth largely depends on the activity of both GH and thyroid hormone.[6,57] The start of puberty (11-12 years) is a function of the hypothalamic–pituitary–gonadal (HPG) axis maturation, resulting in the production of gonadal steroids that cause the typical growth spurt of adolescense by both a direct effect on bone formation and also by a synergistic effect since sex hormones stimulate GH secretion.[5,6,54-57]

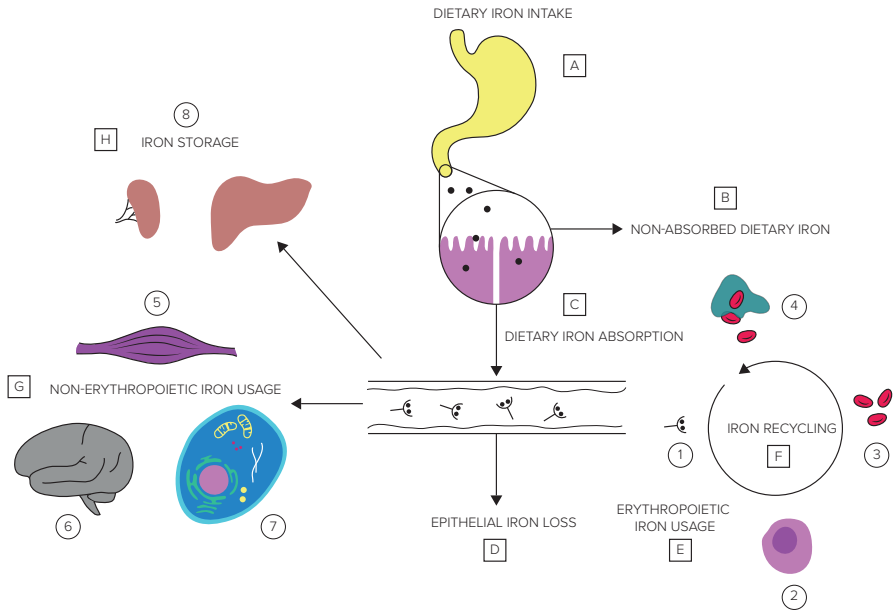
The hormonal mechanisms involved in growth and development have to activate physical, psychological and behavioural traits at the right time in the right context as the child transits through the different phases from infant to adult. Adaptive plasticity plays an important role in this timing, in order to cope with environmental challenges.[58] Both organic (e.g. undernutrition) and non-organic (e.g. child abuse) threats may result in a delay in transition from infancy to childhood or from childhood to adolescense, leading to a short stature at reaching adulthood, outside the target

height range based on the length of the parents.[58] ID, whether or not in the context of undernutrition, is also such an organic threat that may have longlasting effects on growth and development.

Growth implies a huge expansion of blood volume requiring large amounts of iron
The enormous growth that occurs from fetus to adult is accompanied by a 15-20 times expansion of the blood volume from an average of 240-250 mL at birth to an average of 4-5 liters in adulthood. To meet the iron demand for both growth-related increase of the blood volume and for the substitution of the recycled erythrocytes, the daily requirements for nutrients and also for iron is therefore relatively greater for children than for adults that only have to replace senescent erythrocytes.[59] For example, a 3 kg neonate requires approximately 0.2-0.3 mg iron (0.07-0.1 mg/kg) per day, while a 70 kg male adult requires 0.4-1.6 mg (0.01-0.02 mg/kg) per day (**Figure 1, Table 2**).This makes children vulnerable to IDA, affecting hundreds of millions of children.[60,61]

Iron is essential for bone formation
Bone is a metabolically highly active tissue that is continously being remodeled, enabling growth in children as well as repair and adaptation of the skeleton in adults. Increase of the bone mass in childhood is paramount in order to support the growing body, but also to expand the environment for hematopoiesis in the red marrow inside the bones.[62] During the first two decades of life, the skeleton grows tremendously.[63] Around the age of 30, bones reach their maximum size, strenght and density, known as peak bone mass.[63] Since later in life the loss of bone exceeds the rate of bone replacement, lifelong bone health is strongly dependent on this maximum bone mass that is formed during the critical period of growth and maturation in childhood, adolescence and early adulthood.[63] A complex interaction of genetic, behavioral and environmental factors determine the bone mass.[64] Genetic traits contribute to approximately 60-80%, while physical activity and nutrition are responsible for the remaining 20-40% of variation in peak bone mass.[64] Although in particular calcium, phosphate and vitamin D play a crucial role in the formation of bone, other nutrients as vitamin K, copper, magesium, manganese, zinc and also iron are important for bone health.[64,65]

Figure 1. Iron content and iron flows in the human body



Iron content and iron flows for the different compartments during the developmental stages from fetus to adulthood in iron replete individuals. Iron content and flows are based on the infancy-childhood-puberty model. Numbers refer to the various human body iron compartments of Table 2A. Capital letters refer to the systemic iron flows in the human body as given in Table 2B.

Table 2. Iron content (per compartment in mg, A) and iron flows (mg/day, B)

Numbers for the compartments and capital letter for the iron flows refer to those in Figure 1.

A. Compartment	Neonate[a]	Infant[b]	Child[c]	Adolescent[d]	Adult
1. Transferrin	0.2[211]	0.2[212]	1[213]	2.5[213]	4[1,201,202]
2. Erythroblasts in bone marrow[1,201]	20	35	85	215	300[201,202]
3. Red blood cells in circulation[1,201]	190[146,214]	280[147]	725[60,215]	1800[60]	2500[1,201,202]
4. Circulating macrophages	45	65	175	430	600[202,205]
5. Muscular system	16[214]	5[214]	35	95	130[1,201]
6. Central nervous system	?	?	?	?	?
7. Cellular level	10	15	45	110	150[201]
8. Liver, spleen	65[214]	100[147]	400[215]	700[60,198]	1000[1,201,202]
Total	270-340[125,214]	450-500[147]	1500	3500	4000-5000

Table 2. Continued

B. Iron flows	Neonate[a]	Infant[b]	Child[c]	Adolescent[d]	Adult[e]
A. Dietary intake (mg/d)	0.2-0.3 [h,l] [160,216]	10[217]	13 [147,160,217,218]	15-18 [147,218,219]	10-14[1]
B. Non-absorbed dietary iron (mg/day)	0.1[216]	9.5[217]	12.5[220]	10-17[219]	8-13.5[1]
C. (Dietary) iron absorption (mg/day)[f]	0.2-0.3 [9,157,216]	0.5-1.0 [217,221]	0.5[220]	0.8-5.0[197,219]	0.5-2.0[1]
D. Epithelial iron loss (mg/day)	0.2[214]	0.2[214]	?	0.7[219]	1-2[1]
E. Erythropoietic iron usage	0.2[97,216]	?	?	?	0.4-1.6[1]
F. Iron recycling from senescent erythrocytes (mg/day)	2	3	7	18	20-25[1]
G. Non-erythropoietic iron usage[g]	0.1	?	?	?	0.1-0.4
H. Iron storage	?	?	?	?	?

Categorization in the Figure 1 and Table 2 is based on the infancy-childhood-puberty model.[55,56] For each phase a certain age was chosen as an example for iron contents and iron flows.

[a] Based on term neonate, assuming a weight of 3 kg, and a circulating volume of 80-85 mL/kg, hence approximately 240-250 mL[222]

[b] Based on infant aged 1 year, assuming a weight of 10 kg,[53] and a circulating volume of 75-80 mL/kg, hence approximately between 750-800 mL.[223]

[c] Based on child aged 6 years, assuming a weight of 23 kg,[53] and a circulating volume of 70-75 mL/kg, hence approximately between 1500- 1750 mL.[223]

[d] Based on male adolescent, aged 15 years, assuming a weight of 50 kg,[53] and a circulating of volume 65-70 mL/kg, hence approximately between 3250- 3500 mL.[223]

[e] Based on male adult, assuming a weight of 70 kg, and circulating volume of 4000-5000 mL.[1]

f Absorption of iron is highly dependent on iron status and composition of diet; iron deficiency and a diet rich of vitamin C and animal protein enhance iron absorption,[217] while iron loading and the existence of calcium and phytates and polyphenols decrease ion absorption.[224]

[g] Non-erythropoietic iron usage for the neonate and adults were calculated by subtracting erythropoietic iron usage (E) from the amount of absorbed iron (D). For the other age groups this deduction was not possible since data on E are lacking.

[h] Based on calculation of breastfed term neonate of 3 kg with an intake of on average 500 mL breast milk per day. Breast milk has a relatively low iron content; colostrum contains approximately 0.8 mg/L iron and the concentration in mature milk declines over time from 0.6 mg/L at 2 weeks to 0.3 mg/l at 5 months postpartum.[174,225] The iron content of formula feeding is on average 4-12 mg/L, which is 7-40 times greater than the iron levels in breast milk in order to compensate for the poorer availability of iron in formula milk compared to breast milk.[8]

[l] Of note, daily iron requirement for a neonate is 0.55-0.75 mg/day, meaning that iron stores are drawn on during breastfeeding.[217] However, after birth, iron stores significantly increase due to the fetal-to-adult hemoglobin switch, i.e. the breakdown of fetal Hb (HbF) that is replaced by adult Hb (HbA) by endogenous erythropoiesis, which typically resumes when Hb has decreased from 17-12 g/dL.[8,2]

Iron is critically involved in the production of collegen I, a crucial component of bone, since its synthesis is dependent of hydroxylases requiring iron for their catalytic activity.[62] Iron is also essential for the activation of vitamin D that is indispensable for bone formation, by enzymes of the cytochrome P450 family containing heme.[62] Furthermore, iron influences the differentiation from mesenchymal stem cells to osteoblasts and also the production and activity of osteoclasts[62] (Table 1).

Accumulating clinical evidence exists that IO conditions as hereditary hemochromatosis and thalassemia syndromes are associated with decreased bone mineral density.[66] *In vitro* studies indicate that iron excess inhibits osteoblast formation[67] and enhances osteoclast differenation and activity.[68] Inceased bone resorption in IO conditions is thought to be at least partially mediated by oxidative stress inducing inflammatory changes that affect bone composition with altered microarchitecture.[69] Furthermore, hepcidin deficiency relative to ferritin, which is characteristic for most forms of hereditary hemochromatosis and iron loading anemias, is associated with decreased osteoblast activity.[70] Of note, clinical reports indicate that parenteral iron treatment induces renal phosphate wasting, thereby suggesting that iron, and especially iron treatment, may also influence bone health in an indirect manner.[71]

Conversely, the effect of ID on bone health is less clear; *in vitro* data suggest a biphasic effect of low iron with osteoblast acitivy increasing in case of mild ID and decreasing in case of severe ID.[72] Clinical data regarding the possible association between ID and bone mass and bone density are scarce but indicate that low iron status results in increased bone resorption[73] that might be reversed by iron treatment.[74]

Data from child studies exploring the effects of ID and iron excess on peak bone mass are lacking. One study on bone disease in children suffering from β-thalassemia major shows osteoporosis of multifactorial origin, whereby iron loading may play a role.[75]

Iron and development of the brain

Iron is essential for the development of the central nervous system.[76] Iron and iron-containing enzymes are crucial for neuronal and glial energy metabolism, myelin and neurotransmitter synthesis.[1] Iron demands of the growing and developing brain are temporally and spatially sensitive. [12,77] MRI imaging in children suggests that the iron content of the brain increases considerably during childhood and adolescence, although quantities are unknown.[78]

Animal studies indicate that both ID and iron excess early in life negatively influence the growth and maturation of the brain, whereby the severity of the neurodevelopmental deficit depends on timing, severity and duration of aberrant iron levels.[79,80] There are also growing concerns about the effects of excessive iron exposure early in life on brain ageing and the development of neurodegenerative disease.[81-83] Based on these animal studies, ID with and without IDA but also iron excess that occurs during the brain growth spurt in early life is thought to have negative and longlasting effects on the mental and psychomotor development of the child.[84-91]

Placebo-controlled randomized clinical studies on the effect of iron supplementationin infants and toddlers with IDA show no convincing evidence for a positive effect on psychomotor or coginitive skills within 1-4 months.[92,93] This might indicate that data from rodent studies regarding the negatieve effects of ID on the central nervous system cannot be extrapolated to human infants directly, or that critical iron-dependent windows of brain development have already passed at this age, making it inpossible to correct the ID-related neurodevelopmental deficits, consistent with the above-mentioned theory. Of note, exposure to iron-fortified formula at infant age might even result[94] in poorer cognitive outcomes than exposure to low-iron formula feeding.[95,96] Theses data underscore the importance of accurate iron supplementation in infancy. However, the optimal level of iron suppletion is unclear for this age.

Another important question is if and how the human body prioritizes the available iron between bone marrow, brain and other iron-dependent tissues during critical periods of growth and development and during periods of scarcity of micronutrients including iron. Isotope studies show that in adults approximately 80% of the absorbed iron is incorporated into the red blood cells, while this percentage is much lower (~50%) in infants, suggesting that the distribution of iron between the different iron-demanding organ systems is age dependent.[97] Furthermore, *in vitro* studies suggest partly suppression of erythropoiesis and return of iron from the bone marrow to the circulation during periods of limited iron supply in order te protect the brain and other vital iron-dependent organs with iron.[89,98-100]

However, several animal and human studies suggest that in case of a negative iron balance during the fetal and infant period, iron is still prioritized to the erythroblasts over all other organ systems, including the central nervous system.[77] Importantly, accesible biomarkers in serum, saliva, urine of CSF that

give information on brain iron are lacking until now, hampering the assessment of brain dysfunction due to both cerebral ID -before the occurrence of IDA- and of cerebral iron excess.[77] Moreover, how iron enters the central nervous system, still needs elucidation. Rodent studies indicate that the brain acquires iron by diferric transferrin, since hypo-transferrinemia in mice affects neurologic development.[101] However, other mice studies suggest that NTBI, acquired by ZIP8, DMT1 and possibly other, still unidentified NTBI iron transporters, appears to be the main source of iron for astrocytes, oligodendrocytes, and microglia, since these cell types do not express transferrin receptors.[13]

Iron and the immune system

The human immune system is a dynamic system that varies with age refelecting the different challenges during the different phases of fetal life, childhood and adulthood, a concept known as immune ontogeny.[102] The immune system in early life is programmed to tolerate foreign antigenic influences of the mother *in utero* but has to adapt rapidly to a functional system that is able to distuinguish harmful pathogens from helpful microbes right after birth.[102] This ability is thought to be critical for health and failure to regulate these responses may result in recurrent infections, auto-inflammatory diseases and allergies.[103,104] Because the intestine houses the majority of lymphocytes and other immune effector cells of the human body, nutritional status and also iron is very likely to contribute to the education of the immature immune system, by directly influencing the function of the intestinal immune cells [103,105] and by indirectly modifying the intestinal microbiota.[106]

Apart from the developmental aspects, both the innate [107-109] and adaptive [110] immune system need iron in order to mount a proper response against pathogens. Regarding the innate immune system, iron is essential for the antimicrobial oxidative burst inside neutrophils, the binding activity of pro-inflammatory transcription factors and the polarisation and differentiaton of macrophages during infections.[107,111-113] For the adaptive immune sytem, *in vitro* studies and anecdotal data indicate that the proliferation and function of lymphocytes also depend on iron.[110,114] Impaired cellular iron acquisition of lymphocytes caused by a defect of TfR1 caused compromised T and B cell proliferation and subsequent antibody class switching, resulting in a severe immunodeficiency with life threatening and even fatal infections in two affected families in the Middle East.[110,114] This is clinically important since ID is highly prevalent in young

children both in low-middle income and high-income countries,[60,61] rendering them more vulnerable to infections and possibly also to a suboptimal response to vaccinations.[115] Interestingly, high neonatal iron blood count was associated with an increased risk of the development of diabetes mellitus type I before the age of 16 years,[116] suggesting that IO in early life may also negatively affect T cell count and function, setting a trend to auto-immunity. Moreover, high iron body content makes the human body an attractive host for iron-dependent pathogens.[4] Clinical evidence exists that iron restriction is protective against malaria, respiratory tract infections and diarrrhoea in low-income countries. [27,117,118]

Iron influences the development of the gut microbiota

Growing evidence emphasises the important implications of the gut microbiota for human health and disease.[106] The way the infant interacts with intestinal microbes programmes immune and metabolic pathways that persist into adulthood.[106] Inflammatory bowel disease, functional gastro-intestinal disorders, allergies, obesity and liver disease are all thought to be associated with an imbalance or alteration in the composition and/or the function of the intestinal microbiota during early life.[119]

Initiation of the microbial colonisation of the intestine may start *in utero* from the mother to the fetus, challenging the idea of a sterile colon at birth.[120] However, the development of the gut microbiota primarily occurs after birth, during infancy. Environmental factors as gestational age, mode of delivery, type of feeding (breast- versus formula feeding), exposure to antibiotics and to proton pump inhibitors all influence the variability of microbiome development in children.[106,119]

Iron also influences the gut microbiotica. Accumulating preclinical and clinical evidence indicates that unabsorbed nutritional iron enters the colon and may have unfavorable effects on the intestinal microbiotica-host interface.[38,121] Pathogenic species as *Enterobacteriaceae* can take advantage of an iron-rich intestinal environment at the expense of beneficial species as *Bifidobacteria and Lactobacilli* species, important for the production of short chain fatty-acids and for the integrity of the gut barrier, resulting in an unfavorable shift towards an adverse microbial profile.[38] Especially preterm infants are at risk for the occurrence of this dysbiosis.[122] Notably, nutritional guidelines that balance the possible benefits or iron supplementation against the risk of perturbation of the gut microbiota, are lacking until now.[33,122]

DEVELOPMENTAL ASPECTS OF IRON AND IRON HANDLING FROM FETUS TO ADULT

Maturation of iron handling during growth and development

As reviewed in the second section, hepcidin plays a crucial role in systemic iron homeostasis. Noteworthy, serum hepcidin levels reflect the integration of various (opposing) signals important for systemic iron homeostasis, and the relative contribution of these stimuli, such as the above-mentioned hormones, might differ from those in adults, requiring further elucidation of iron handling in childhood. In order to assess iron handling in childhood, we recently performed a study on serum hepcidin values in childhood, relative to ferritin and transferrin saturation, in Dutch children aged 0.3-17 years. We found that hepcidin values, hepcidin/ferritin and TSAT/hepcidin ratios in children between the age of 0.3-17 years were age dependent, suggesting a changing set point of serum hepcidin relative to stored and circulating iron. (www.hepcidinanalysis.com[42]).[43]

Furthermore, while in adults the majority of iron is dedicated to hemoglobin synthesis,[11] distribution of iron between the different iron-demanding might differ and mature between during childhood. One clinical study indicated that iron distribution between the erythropoietic system and storage compartment changes during childhood, since dietary iron intake was positively associated with hemoglobin levels in infants aged 9-12 months, while it was positively associated with serum ferritin levels in toddlers aged 12-17 months.[9] This suggests a maturation process regarding the channeling of iron inside the human body to either the bone marrow or the liver and spleen.[9]

Moreover, *in vitro* studies suggest the existence of protection mechanisims during periods of low iron supply; in case of ID erythropoiesis was suppressed with return of iron from the bone marrow into the circulation, probably in order to protect other vital iron-dependent organ systems from an iron deficit.[89,98-100] Whether such survival strategies also occur during human early life is unclear. However, several animal and human studies suggest that during the fetal and infant period, iron is prioritized to the erythroblasts over all other organ systems, including the central nervous system, irrespective of a negative iron balance.[77]

In this fourth section we review the various challenges regarding iron and the known insights regarding iron handling for the fetus, infant (0 – 2-3 years), child (2-3 – 11-12 years) and adolescent (11-12 years – 17 years).

Iron and the fetal period

Iron requirements in utero

The greatest increase in body weight, length and brain mass occurs during fetal life; the velocity of *in utero* linear growth is maximal at about 18 weeks of gestational age in the human. At this time, the fetus grows four times more rapidly than at any time postnatal. Increases in body weight follow a similar temporal pattern. The maternal-placental environment in which the fetus develops is a critical factor in this process. A sufficient supply of micronutrients, including iron, from the mother to the fetus is a prerequisite to obtain this phenomenal growth. [5,6,123,124] A term fetus requires approximately 270 mg[125,126] of iron and the placenta 90 mg[127] (reviewed in[128]). Approximately 80% of fetal iron accrues in the last trimester of pregnancy by active placental transport from the mother to the fetus, rendering premature infants susceptible to ID and IDA at birth.[129] (**Figure 1**)

Maternal and fetal hepcidin are involved in iron sensing and iron transport in utero

Under most circumstances, maternal iron status modulates the expression of placental iron transport in favor of the fetal demands. In healthy pregnancy, hepcidin increases in the first[130] trimester but then decreases till nearly undetectable levels during the second[130] and third trimester[131,132] in order to enhance iron availability. However, evidence exists that the regulatory system can no longer sustain sufficient transfer of iron from the mother to the fetus in case of severely exhausted maternal iron status, resulting in fetal ID that is associated with preterm labor, low birth weight or even fetal death.[133-136] This is a major problem in low- and middle income countries,[60] but might also occur in high income countries due to vegetarian and vegan diet habits during pregnancy.[137]

Besides maternal also placental and fetal signals play a role in iron sensing and transport from the mother to the fetus.[128] Fetal hepcidin could influence placental iron transfer,[126,128] but how the systemic and local mechanisms regulate this is largely unknown,[126,128,129,131,135,138] as well as the molecular pathways of iron trafficking in the placenta on the cellular level.[126,128,138]

Iron and fetal epigenetic programming

Importantly, environmental factors (maternal health, placental function, stress, life style, nutrients and also paternal diet[139]) that affect growth and development in early life can profoundly influence human biology and long-term health by epigenetic mechanisms of gene regulation that may be inheritable and passed on to the next generation.[124] Undernutrition *in utero* and stress are well-documented examples of conditions that increase the risk of metabolic disease as type II diabetes and cardiovascular disease, known as the Barker-hypothesis or Barker mismatch paradigm.[140,141] The fetus or young infants adequately adapts to maternally transmitted environmental challenges as undernutrition or stress by changing metabolic set points via epigenetic programming of the expression of relevant genes, which persist into adulthood (i.e. metabolic memory). If these adaptations are functional in the mature environment of the older child and adult, the risk of metabolic disease is low. However, if these adaptations are not functional, e.g. the mature environment does not match the prenatal and early life environment, the risk of metabolic disease increases.[124,140] Since both maternal anemia and ID negatively influence fetal growth, these conditions are suggested to increase the risk of adult hypertension of the offspring.[123,142,143]

Furthermore, in rodents, several genes involved in hippocampal plasticity, change their expression profiles in response to early ID.[79,144,145] Whether exposure to inadequately low or high iron status in early life affects the expression of systemic iron regulation genes is unclear.

Iron and infancy

Iron status during the neonatal period

At birth, maternal iron status accounts for 6% of the variants in infant stores and birth, and the remaining causes in the highly variable size of birth iron endowment are unknown, but likely include gestational age, intrauterine growth restriction, time of cord clamping, maternal smoking habits and diabetes.[125,146,147]

After the fetal period, characterized by large iron requirements, iron demands remain high to meet the quantities needed for the rapid growth of the newborn and the concomitant increase of circulating blood volume. Weight and height increase approximately 15-20 mg/kg/day during the first months and approximately 25 cm in the first year and 12.5 cm in the second year of life, respectively, with height velocity

adjusting towards the genetically predicted trajectory.[54] Preterm babies have overall greater nutritional needs and higher iron requirements than healthy full-term babies, since their iron stores are smaller at birth and extra iron is needed for catch-up growth.[148] To meet these substantial physiological needs, iron is mobilized from iron stores, recycled from hemoglobin breakdown and absorbed from dietary sources.

Human infants are born with relatively high iron contents per kilogram; a full term infant of 3 kg is endowed with approximately 330 mg iron (110 mg/kg), which is per kg roughly 1.5 times more than the iron content of a male adult of 70 kg containing approximately 5000 mg (70 mg/kg) (**Figure 1, Table 2**).[147] Moreover, the normal decline in Hb due to the breakdown of fetal Hb significantly increases iron stores in newborns. Therefore, healthy, term and breastfed infants are initially independent of additional external iron and can double their birth weight before iron stores are depleted.[8,9,149]

During infancy, ID and IDA may negatively affect growth that might improve after correction of the iron deficit according to one observational study and one RCT,[150,151] although meta-analyses fail to demonstrate such a beneficial effect.[93,152] Moreover, iron supplementation in iron-replete infants may have detrimental effects on growth,[93] possibly by competition with the absorption of zinc and other micronutrients.[9] Other mechanisms include the induction of oxidative stress via NTBI[153], or perturbation of the gut microbiota.[154]

Iron and lactation

For suckling infants, breast milk and/or infant formula are the only sources of dietary iron. Although relatively low in iron content, the bioavailibility of iron in breast milk is higher (~50%) than in formula milk and cowmilk (~3-4%), explaining the need for a higher iron content in infant formula.[155-158] This relatively high bioavailability of breast milk iron is explained by the unique properties of lactoferrin, a single polypeptide chain that is closely related to transferrin, the iron-carrier protein in plasma, also capable of binding 2 iron atoms.[159-162] In comparison to transferrin, lactoferrin has a 300 times greater affinity for iron and can retain iron down to more acidic environments (~pH 3), e.g. in the stomach or in infected tissues, as transferrin releases iron already at a pH below 5.5.[160,162-164] Lactoferrin is relatively resistant against proteolysis since it appears in the stools of breastfed infants in intact form.[165] Lactoferrin receptors have been identified in the small intestine

of infants and neonates, suggesting that the import of iron across the apical membrane of the enterocyte is mediated by the absorption of lactoferrin-bound iron. [8,155,166-170] Importantly, both *in vitro* and *in vivo* studies have demonstrated that lactoferrin has crucial antimicrobial, immune modulating and anti-inflammatory properties, apart from its capacity to absorb iron.[171] The antimicrobial function involves the withdrawal of iron from iron-stealing pathogens in combination with specific interactions with the bacterial wall but also with viruses and parasites, while the immune modulating and anti-inflammatory characters can be attributed to a direct effect on the migration, maturation and function of various immune cells.[171]

Noteworthy, clinical trials with bovine lactoferrin added to infant formula have not shown any enhancing effect on iron absorption or iron status, which may be because bovine lactoferrin does not bind to the human lactoferrin receptor or because other constituents of the formula may interfere with iron utilization from lactoferrin.[166,172,173]

An important question is if and how the human female body regulates the iron content of breast milk. Until now, a membrane iron exporter protein has not been identified in human lactacting epithelial cells, suggesting that iron reaches the breast milk by passive desquamation and not by active excretion.[158,174] This hypothesis is supported by the occurrence of iron-containing mammary epithelial cells in human milk.[158]

A next clinically relevant knowledge gap concerns the influence of maternal iron supplementation on the iron content of breast milk.[175,176] A recent study showed that a single dose of intravenous iron temporally enhanced iron concentration of breast milk, but more research is warranted on this topic.[177]

Iron homeostasis in infancy

During the first year, the infant weight and height increase on average from 3 kg to 10 kg and from 50 cm to 75 cm respectively,[53] accompanied by an approximate increase of body iron from 270 mg to 450 mg (**Figure 1, Table 2**). The ongoing development of the neonatal brain specifically requires high quantities of iron, although precise amounts are unknown; more than 50% of resting metabolic rate is devoted to brain growth and function during the neonatal period. [5]

Throughout the first 48 hours of life an acute hypo-ferremia occurs in children,[178,179] possibly due to a birth-related stress response with an increase of cytokines. The decline of serum ferritin levels is associated with increased serum hepcidin levels, suggesting intact hepcidin-mediated regulation of iron status in young infants.[10] This is supported by rodent studies in suckling rats.[180,181] However, both animal and human studies suggest that, despite intact hepcidin signaling, the interaction between hepcidin and ferroportin and the *netto* effect on enteral iron absorption changes and matures during early life. At the age of 10 days, when fully nursing, iron absorption was independent of iron status in suckling rat pups, suggesting a lack of sensing and/or regulation of iron import. Nonetheless, at the age of 20 days, when pups were partially weaned from breast milk, ID strongly up-regulated and iron supplementation strongly down-regulated the expression of both DMT1 and ferroportin, supporting the hypothesis of developmental iron homeostasis.[182] Interestingly, these studies also showed that DMT1 and ferroportin are expressed in all areas of the small intestines and colon of the suckling rat, in contrast to adult rats in whom iron absorption predominantly occurs in the duodenum, suggesting that surface area and time available to absorb iron are age dependent.[183] Of note, ferroportin was relatively insensitive to hepcidin during suckling, supporting the above-mentioned inability to regulate iron absorption by the gut according to physiologic needs in early life. Conversely it might suggest an adaptive mechanism to absorb as much iron as possible during the suckling period when milk, which has a low iron content, is the main food.

In line with the first-mentioned hypothesis, studies in human infants showed that fractional iron absorption was independent of oral iron supplementation in breastfed infants until the age of nine months, indicating that the ability to decrease iron absorption in case of a high iron supply matures during infancy.[184] This will render the infant susceptible to iron excess and its undesired consequences such as compromised growth, predisposition to bacterial infections and a disturbance of the gut microbiota.[106,119,185,186]

Extracellular iron transport in infants

Infants have low levels of ceruloplasmin that is required to oxidize Fe^{2+} released from cells to Fe^{3+} to enabling binding to circulating transferrin. Clinical studies demonstrate that also transferrin is relatively low in the infant, especially in the premature child, suggesting that both the affinity and the capacity to bind free iron is reduced, making especially the premature infant vulnerable to damage by NTBI.[187-189]

Iron and childhood

The phase of childhood is characterized by a decreasing growth velocity compared to the period of infancy. The annual height velocity decreases to 8 cm (ages 2–4 years) and 6 cm (ages 4–6 year) during childhood. A plateau-like phase emerges in mid-childhood, wherein height velocity approaches 5.5 cm/year before puberty.[54] From the age of one year (infant) to 6 years (child), total body iron roughly triples from 500 mg to 1500 mg, especially due to the expanding blood volume from approximately 750 mL to 1500 mL (**Figure 1, Table 2**).

During childhood, growth largely depends on the activity of thyroid hormone and growth hormone (GH).[6,57] Noteworthy, iron is crucial for thyroid function, whereas IDA is associated with thyroid dysfunction, especially (subclinical) hypothyroidism function.[190-192] The mechanism by which iron influences thyroid function is still incompletely understood, but involves the activity of the heme-containing enzyme thyroid peroxidase (TPO), the tissue conversion of T4 to T3 and the pituitary TSH secretion.[192] A small case-control study comparing nine IDA children with matched controls showed no difference regarding TSH, FT4 and FT3 between cases and controls, and also no changes in thyroid function before and after correction of IDA in the IDA group.[193]

Vice versa, GH influences iron status and distribution.[194,195] Children receiving GH treatment show decreasing serum ferritin and increasing serum transferrin levels, while Hb increases, suggesting accelerated erythropoiesis.[194] In a small study (n=15), including GH replete children and adults, GH administration decreased serum hepcidin levels, indicating that GH suppresses hepcidin production, possibly indirectly via erythropoietin induced enhanced erythropoiesis[195] However, in a next study (n=10) of the same authors, including GH-deficient adults, serum hepcidin levels did not change after correction of the GH deficit in the GH-deficient individuals.[196] However, given the small sample sizes of the above-mentioned studies, more clinical studies are required in order to explore the association between iron status and both thyroid and growth hormone in childhood.

Iron and puberty

During puberty, boys and girls experience a growth velocity that is greater than at any postnatal age since infancy.[6] Boys achieve a peak height velocity (PHV) of 9.5 cm/year at a mean of about 13.5 years; the PHV in girls is approximately 8.3 cm/year at

a mean chronologic age of 11.5 years. Boys grow a mean of 28 cm, and girls grow 25 cm between takeoff and cessation of growth, according to a study performed in the United Kingdom. [6] The pubertal growth spurt is accompanied by a large increase of circulating blood volume, from on average 2800 mL in an 11-years male to 4500 mL in a 17-years male, requiring an increase of approximately 1000 mg iron dedicated to erythopoiesis alone (see also **Figure 1, Table 2**). Iron stores relatively decrease during adolescence related to the progression of genital stages, as concluded from a decrease in serum ferritin levels and an increase in the ratio soluble transferrin receptor/ferritin.[197,198]

Growth during the pubertal phase largely depends on the direct and indirect effects of the gonadal steroids estrogen and testosterone. Direct effect of the gonadal steroids involve the stimulation of the production of IGF1 and other growth factors locally in the bone. Indirect effects involve the induction of GH secretion by sex steroids. Both mechanisms result in a considerable increase in length during puberty. [6,57]

During adolescence hepcidin levels decrease in response to stimulation of endogenous production of both estrogens and testosterone.[7] This suggests a regulatory mechanism in order to adapt to increased iron demands due to rapid growth and development during puberty in both boys and girls and due to menstrual blood loss in girls.[196,199] This corroborates our results of a study on serum hepcidin values recently performed in Dutch children, showing a significant decrease of serum hepcidin relative to indicators of stored and circulating iron after the age of 12 years (www.hepcidinanalysis.com[42]).[43]

Overall aspects of developmental aspects of iron metabolism from fetus to adult

Iron is crucial during the journey from fetus to adult. The growing child is dependent on iron for the huge expansion of the blood volume during childhood and for the maturation of many organs and physiological systems including the skeleton, the brain, the immune system and the gut microbiota. According to results of MRI imaging, especially the iron content of the brain is thought to considerably increase during childhood and adolescence.[78] Morover, hormonal systems that highly influence growth during childhood, as the thyroid and GH system, are associated with iron status. Therefore, in children iron demands are relatively high, compared to adults (**Figure 1, Table 2**). Unbalanced iron status during critical iron-dependent windows

of growth and development may have unfavorable and long-lasting effects on the function of the involved organs or physiological systems. Both *in vitro* and *in vivo* data suggest that iron handling matures during childhood, corroborating our recent study on serum hepcdin values in healthy children (www.hepcidinanalysis.com[42]).[43] However, how the human body balances between the opposite forces regarding iron metabolism, e.g. the need to acquire large amounts of iron in order to ensure the enormous growth that occurs during childhood versus the need to withhold iron from iron-dependent pathogens that are especially threatening for infants, is still largely unknown. Although both *in vitro* and *in vivo* data suggest intact hepcidin signaling in early life, the relative contribution of both stimulating and inhibiting stimuli to hepcidin secretion is unclear. Other *in vitro* data suggest that the interaction between hepcidin and ferroportin inside the enterocyte changes after weaning from breastfeeding, favoring enhanced iron absorption during the suckling period, which is characterized by a low iron intake. To the best of our knowledge, no *in vivo* data are available on this topic.

Furthermore, understanding how the human body distributes iron between the various iron-demanding compartments during the period from fetus to adult, both in periods of iron homeostasis, iron deficit and iron excess, is limited. Moreover, knowledge on the optimal iron status for the different iron-demanding tissues during human growth and development is largely missing. Finally, the lack of adequate biomarkers of iron status of the various iron-consuming organs in the developing child, and especially the central nervous system, hampers both clinicians and researchers in assessing, possibly treating and exploring (dys)regulation of iron homeostasis.

CONCLUSIONS

Knowledge on systemic and cellular iron homeostasis has greatly improved during the last decades, but many questions remain on the physiology of iron regulation in childhood. Our recent study in healthy children indicate that serum hepcidin relative to indicators of body iron is age dependent, suggesting a changing set point regarding systemic iron homeostasis during childhood. However, understanding of the optimal iron status for the different iron-demanding tissues is limited. Also mechanisms underlying the human body prioritorization and distribution of iron between the erythoblasts in the bone marrow, the brain and other iron-dependent organs systems during the different phases of development from fetus to adult are largely unknown. In order to get more insight into these processes, adequate assessment of iron status of the various iron-consuming organs in the developing child, and especially the central nervous system, is paramount, but appropriate (noninvasive) biomarkers are lacking until now. In this review we identified several knowledge gaps (summarized in Table 3). When these gaps have been succesfully addressed, the newly acquired insights will contribute to the development of clinical guidelines on a balanced iron supply and on the accurate diagnosis and treatment of iron disorders of the growing and developing child.

For now, the limited understanding of iron physiology of childhood hampers the diagnosis and management of both acquired iron disorders as ID (anemia), anemia of inflammation or iron loading due to ineffective erythropoiesis and also anemias due to genetic disorders of iron metabolism.

Table 3. Future research challenges

Knowledge gaps	
Physiology of iron and iron handling in childhood	
Critical roles of iron during the different phases of childhood	The relation between iron status in children and with adult peak bone mass The effect of iron status in infants on vaccination response?
Regulation of iron handling *in utero*	Systemic fetal iron homeostasis, role of maternal and fetal hepcidin Hepcidin regulation *in utero* Cellular iron trafficking from mother to fetus
Iron and epigenetic programming	The relevance of the Barker hypothesis as described for metabolic syndrome for iron deficiency and iron excess in utero The existence of epigenetic programming of iron regulating genes
Iron and lactation	Factors contributing to iron content of mother milk and its bio-availability for neonate
Developmental aspects of iron handling	Survival advantage of relatively high hepcidin in relation to body iron parameters due to the role of hepcidin in the innate immune system Priorisation of iron acquiring versus iron withholding in children during inflammation Priorisation of iron between erytroblasts, brain, other iron requiring organs age dependent, does it change during childhood. Hierarchical distrubution of iron among different organ systems
Diagnosis of iron status	
Diagnosis of iron deficiency and iron deficiency anemia	Hepcidin as a possible marker for ID(A) in both adults and children? The value of hepcidin as a marker of ID before IDA occurs Hepcidin, in combination with other iron and inflammatory parameters as a marker to differentiate between ID, iron restriction in inflammation (recurrent upper airway infections, very common in childhood) and iron restriction in combination with ID. Hepcidin as a marker to personalize iron treatment in accordance with individual characteristics
Diagnosis of ID of the brain	Brain iron status indicators, i.e. serum biomarkers that provide information on brain iron status and detect brain iron deficiency before IDA occurs
Hepcidin measurements	Worldwide implementation of standardized hepcidin assays. Standardized serum hepcidin reference ranges for different age groups from different ethnicities. Development and analytical and clinical validation of non-invasive hepcidin measurements (saliva, urine)
Treatment of iron deficiency	Guidelines on iron treatment in premature and small for gestational age children balancing the risk of iron deficiency against the risk of dysbiosis/perturbation of the gut microbiotca

Author Contributions

Conceptualization: A.E.D. and D.W.S.
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Reviewing and editing: D.W.S.

Conflict-of-interest disclosure

DWS is an employee of Radboudumc that offers hepcidin assays and hepcidin reference material to the research, clinical and Pharmaceutical community at a fee for service via the Hepcidinanalysis initiative (www.hepcidinanalysis.com).[42]

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REFERENCES

1. Beard JL. Iron biology in immune function, muscle metabolism and neuronal functioning. *The Journal of nutrition.* 2001;131(2s-2):568S-579S; discussion 580S.
2. Fleming RE, Ponka P. Iron overload in human disease. *The New England journal of medicine.* 2012;366(4):348-359.
3. Armitage AE, Drakesmith H. Genetics. The battle for iron. *Science.* 2014;346(6215):1299-1300.
4. Pietrangelo A. Pathogens, Metabolic Adaptation, and Human Diseases--An Iron-Thrifty Genetic Model. *Gastroenterology.* 2015;149(4):834-838.
5. Hochberg Z. Evo-devo of child growth II: human life history and transition between its phases. *European journal of endocrinology.* 2009;160(2):135-141.
6. Williams Textbook of Endocrinology. Styne DM, Grumbach MM. Chapter 25. Physiology and Disorders of Puberty. 13th Edition 2016. Elsevier.
7. Girelli D, Nemeth E, Swinkels DW. Hepcidin in the diagnosis of iron disorders. *Blood.* 2016;127(23):2809-2813.
8. Lonnerdal B, Georgieff MK, Hernell O. Developmental Physiology of Iron Absorption, Homeostasis, and Metabolism in the Healthy Term Infant. *The Journal of pediatrics.* 2015;167(4 Suppl):S8-14.
9. Lonnerdal B. Development of iron homeostasis in infants and young children. *The American journal of clinical nutrition.* 2017;106(Suppl 6):1575s-1580s.
10. Armitage AE, Moretti D. The Importance of Iron Status for Young Children in Low- and Middle-Income Countries: A Narrative Review. *Pharmaceuticals (Basel).* 2019;12(2).
11. Muckenthaler MU, Rivella S, Hentze MW, Galy B. A Red Carpet for Iron Metabolism. *Cell.* 2017;168(3):344-361.
12. Beard J. Iron deficiency alters brain development and functioning. *The Journal of nutrition.* 2003;133(5 Suppl 1):1468s-1472s.
13. Knutson MD. Non-transferrin-bound iron transporters. *Free radical biology & medicine.* 2019;133:101-111.
14. Hentze MW, Muckenthaler MU, Galy B, Camaschella C. Two to tango: regulation of Mammalian iron metabolism. *Cell.* 2010;142(1):24-38.
15. Muckenthaler MU, Galy B, Hentze MW. Systemic iron homeostasis and the iron-responsive element/iron-regulatory protein (IRE/IRP) regulatory network. *Annual review of nutrition.* 2008;28:197-213.
16. Wilkinson N, Pantopoulos K. The IRP/IRE system in vivo: insights from mouse models. *Frontiers in pharmacology.* 2014;5:176.
17. Dev S, Babitt JL. Overview of iron metabolism in health and disease. *Hemodialysis international International Symposium on Home Hemodialysis.* 2017;21 Suppl 1:S6-s20.
18. Semenza GL. Hypoxia-inducible factors in physiology and medicine. *Cell.* 2012;148(3):399-408.
19. Anderson SA, Nizzi CP, Chang YI, et al. The IRP1-HIF-2alpha axis coordinates iron and oxygen sensing with erythropoiesis and iron absorption. *Cell metabolism.* 2013;17(2):282-290.
20. Drakesmith H, Nemeth E, Ganz T. Ironing out Ferroportin. *Cell metabolism.* 2015;22(5):777-787.
21. Silvestri L, Nai A, Dulja A, Pagani A. Hepcidin and the BMP-SMAD pathway: An unexpected liaison. *Vitamins and hormones.* 2019;110:71-99.
22. Pak M, Lopez MA, Gabayan V, Ganz T, Rivera S. Suppression of hepcidin during anemia requires erythropoietic activity. *Blood.* 2006;108(12):3730-3735.
23. Kroot JJ, Tjalsma H, Fleming RE, Swinkels DW. Hepcidin in human iron disorders: diagnostic implications. *Clinical chemistry.* 2011;57(12):1650-1669.
24. Kautz L, Nemeth E. Molecular liaisons between

erythropoiesis and iron metabolism. *Blood.* 2014;124(4):479-482.
25. Nicolas G, Chauvet C, Viatte L, et al. The gene encoding the iron regulatory peptide hepcidin is regulated by anemia, hypoxia, and inflammation. *The Journal of clinical investigation.* 2002;110(7):1037-1044.
26. Michels K, Nemeth E, Ganz T, Mehrad B. Hepcidin and Host Defense against Infectious Diseases. *PLoS pathogens.* 2015;11(8):e1004998.
27. Ganz T. Iron and infection. *International journal of hematology.* 2017.
28. Burte F, Brown BJ, Orimadegun AE, et al. Circulatory hepcidin is associated with the anti-inflammatory response but not with iron or anemic status in childhood malaria. *Blood.* 2013;121(15):3016-3022.
29. Donker AE, Schaap CC, Novotny VM, et al. Iron Refractory Iron Deficiency Anemia (IRIDA): A heterogeneous disease that is not always iron refractory. *American journal of hematology.* 2016.
30. Suchdev PS, Williams AM, Mei Z, et al. Assessment of iron status in settings of inflammation: challenges and potential approaches. *The American journal of clinical nutrition.* 2017;106(Suppl 6):1626s-1633s.
31. Ambroszkiewicz J, Klemarczyk W, Mazur J, et al. Serum Hepcidin and Soluble Transferrin Receptor in the Assessment of Iron Metabolism in Children on a Vegetarian Diet. *Biol Trace Elem Res.* 2017;180(2):182-190.
32. Uijterschout L, Swinkels DW, Akkermans MD, et al. The value of soluble transferrin receptor and hepcidin in the assessment of iron status in children with cystic fibrosis. *J Cyst Fibros.* 2014;13(6):639-644.
33. Brannon PM, Stover PJ, Taylor CL. Integrating themes, evidence gaps, and research needs identified by workshop on iron screening and supplementation in iron-replete pregnant women and young children. *The American journal of clinical nutrition.* 2017;106(Suppl 6):1703s-1712s.
34. Sdogou T, Tsentidis C, Gourgiotis D, et al. Immunoassay-based serum hepcidin reference range measurements in healthy children: differences among age groups. *J Clin Lab Anal.* 2015;29(1):10-14.
35. Uijterschout L, Swinkels DW, Domellöf M, et al. Serum hepcidin measured by immunochemical and mass-spectrometric methods and their correlation with iron status indicators in healthy children aged 0.5-3 y. *Pediatr Res.* 2014;76(4):409-414.
36. Mupfudze TG, Stoltzfus RJ, Rukobo S, et al. Hepcidin decreases over the first year of life in healthy African infants. *British journal of haematology.* 2014;164(1):150-153.
37. Mupfudze TG, Stoltzfus RJ, Rukobo S, et al. Plasma Concentrations of Hepcidin in Anemic Zimbabwean Infants. *PloS one.* 2015;10(8):e0135227.
38. Jaeggi T, Moretti D, Kvalsvig J, et al. Iron status and systemic inflammation, but not gut inflammation, strongly predict gender-specific concentrations of serum hepcidin in infants in rural Kenya. *PloS one.* 2013;8(2):e57513.
39. Choi HS, Song SH, Lee JH, Kim HJ, Yang HR. Serum hepcidin levels and iron parameters in children with iron deficiency. *Korean J Hematol.* 2012;47(4):286-292.
40. Mahajan G, Sharma S, Chandra J, Nangia A. Hepcidin and iron parameters in children with anemia of chronic disease and iron deficiency anemia. *Blood Res.* 2017;52(3):212-217.
41. Sanad M, Osman M, Gharib A. Obesity modulate serum hepcidin and treatment outcome of iron deficiency anemia in children: a case control study. *Ital J Pediatr.* 2011;37:34.
42. http://www.hepcidinanalysis.com. Accessed 9 October 2019.
43. Donker AE, Galesloot TE, Laarakkers CM, Klaver SM, Bakkeren DL, Swinkels DW. Standardized serum hepcidin values in Dutch children: Set point relative to body iron changes during childhood. *Pediatric blood & cancer.* 2020;67(3):28038.
44. Diepeveen LE, Laarakkers CMM, Martos G, et al. Provisional standardization of hepcidin assays: creating a traceability chain with

a primary reference material, candidate reference method and a commutable secondary reference material. *Clin Chem Lab Med.* 2018.

45. Kemna EH, Kartikasari AE, van Tits LJ, Pickkers P, Tjalsma H, Swinkels DW. Regulation of hepcidin: insights from biochemical analyses on human serum samples. *Blood cells, molecules & diseases.* 2008;40(3):339-346.
46. Lasocki S, Baron G, Driss F, et al. Diagnostic accuracy of serum hepcidin for iron deficiency in critically ill patients with anemia. *Intensive Care Med.* 2010;36(6):1044-1048.
47. Sangkhae V, Nemeth E. To induce or not to induce: the fight over hepcidin regulation. *Haematologica.* 2019;104(6):1093-1095.
48. Stoffel NU, Lazrak M, Bellitir S, et al. The opposing effects of acute inflammation and iron deficiency anemia on serum hepcidin and iron absorption in young women. *Haematologica.* 2019;104(6):1143-1149.
49. Jonker FA, Calis JC, Phiri K, et al. Low hepcidin levels in severely anemic malawian children with high incidence of infectious diseases and bone marrow iron deficiency. *PloS one.* 2013;8(12):e78964.
50. Prentice AM, Bah A, Jallow MW, et al. Respiratory infections drive hepcidin-mediated blockade of iron absorption leading to iron deficiency anemia in African children. *Science advances.* 2019;5(3):eaav9020.
51. Weiss G, Ganz T, Goodnough LT. Anemia of inflammation. *Blood.* 2019;133(1):40-50.
52. Hoftiezer L, Hof MHP, Dijs-Elsinga J, Hogeveen M, Hukkelhoven C, van Lingen RA. From population reference to national standard: new and improved birthweight charts. *American journal of obstetrics and gynecology.* 2019;220(4):383.e381-383.e317.
53. TNO. Factsheet Resultaten Vijfde Landelijke Groeistudie TNO, 10 juni 2010.
54. Veldhuis JD, Roemmich JN, Richmond EJ, et al. Endocrine control of body composition in infancy, childhood, and puberty. *Endocrine reviews.* 2005;26(1):114-146.
55. Karlberg J. A biologically-oriented mathematical model (ICP) for human growth. *Acta paediatrica Scandinavica Supplement.* 1989;350:70-94.
56. Karlberg J. On the construction of the infancy-childhood-puberty growth standard. *Acta paediatrica Scandinavica Supplement.* 1989;356:26-37.
57. Tse WY, Hindmarsh PC, Brook CG. The infancy-childhood-puberty model of growth: clinical aspects. *Acta paediatrica Scandinavica Supplement.* 1989;356:38-43; discussion 44-35.
58. Hochberg Z. Evolutionary perspective in child growth. *Rambam Maimonides medical journal.* 2011;2(3):e0057.
59. Domellof M, Braegger C, Campoy C, et al. Iron requirements of infants and toddlers. *Journal of pediatric gastroenterology and nutrition.* 2014;58(1):119-129.
60. http://www.who.int/vmnis/anaemia/prevalence/summary/anaemia_data_status_t2/en/ Accessed 9 October 2019.
61. Vos T, Abajobir AA, Abate KH, et al. Global, regional, and national incidence, prevalence, and years lived with disability for 328 diseases and injuries for 195 countries, 1990–2016: a systematic analysis for the Global Burden of Disease Study 2016. *The Lancet.* 2017;390(10100):1211-1259.
62. Balogh E, Paragh G, Jeney V. Influence of Iron on Bone Homeostasis. *Pharmaceuticals (Basel).* 2018;11(4).
63. Levine MA. Assessing bone health in children and adolescents. *Indian journal of endocrinology and metabolism.* 2012;16(Suppl 2):S205-212.
64. Heaney RP, Abrams S, Dawson-Hughes B, et al. Peak bone mass. *Osteoporosis international : a journal established as result of cooperation between the European Foundation for Osteoporosis and the National Osteoporosis Foundation of the USA.* 2000;11(12):985-1009.
65. Rauner M, Baschant U, Roetto A, et al. Transferrin receptor 2 controls bone mass and pathological bone formation via BMP and Wnt signaling. *Nature metabolism.* 2019;1(1):111-124.

66. Jeney V. Clinical Impact and Cellular Mechanisms of Iron Overload-Associated Bone Loss. *Frontiers in pharmacology.* 2017;8:77.
67. Yang Q, Jian J, Abramson SB, Huang X. Inhibitory effects of iron on bone morphogenetic protein 2-induced osteoblastogenesis. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research.* 2011;26(6):1188-1196.
68. Ishii KA, Fumoto T, Iwai K, et al. Coordination of PGC-1beta and iron uptake in mitochondrial biogenesis and osteoclast activation. *Nature medicine.* 2009;15(3):259-266.
69. Tsay J, Yang Z, Ross FP, et al. Bone loss caused by iron overload in a murine model: importance of oxidative stress. *Blood.* 2010;116(14):2582-2589.
70. Jiang Y, Yan Y, Wang X, Zhu G, Xu YJ. Hepcidin inhibition on the effect of osteogenesis in zebrafish. *Biochemical and biophysical research communications.* 2016;476(1):1-6.
71. Zoller H, Schaefer B, Glodny B. Iron-induced hypophosphatemia: an emerging complication. *Current opinion in nephrology and hypertension.* 2017;26(4):266-275.
72. Zhao GY, Zhao LP, He YF, et al. A comparison of the biological activities of human osteoblast hFOB1.19 between iron excess and iron deficiency. *Biol Trace Elem Res.* 2012;150(1-3):487-495.
73. Toxqui L, Perez-Granados AM, Blanco-Rojo R, Wright I, de la Piedra C, Vaquero MP. Low iron status as a factor of increased bone resorption and effects of an iron and vitamin D-fortified skimmed milk on bone remodelling in young Spanish women. *Eur J Nutr.* 2014;53(2):441-448.
74. Wright I, Blanco-Rojo R, Fernandez MC, et al. Bone remodelling is reduced by recovery from iron-deficiency anaemia in premenopausal women. *Journal of physiology and biochemistry.* 2013;69(4):889-896.
75. Skordis N, Toumba M. Bone disease in thalassaemia major: recent advances in pathogenesis and clinical aspects. *Pediatric endocrinology reviews : PER.* 2011;8 Suppl 2:300-306.
76. Georgieff MK. Long-term brain and behavioral consequences of early iron deficiency. *Nutrition reviews.* 2011;69 Suppl 1:S43-48.
77. Georgieff MK. Iron assessment to protect the developing brain. *The American journal of clinical nutrition.* 2017;106(Suppl 6):1588s-1593s.
78. Hect JL, Daugherty AM, Hermez KM, Thomason ME. Developmental variation in regional brain iron and its relation to cognitive functions in childhood. *Developmental cognitive neuroscience.* 2018;34:18-26.
79. Tran PV, Carlson ES, Fretham SJ, Georgieff MK. Early-life iron deficiency anemia alters neurotrophic factor expression and hippocampal neuron differentiation in male rats. *The Journal of nutrition.* 2008;138(12):2495-2501.
80. Felt BT, Beard JL, Schallert T, et al. Persistent neurochemical and behavioral abnormalities in adulthood despite early iron supplementation for perinatal iron deficiency anemia in rats. *Behavioural brain research.* 2006;171(2):261-270.
81. Kaur D, Peng J, Chinta SJ, et al. Increased murine neonatal iron intake results in Parkinson-like neurodegeneration with age. *Neurobiology of aging.* 2007;28(6):907-913.
82. Berggren KL, Lu Z, Fox JA, Dudenhoeffer M, Agrawal S, Fox JH. Neonatal Iron Supplementation Induces Striatal Atrophy in Female YAC128 Huntington's Disease Mice. *Journal of Huntington's disease.* 2016;5(1):53-63.
83. Hare DJ, Arora M, Jenkins NL, Finkelstein DI, Doble PA, Bush AI. Is early-life iron exposure critical in neurodegeneration? *Nature reviews Neurology.* 2015;11(9):536-544.
84. Lozoff B. Iron deficiency and child development. *Food and nutrition bulletin.* 2007;28(4 Suppl):S560-571.
85. Grantham-McGregor S, Ani C. A review of studies on the effect of iron deficiency on cognitive development in children. *The Journal of nutrition.* 2001;131(2s-2):649S-666S; discussion 666S-668S.

86. Iglesias L, Canals J, Arija V. Effects of prenatal iron status on child neurodevelopment and behavior: A systematic review. *Critical reviews in food science and nutrition.* 2018;58(10):1604-1614.
87. Wessling-Resnick M. Excess iron: considerations related to development and early growth. *The American journal of clinical nutrition.* 2017;106(Suppl 6):1600s-1605s.
88. Lozoff B, Beard J, Connor J, Barbara F, Georgieff M, Schallert T. Long-lasting neural and behavioral effects of iron deficiency in infancy. *Nutrition reviews.* 2006;64(5 Pt 2):S34-43; discussion S72-91.
89. Nai A, Lidonnici MR, Rausa M, et al. The second transferrin receptor regulates red blood cell production in mice. *Blood.* 2015;125(7):1170-1179.
90. Lozoff B, Georgieff MK. Iron deficiency and brain development. *Seminars in pediatric neurology.* 2006;13(3):158-165.
91. Cusick SE, Georgieff MK, Rao R. Approaches for Reducing the Risk of Early-Life Iron Deficiency-Induced Brain Dysfunction in Children. *Nutrients.* 2018;10(2).
92. Wang B, Zhan S, Gong T, Lee L. Iron therapy for improving psychomotor development and cognitive function in children under the age of three with iron deficiency anaemia. *The Cochrane database of systematic reviews.* 2013(6):Cd001444.
93. Pasricha SR, Hayes E, Kalumba K, Biggs BA. Effect of daily iron supplementation on health in children aged 4-23 months: a systematic review and meta-analysis of randomised controlled trials. *The Lancet Global health.* 2013;1(2):e77-e86.
94. Turker G, Ergen K, Karakoc Y, Arisoy AE, Barutcu UB. Concentrations of toxic metals and trace elements in the meconium of newborns from an industrial city. *Biology of the neonate.* 2006;89(4):244-250.
95. Gahagan S, Delker E, Blanco E, Burrows R, Lozoff B. Randomized Controlled Trial of Iron-Fortified versus Low-Iron Infant Formula: Developmental Outcomes at 16 Years. *The Journal of pediatrics.* 2019.
96. Lozoff B, Castillo M, Clark KM, Smith JB. Iron-fortified vs low-iron infant formula: developmental outcome at 10 years. *Archives of pediatrics & adolescent medicine.* 2012;166(3):208-215.
97. Fomon SJ, Ziegler EE, Serfass RE, Nelson SE, Rogers RR, Frantz JA. Less than 80% of absorbed iron is promptly incorporated into erythrocytes of infants. *The Journal of nutrition.* 2000;130(1):45-52.
98. Zhang DL, Senecal T, Ghosh MC, Ollivierre-Wilson H, Tu T, Rouault TA. Hepcidin regulates ferroportin expression and intracellular iron homeostasis of erythroblasts. *Blood.* 2011;118(10):2868-2877.
99. Zhang DL, Ghosh MC, Ollivierre H, Li Y, Rouault TA. Ferroportin deficiency in erythroid cells causes serum iron deficiency and promotes hemolysis due to oxidative stress. *Blood.* 2018;132(19):2078-2087.
100. Khalil S, Delehanty L, Grado S, et al. Iron modulation of erythropoiesis is associated with Scribble-mediated control of the erythropoietin receptor. *The Journal of experimental medicine.* 2018;215(2):661-679.
101. Levy JE, Jin O, Fujiwara Y, Kuo F, Andrews NC. Transferrin receptor is necessary for development of erythrocytes and the nervous system. *Nature genetics.* 1999;21(4):396-399.
102. Dowling DJ, Levy O. Ontogeny of early life immunity. *Trends in immunology.* 2014;35(7):299-310.
103. Kelly D, Coutts AG. Early nutrition and the development of immune function in the neonate. *The Proceedings of the Nutrition Society.* 2000;59(2):177-185.
104. Kollmann TR, Kampmann B, Mazmanian SK, Marchant A, Levy O. Protecting the Newborn and Young Infant from Infectious Diseases: Lessons from Immune Ontogeny. *Immunity.* 2017;46(3):350-363.
105. Olin A, Henckel E, Chen Y, et al. Stereotypic Immune System Development in Newborn Children. *Cell.* 2018;174(5):1277-1292.e1214.
106. Arrieta MC, Stiemsma LT, Amenyogbe N,

Brown EM, Finlay B. The intestinal microbiome in early life: health and disease. *Frontiers in immunology.* 2014;5:427.
107. Martins AC, Almeida JI, Lima IS, Kapitao AS, Gozzelino R. Iron Metabolism and the Inflammatory Response. *IUBMB life.* 2017;69(6):442-450.
108. Soares MP, Hamza I. Macrophages and Iron Metabolism. *Immunity.* 2016;44(3):492-504.
109. Roos D, van Bruggen R, Meischl C. Oxidative killing of microbes by neutrophils. *Microbes and infection.* 2003;5(14):1307-1315.
110. Jabara HH, Boyden SE, Chou J, et al. A missense mutation in TFRC, encoding transferrin receptor 1, causes combined immunodeficiency. *Nature genetics.* 2016;48(1):74-78.
111. Soares MP, Weiss G. The Iron age of host-microbe interactions. *EMBO reports.* 2015;16(11):1482-1500.
112. Nairz M, Dichtl S, Schroll A, et al. Iron and innate antimicrobial immunity-Depriving the pathogen, defending the host. *Journal of trace elements in medicine and biology : organ of the Society for Minerals and Trace Elements (GMS).* 2018;48:118-133.
113. Nairz M, Schroll A, Demetz E, Tancevski I, Theurl I, Weiss G. 'Ride on the ferrous wheel'--the cycle of iron in macrophages in health and disease. *Immunobiology.* 2015;220(2):280-294.
114. Seligman PA, Kovar J, Gelfand EW. Lymphocyte proliferation is controlled by both iron availability and regulation of iron uptake pathways. *Pathobiology : journal of immunopathology, molecular and cellular biology.* 1992;60(1):19-26.
115. Kollmann TR. Variation between Populations in the Innate Immune Response to Vaccine Adjuvants. *Frontiers in immunology.* 2013;4:81.
116. Kyvsgaard JN, Overgaard AJ, Thorsen SU, et al. High Neonatal Blood Iron Content Is Associated with the Risk of Childhood Type 1 Diabetes Mellitus. *Nutrients.* 2017;9(11).
117. van Santen S, de Mast Q, Swinkels DW, van der Ven AJ. The iron link between malaria and invasive non-typhoid Salmonella infections. *Trends in parasitology.* 2013;29(5):220-227.
118. Soofi S, Cousens S, Iqbal SP, et al. Effect of provision of daily zinc and iron with several micronutrients on growth and morbidity among young children in Pakistan: a cluster-randomised trial. *Lancet.* 2013;382(9886):29-40.
119. Goulet O, Hojsak I, Kolacek S, et al. Paediatricians play a key role in preventing early harmful events that could permanently influence the development of the gut microbiota in childhood. *Acta paediatrica (Oslo, Norway : 1992).* 2019.
120. Collado MC, Rautava S, Aakko J, Isolauri E, Salminen S. Human gut colonisation may be initiated in utero by distinct microbial communities in the placenta and amniotic fluid. *Scientific reports.* 2016;6:23129.
121. Kortman GA, Raffatellu M, Swinkels DW, Tjalsma H. Nutritional iron turned inside out: intestinal stress from a gut microbial perspective. *FEMS microbiology reviews.* 2014;38(6):1202-1234.
122. Lapillonne A, Bronsky J, Campoy C, et al. Feeding the Late and Moderately Preterm Infant: A Position Paper of the European Society for Paediatric Gastroenterology, Hepatology and Nutrition Committee on Nutrition. *Journal of pediatric gastroenterology and nutrition.* 2019.
123. Gambling L, Kennedy C, McArdle HJ. Iron and copper in fetal development. *Seminars in cell & developmental biology.* 2011;22(6):637-644.
124. Hochberg Z, Feil R, Constancia M, et al. Child health, developmental plasticity, and epigenetic programming. *Endocrine reviews.* 2011;32(2):159-224.
125. Widdowson EM, Spray CM. Chemical development in utero. *Arch Dis Child.* 1951;26(127):205-214.
126. Fisher AL, Nemeth E. Iron homeostasis during pregnancy. *The American journal of clinical nutrition.* 2017;106(Suppl 6):1567s-1574s.
127. Bothwell TH. Iron requirements in pregnancy and strategies to meet them. *The American journal of clinical nutrition.* 2000;72(1 Suppl):257s-264s.

128. Sangkhae V, Nemeth E. Placental iron transport: The mechanism and regulatory circuits. *Free radical biology & medicine.* 2019;133:254-261.
129. Cao C, Fleming MD. The placenta: the forgotten essential organ of iron transport. *Nutrition reviews.* 2016;74(7):421-431.
130. Guo Y, Zhang N, Zhang D, et al. Iron homeostasis in pregnancy and spontaneous abortion. *American journal of hematology.* 2019;94(2):184-188.
131. Koenig MD, Tussing-Humphreys L, Day J, Cadwell B, Nemeth E. Hepcidin and iron homeostasis during pregnancy. *Nutrients.* 2014;6(8):3062-3083.
132. van Santen S, Kroot JJ, Zijderveld G, Wiegerinck ET, Spaanderman ME, Swinkels DW. The iron regulatory hormone hepcidin is decreased in pregnancy: a prospective longitudinal study. *Clin Chem Lab Med.* 2013;51(7):1395-1401.
133. Kumar A, Rai AK, Basu S, Dash D, Singh JS. Cord blood and breast milk iron status in maternal anemia. *Pediatrics.* 2008;121(3):e673-677.
134. Singla PN, Tyagi M, Shankar R, Dash D, Kumar A. Fetal iron status in maternal anemia. *Acta paediatrica (Oslo, Norway : 1992).* 1996;85(11):1327-1330.
135. Basu S, Kumar N, Srivastava R, Kumar A. Maternal and Cord Blood Hepcidin Concentrations in Severe Iron Deficiency Anemia. *Pediatrics and neonatology.* 2016;57(5):413-419.
136. Allen LH. Anemia and iron deficiency: effects on pregnancy outcome. *The American journal of clinical nutrition.* 2000;71(5 Suppl):1280s-1284s.
137. Sebastiani G, Herranz Barbero A, Borras-Novell C, et al. The Effects of Vegetarian and Vegan Diet during Pregnancy on the Health of Mothers and Offspring. *Nutrients.* 2019;11(3).
138. Lipinski P, Stys A, Starzynski RR. Molecular insights into the regulation of iron metabolism during the prenatal and early postnatal periods. *Cellular and molecular life sciences : CMLS.* 2013;70(1):23-38.
139. Vanhees K, Vonhogen IG, van Schooten FJ, Godschalk RW. You are what you eat, and so are your children: the impact of micronutrients on the epigenetic programming of offspring. *Cellular and molecular life sciences : CMLS.* 2014;71(2):271-285.
140. Barker DJ. Fetal origins of coronary heart disease. *Bmj.* 1995;311(6998):171-174.
141. Junien C. Impact of diets and nutrients/drugs on early epigenetic programming. *Journal of inherited metabolic disease.* 2006;29(2-3):359-365.
142. Godfrey KM, Redman CW, Barker DJ, Osmond C. The effect of maternal anaemia and iron deficiency on the ratio of fetal weight to placental weight. *British journal of obstetrics and gynaecology.* 1991;98(9):886-891.
143. Godfrey KM, Barker DJ. Maternal nutrition in relation to fetal and placental growth. *European journal of obstetrics, gynecology, and reproductive biology.* 1995;61(1):15-22.
144. Clardy SL, Wang X, Zhao W, et al. Acute and chronic effects of developmental iron deficiency on mRNA expression patterns in the brain. *Journal of neural transmission Supplementum.* 2006(71):173-196.
145. Tran PV, Kennedy BC, Lien YC, Simmons RA, Georgieff MK. Fetal iron deficiency induces chromatin remodeling at the Bdnf locus in adult rat hippocampus. *American journal of physiology Regulatory, integrative and comparative physiology.* 2015;308(4):R276-282.
146. Siddappa AM, Rao R, Long JD, Widness JA, Georgieff MK. The assessment of newborn iron stores at birth: a review of the literature and standards for ferritin concentrations. *Neonatology.* 2007;92(2):73-82.
147. Oski FA. Iron deficiency in infancy and childhood. *The New England journal of medicine.* 1993;329(3):190-193.
148. Martin CR, Ling PR, Blackburn GL. Review of Infant Feeding: Key Features of Breast Milk and Infant Formula. *Nutrients.* 2016;8(5).
149. Neumann CG, Alpaugh M. Birthweight doubling time: a fresh look. *Pediatrics.* 1976;57(4):469-473.
150. Majumdar I, Paul P, Talib VH, Ranga S. The

effect of iron therapy on the growth of iron-replete and iron-deplete children. *Journal of tropical pediatrics.* 2003;49(2):84-88.
151. Angeles IT, Schultink WJ, Matulessi P, Gross R, Sastroamidjojo S. Decreased rate of stunting among anemic Indonesian preschool children through iron supplementation. *The American journal of clinical nutrition.* 1993;58(3):339-342.
152. McDonagh M, Blazina I, Dana T, Cantor A, Bougatsos C. U.S. Preventive Services Task Force Evidence Syntheses, formerly Systematic Evidence Reviews. *Routine Iron Supplementation and Screening for Iron Deficiency Anemia in Children Ages 6 to 24 Months: A Systematic Review to Update the U.S. Preventive Services Task Force Recommendation*. Rockville (MD): Agency for Healthcare Research and Quality (US); 2015.
153. Brittenham GM, Andersson M, Egli I, et al. Circulating non-transferrin-bound iron after oral administration of supplemental and fortification doses of iron to healthy women: a randomized study. *The American journal of clinical nutrition.* 2014;100(3):813-820.
154. Jaeggi T, Kortman GA, Moretti D, et al. Iron fortification adversely affects the gut microbiome, increases pathogen abundance and induces intestinal inflammation in Kenyan infants. *Gut.* 2015;64(5):731-742.
155. Suzuki YA, Shin K, Lonnerdal B. Molecular cloning and functional expression of a human intestinal lactoferrin receptor. *Biochemistry.* 2001;40(51):15771-15779.
156. Yalcin SS, Baykan A, Yurdakok K, Yalcin S, Gucus AI. The factors that affect milk-to-serum ratio for iron during early lactation. *Journal of pediatric hematology/oncology.* 2009;31(2):85-90.
157. Qasem WA, Friel JK. An Overview of Iron in Term Breast-Fed Infants. *Clinical medicine insights Pediatrics.* 2015;9:79-84.
158. Friel J, Qasem W, Cai C. Iron and the Breastfed Infant. *Antioxidants (Basel, Switzerland).* 2018;7(4).
159. Mayeur S, Spahis S, Pouliot Y, Levy E. Lactoferrin, a Pleiotropic Protein in Health and Disease. *Antioxidants & redox signaling.* 2016;24(14):813-836.
160. Cerami C. Iron Nutriture of the Fetus, Neonate, Infant, and Child. *Annals of nutrition & metabolism.* 2017;71 Suppl 3:8-14.
161. Lonnerdal B, Iyer S. Lactoferrin: molecular structure and biological function. *Annual review of nutrition.* 1995;15:93-110.
162. Ganz T. Iron and infection. *International journal of hematology.* 2018;107(1):7-15.
163. Jiang R, Lopez V, Kelleher SL, Lonnerdal B. Apo- and holo-lactoferrin are both internalized by lactoferrin receptor via clathrin-mediated endocytosis but differentially affect ERK-signaling and cell proliferation in Caco-2 cells. *Journal of cellular physiology.* 2011;226(11):3022-3031.
164. Sánchez L, Calvo M, Brock JH. Biological role of lactoferrin. *Archives of disease in childhood.* 1992;67(5):657-661.
165. DAVIDSON LA, LÖNNERDAL B. Persistence of Human Milk Proteins in the Breast-Fed Infant. *Acta Paediatrica.* 1987;76(5):733-740.
166. Lonnerdal B. Nutritional and physiologic significance of human milk proteins. *The American journal of clinical nutrition.* 2003;77(6):1537s-1543s.
167. Lonnerdal B, Bryant A. Absorption of iron from recombinant human lactoferrin in young US women. *The American journal of clinical nutrition.* 2006;83(2):305-309.
168. Kawakami H, Lonnerdal B. Isolation and function of a receptor for human lactoferrin in human fetal intestinal brush-border membranes. *The American journal of physiology.* 1991;261(5 Pt 1):G841-846.
169. Lawrence RA, Lawrence RM. Breastfeeding : a guide for the medical profession. 2016.
170. Andreas NJ, Kampmann B, Mehring Le-Doare K. Human breast milk: A review on its composition and bioactivity. *Early human development.* 2015;91(11):629-635.
171. Giansanti F, Panella G, Leboffe L, Antonini G. Lactoferrin from Milk: Nutraceutical and Pharmacological Properties. *Pharmaceuticals (Basel).* 2016;9(4).

172. Fairweather-Tait SJ, Balmer SE, Scott PH, Minski MJ. Lactoferrin and iron absorption in newborn infants. *Pediatr Res.* 1987;22(6):651-654.
173. Chierici R, Sawatzki G, Tamisari L, Volpato S, Vigi V. Supplementation of an adapted formula with bovine lactoferrin. 2. Effects on serum iron, ferritin and zinc levels. *Acta paediatrica (Oslo, Norway : 1992).* 1992;81(6-7):475-479.
174. Cai C, Eck P, Friel JK. Gene Expression Profiles Suggest Iron Transport Pathway in the Lactating Human Epithelial Cell. *Journal of pediatric gastroenterology and nutrition.* 2017;64(3):460-464.
175. Allen LH. Current Information Gaps in Micronutrient Research, Programs and Policy: How Can We Fill Them? *World review of nutrition and dietetics.* 2016;115:109-117.
176. Castillo-Castaneda PC, Garcia-Gonzalez A, Bencomo-Alvarez AE, et al. Micronutrient content and antioxidant enzyme activities in human breast milk. *Journal of trace elements in medicine and biology : organ of the Society for Minerals and Trace Elements (GMS).* 2019;51:36-41.
177. Holm C, Thomsen LL, Norgaard A, Markova V, Michaelsen KF, Langhoff-Roos J. Iron concentration in breast milk normalised within one week of a single high-dose infusion of iron isomaltoside in randomised controlled trial. *Acta paediatrica (Oslo, Norway : 1992).* 2017;106(2):256-260.
178. Sturgeon P. Studies of iron requirements in infante and children. I. Normal values for serum iron, copper and free erythrocyte protoporphyrin. *Pediatrics.* 1954;13(2):107-125.
179. Szabo M, Vasarhelyi B, Balla G, Szabo T, Machay T, Tulassay T. Acute postnatal increase of extracellular antioxidant defence of neonates: the role of iron metabolism. *Acta paediatrica (Oslo, Norway : 1992).* 2001;90(10):1167-1170.
180. Frazer DM, Darshan D, Anderson GJ. Intestinal iron absorption during suckling in mammals. *Biometals.* 2011;24(3):567-574.
181. Darshan D, Wilkins SJ, Frazer DM, Anderson GJ. Reduced expression of ferroportin-1 mediates hyporesponsiveness of suckling rats to stimuli that reduce iron absorption. *Gastroenterology.* 2011;141(1):300-309.
182. Leong WI, Bowlus CL, Tallkvist J, Lonnerdal B. DMT1 and FPN1 expression during infancy: developmental regulation of iron absorption. *American journal of physiology Gastrointestinal and liver physiology.* 2003;285(6):G1153-1161.
183. Frazer DM, Wilkins SJ, Darshan D, Mirciov CSG, Dunn LA, Anderson GJ. Ferroportin Is Essential for Iron Absorption During Suckling, But Is Hyporesponsive to the Regulatory Hormone Hepcidin. *Cellular and molecular gastroenterology and hepatology.* 2017;3(3):410-421.
184. Domellof M, Lonnerdal B, Abrams SA, Hernell O. Iron absorption in breast-fed infants: effects of age, iron status, iron supplements, and complementary foods. *The American journal of clinical nutrition.* 2002;76(1):198-204.
185. Lind T, Seswandhana R, Persson LA, Lonnerdal B. Iron supplementation of iron-replete Indonesian infants is associated with reduced weight-for-age. *Acta paediatrica (Oslo, Norway : 1992).* 2008;97(6):770-775.
186. Lonnerdal B. Excess iron intake as a factor in growth, infections, and development of infants and young children. *The American journal of clinical nutrition.* 2017;106(Suppl 6):1681s-1687s.
187. Scott PH, Berger HM, Kenward C, Scott P, Wharton BA. Effect of gestational age and intrauterine nutrition on plasma transferrin and iron in the newborn. *Arch Dis Child.* 1975;50(10):796-798.
188. Fryer AA, Jones P, Strange R, Hume R, Bell JE. Plasma protein levels in normal human fetuses: 13 to 41 weeks' gestation. *British journal of obstetrics and gynaecology.* 1993;100(9):850-855.
189. Collard KJ. Iron homeostasis in the neonate. *Pediatrics.* 2009;123(4):1208-1216.
190. Luo J, Hendryx M, Dinh P, He K. Association of Iodine and Iron with Thyroid Function. *Biol Trace Elem Res.* 2017;179(1):38-44.
191. Zimmermann MB. The influence of iron status on iodine utilization and thyroid function.

Annual review of nutrition. 2006;26:367-389.
192. Soliman AT, De Sanctis V, Yassin M, Wagdy M, Soliman N. Chronic anemia and thyroid function. *Acta bio-medica : Atenei Parmensis.* 2017;88(1):119-127.
193. Tienboon P, Unachak K. Iron deficiency anaemia in childhood and thyroid function. *Asia Pacific journal of clinical nutrition.* 2003;12(2):198-202.
194. Vihervuori E, Cook JD, Siimes MA. Iron status of children with short stature during accelerated growth due to growth hormone treatment. *Acta paediatrica (Oslo, Norway : 1992).* 1997;86(6):588-593.
195. Troutt JS, Rudling M, Persson L, et al. Circulating human hepcidin-25 concentrations display a diurnal rhythm, increase with prolonged fasting, and are reduced by growth hormone administration. *Clinical chemistry.* 2012;58(8):1225-1232.
196. Lehtihet M, Bonde Y, Beckman L, et al. Circulating Hepcidin-25 Is Reduced by Endogenous Estrogen in Humans. *PloS one.* 2016;11(2):e0148802.
197. Anttila R, Cook JD, Siimes MA. Body iron stores in relation to growth and pubertal maturation in healthy boys. *British journal of haematology.* 1997;96(1):12-18.
198. Anttila R, Siimes MA. Serum transferrin and ferritin in pubertal boys: relations to body growth, pubertal stage, erythropoiesis, and iron deficiency. *The American journal of clinical nutrition.* 1996;63(2):179-183.
199. Bachman E, Feng R, Travison T, et al. Testosterone suppresses hepcidin in men: a potential mechanism for testosterone-induced erythrocytosis. *J Clin Endocrinol Metab.* 2010;95(10):4743-4747.
200. Finch C. Regulators of iron balance in humans. *Blood.* 1994;84(6):1697-1702.
201. Tietz textbook of Clinical chemistry and molecular diagnostics. Cappellini MD, Lo S, Swinkels DW. Chapter 38 Hemoglobin, Iron and Bilirubin. 6th Edition, 2018. Elsevier.
202. Hentze MW, Muckenthaler MU, Andrews NC. Balancing acts: molecular control of mammalian iron metabolism. *Cell.* 2004;117(3):285-297.
203. Olsson KS, Norrby A. Comment to: Hepcidin: from discovery to differential diagnosis. Haematologica 2008; 93:90-7. *Haematologica.* 2008;93(6):e51; discussion e52.
204. Cook JD, Skikne BS, Lynch SR, Reusser ME. Estimates of iron sufficiency in the US population. *Blood.* 1986;68(3):726-731.
205. Swinkels DW, Janssen MC, Bergmans J, Marx JJ. Hereditary hemochromatosis: genetic complexity and new diagnostic approaches. *Clinical chemistry.* 2006;52(6):950-968.
206. Todorich B, Pasquini JM, Garcia CI, Paez PM, Connor JR. Oligodendrocytes and myelination: the role of iron. *Glia.* 2009;57(5):467-478.
207. Badaracco ME, Siri MV, Pasquini JM. Oligodendrogenesis: the role of iron. *BioFactors (Oxford, England).* 2010;36(2):98-102.
208. Smith SM, Johnson PE, Lukaski HC. In vitro hepatic thyroid hormone deiodination in iron-deficient rats: effect of dietary fat. *Life sciences.* 1993;53(8):603-609.
209. Braymer JJ, Lill R. Iron-sulfur cluster biogenesis and trafficking in mitochondria. *The Journal of biological chemistry.* 2017;292(31):12754-12763.
210. Yamamoto S. Mammalian lipoxygenases: molecular structures and functions. *Biochimica et biophysica acta.* 1992;1128(2-3):117-131.
211. Briana DD, Boutsikou T, Baka S, et al. Perinatal role of hepcidin and iron homeostasis in full-term intrauterine growth-restricted infants. *European journal of haematology.* 2013;90(1):37-44.
212. Saarinen UM, Siimes MA. Developmental changes in serum iron, total iron-binding capacity, and transferrin saturation in infancy. *The Journal of pediatrics.* 1977;91(6):875-877.
213. Koerper MA, Dallman PR. Serum iron concentration and transferrin saturation in the diagnosis of iron deficiency in children: normal developmental changes. *The Journal of pediatrics.* 1977;91(6):870-874.
214. Dewey KG, Chaparro CM. Session 4: Mineral metabolism and body composition iron status of breast-fed infants. *The Proceedings of the*

Nutrition Society. 2007;66(3):412-422.
215. Gunnarsson BS, Thorsdottir I, Palsson G. Iron status in 6-y-old children: associations with growth and earlier iron status. *European journal of clinical nutrition.* 2005;59(6):761-767.
216. Fomon SJ, Nelson SE, Ziegler EE. Retention of iron by infants. *Annual review of nutrition.* 2000;20:273-290.
217. Zimmermann MB, Hurrell RF. Nutritional iron deficiency. *Lancet.* 2007;370(9586):511-520.
218. Trumbo P, Yates AA, Schlicker S, Poos M. Dietary reference intakes: vitamin A, vitamin K, arsenic, boron, chromium, copper, iodine, iron, manganese, molybdenum, nickel, silicon, vanadium, and zinc. *Journal of the American Dietetic Association.* 2001;101(3):294-301.
219. Mesias M, Seiquer I, Navarro MP. Iron nutrition in adolescence. *Critical reviews in food science and nutrition.* 2013;53(11):1226-1237.
220. Rodriguez SC, Hotz C, Rivera JA. Bioavailable dietary iron is associated with hemoglobin concentration in Mexican preschool children. *The Journal of nutrition.* 2007;137(10):2304-2310.
221. Saarinen UM, Siimes MA. Iron absorption from infant milk formula and the optimal level of iron supplementation. *Acta paediatrica Scandinavica.* 1977;66(6):719-722.
222. Jegier W, Maclaurin J, Blankenship W, Lind J. COMPARATIVE STUDY OF BLOOD VOLUME ESTIMATION IN THE NEWBORN INFANT USING I-131 LABELED HUMAN SERUM ALBUMIN (IHSA) AND T-1824. *Scand J Clin Lab Invest.* 1964;16:125-132.
223. Raes A, Van Aken S, Craen M, Donckerwolcke R, Vande Walle J. A reference frame for blood volume in children and adolescents. *BMC pediatrics.* 2006;6:3.
224. Petry N, Egli I, Zeder C, Walczyk T, Hurrell R. Polyphenols and phytic acid contribute to the low iron bioavailability from common beans in young women. *The Journal of nutrition.* 2010;140(11):1977-1982.
225. Siimes MA, Vuori E, Kuitunen P. Breast milk iron--a declining concentration during the course of lactation. *Acta paediatrica Scandinavica.* 1979;68(1):29-31.
226. Sankaran VG, Orkin SH. The switch from fetal to adult hemoglobin. *Cold Spring Harb Perspect Med.* 2013;3(1):a011643.

Chapter 3

Practice Guidelines for the Diagnosis and Management of Microcytic Anemias due to Genetic Disorders of Iron Metabolism or Heme Synthesis

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ABSTRACT

During recent years our understanding of the pathogenesis of microcytic inherited anemias has gained from the identification of several genes and proteins involved in systemic and cellular iron metabolism and heme syntheses. Numerous case reports illustrate that the implementation of these novel molecular discoveries in clinical practice has increased our understanding of the presentation, diagnosis and management of these diseases. Integration of these insights in daily clinical practice will reduce delays in time to establish a proper diagnosis, invasive and/or costly diagnostic tests and unnecessary or even detrimental treatments. In order to assist the clinician, we developed an evidence-based multidisciplinary guideline on the management of rare microcytic anemias due to genetic disorders of iron metabolism and heme synthesis. These genetic disorders may present at all ages and therefore this guideline is relevant for pediatricians as well as clinicians treating adults. This article summarizes these clinical practice guidelines and includes i) background on pathogenesis, ii) conclusions and recommendations and iii) a diagnostic flow chart to facilitate its use in the clinical setting.

Key words

anemia, epidemiology, genetic disorder, heme, heme biosynthesis, iron, iron metabolism, iron overload, microcytic anemia, sideroblastic anemia

INTRODUCTION

Anemia in children, adolescents and adults is commonly encountered in general clinical practice. Multiple causes exist, but with a thorough history, physical examination and limited laboratory evaluations a specific diagnosis can often be established. A standard diagnostic approach is to classify anemia as microcytic (MCV< 80 fL), normocytic or macrocytic (MCV> 98 fL). Nutritional iron deficiency, iron loss by gastro-intestinal disease, iron malabsorption, hemoglobinopathies, including some thalassemia syndromes, and severe anemia of chronic disease are the primary causes of microcytic anemias. In case of normocytic anemia, hemolysis, anemia of chronic disease or bone marrow pathology should be considered. Macrocytic anemias are often caused by toxic agents such as alcohol, deficiency of folic acid and/or vitamin B12 or less frequently by myelodysplastic syndrome.

However, some patients with microcytic anemias remain unexplained by the above-mentioned categorization. In these cases: i) elevation of ferritin and/or transferrin saturation (TSAT), ii) low TSAT in combination with low- normal ferritin levels (> 20 µg/L), suggest a genetic disorder of iron metabolism or heme synthesis. The family history, an anemia that is refractory or incompletely responsive to iron supplementation and additional features such as neurologic disease and skin photosensitivity may also be indicative of these disorders.

During recent years, defects in genes with roles in systemic and cellular iron metabolism and heme synthesis have been identified to be involved in the pathogenesis of these genetic anemias.[1-4] We recommend integrating these clinical and molecular insights in daily practice, to avoid unnecessary delay in diagnosis, invasive or costly diagnostic tests and harmful treatments. Importantly, in some genetic anemias, such as the sideroblastic anemias, iron overload is of greater consequence than the anemia itself.[3,5] Unrecognized tissue iron loading might lead to severe morbidity and even mortality, underscoring the need for accurate and timely diagnosis of these disorders.

In this article we present an evidence-based multidisciplinary guideline for the diagnosis and treatment of 12 disorders of microcytic anemia due to defects in 13 different genes leading to genetic disorders of iron metabolism and heme synthesis. We included the disorder associated with a defect in *SLC40A1 (*or *ferroportin-1)* in this guideline, since in

animal models mutations in this gene cause a microcytic anemia,[6] and narrative reviews classified the related human disease as a microcytic anemia,[1] despite the fact that our literature review showed that microcytic anemia is rarely reported in these patients. We also included (normocytic) patients with XLDPP, since this disorder is a variant of erythropoietic protoporphyria, a disease with patients presenting with microcytic anemia. We describe the methodology used, provide a short and illustrated introduction in iron homeostasis (**Figure 1**) and briefly discuss the pathogenesis, epidemiology, clinical presentation, diagnosis, and treatment. We present i) case tables comprising characteristics of the individual patients described in the literature (**Supplement 1, online**), except for patients with loss-of-function defects in *ALAS2* and defects in *SLC40A1*, *UROS* and *FECH* because of their relatively high prevalence, ii) evidence-based conclusions and related references (**Supplement 2, online**), iii) recommendations including advice on family screening (**Table 1**), and iv) results of literature analysis on prevalence of anemia in patients with defects in *SLC40A1* (**Supplement 3**). To facilitate the clinician, a table summarizing the characteristics of the disorders (**Table 2**) and a flow chart (**Figure 2**) to aid the diagnosis of the relevant diseases are included.

Methodology

This guideline has been developed to assist clinicians and patients in the clinical decision-making process for rare anemia due to genetic disorders of iron metabolism and heme synthesis by describing a number of generally accepted approaches for the diagnosis and treatment of these disorders.

For the development of this evidence-based guideline, the working group adopted the methodology described in the Medical specialist guidelines 2.0 of the Netherlands Association of Medical Specialists.[7] This methodology is based upon the international AGREE II criteria for assessing the quality of guidelines (http://www.agreetrust.org).[8] In short, the development of the guideline was started by formulating a number of starting questions (**Supplement 4**). Each question guided a systematic literature review in both Medline and Embase up to December 2010, using MESH headings and free text words related to the name of the predefined genes and disease of interest (see “definitions” below), and disease specific symptoms. In addition, we retrieved more recent original and review articles by using the same key words for searching PubMed and checked the references of the obtained papers. Searches were limited to those written in English, German, French and Dutch. Searches were not limited in time.

The majority of the articles retrieved were case reports or small case series. The searches also showed a number of reviews, however, none of them used a systematic approach. Therefore, we modified the usual therapeutic/intervention-based system for grading the evidence [9,10] of the conclusions in supplement 2 in the following levels: 1. Proven or very likely based on results by numerous investigators, in various populations and settings, 2. Probable based on moderate number of reports 3. Indicative based on small number of reports 4. Expert opinion of members of the working group.

Working group

The working group consisted of the authors of this article.

Definitions

This guideline covers microcytic anemias due to genetic defects in iron metabolism or heme synthesis (Table 2). For the selection of these diseases, we used recent reviews,[1,4] and added disorders that were described in more recent years, i.e. sideroblastic anemia due to defects in *STEAP3* and X-linked dominant protoporphyria (XLDPP).

We excluded microcytic anemia caused by hemoglobinopathies and genetic diseases not predominantly characterized by a primary defect in iron metabolism or heme synthesis (Figure 2 and its legend).

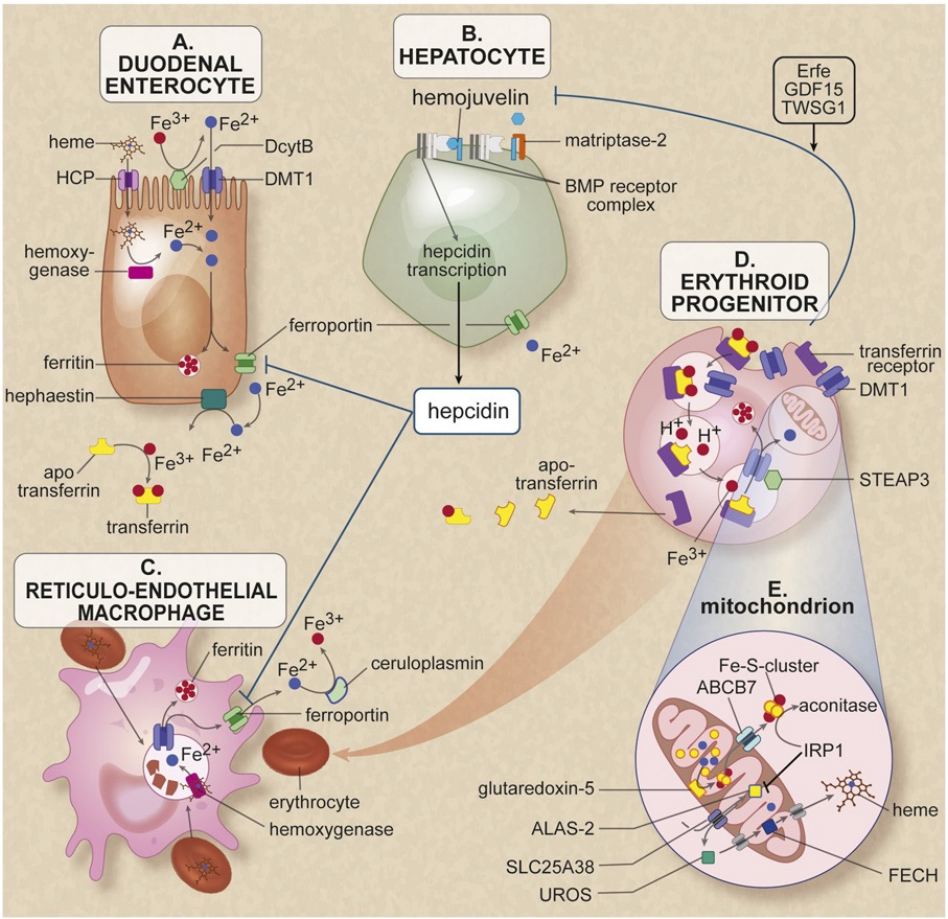
Introduction in iron metabolism and heme synthesis

Iron plays an essential role in many biochemical processes, in particular in the production of heme for the incorporation in hemoglobin and myoglobin, and iron-sulphur clusters, which serve as enzyme cofactors.[11] In case of iron deficiency, cells lose their capacity for electron transport and energy metabolism. Clinically, iron deficiency causes anemia and may result in neurodevelopmental deficits.[12] On the other hand, iron excess leads to complications such as endocrine disorders, liver cirrhosis and cardiac dysfunction.[13]

Therefore, tight regulation of body iron homeostasis on systemic and cellular level is paramount. These processes comprise several proteins, most of which have been discovered in the last 20 years. Defects in these proteins lead to disorders of iron metabolism and heme synthesis that are characterized by iron overload, iron deficiency or iron maldistribution.

Cells involved in iron homeostasis are duodenal enterocytes, hepatocytes, macrophages and erythroid precursors. To illustrate the description on pathophysiology for the different disorders, in Figure 1 we schematically present the function of the above-mentioned cells in iron homeostasis, and the proteins involved.

Figure 1. Cells and proteins involved in iron homeostasis and heme synthesis



◄ A. The duodenal enterocyte: iron enters the body through the diet. Most iron absorption takes place in the duodenum and proximal jejunum. The absorption or iron takes place in different phases. In the luminal phase iron is solubilized and converted from trivalent iron into bivalent iron by duodenal cytochrome B (DcytB). During the mucosal phase iron is bound to the brush border and transported into the mucosal cell by the iron transporter dimetal transporter (DMT1). In the cellular phase iron is either stored in cellular ferritin or transported directly to the opposite side of the mucosal side. In the last phase of iron absorption Fe^{2+} is released into the portal circulation by the basolateral cellular exporter ferroportin. Enterocytic iron export requires hephaestin, a multicopper oxidase homologous to ceruloplasmin, which oxidases Fe^{2+} to Fe^{3+} for loading onto transferrin. This cellular efflux of iron is inhibited by the peptide hormone hepcidin by binding to ferroportin and subsequent degradation of the ferroportin-hepcidin complex.

B. The hepatocyte: serves as the main storage for the iron surplus (most body iron is present in erythrocytes and macrophages). Furthermore, this cell, as the main producer of hepcidin, largely controls the systemic iron regulation. The signal transduction pathway runs from the membrane to the nucleus, where bone morphogenetic protein (BMP) receptor, the membrane protein hemojuvelin (HJV), the HFE protein and transferrin receptor (TfR) -1 and -2, and matriptase 2 play an essential role. Through intracellular pathways, a signal is given to hepcidin transcription. The membrane associated protease matriptase 2 (encoded by *TMPRSS6*), detects iron deficiency and blocks hepcidin transcription by cleaving HJV.

C. The reticulo-endothelial macrophage: belongs to the group of reticulo-endothelial cells and breaks down senescent red blood cells. During this process iron is released from heme proteins. This iron can either be stored in the macrophage as hemosiderin or ferritin or may be delivered to the erythroid progenitor as ingredient for new erythrocytes. The iron exporter ferroportin is responsible for the efflux of Fe^{2+} into the circulation. In both hepatocytes and macrophages this transport requires the multicopper oxidase ceruloplasmin (CP), which oxidases Fe^{2+} to Fe^{3+} for loading unto transferrin.

D. The erythroid progenitor: transferrin saturated with 2 iron molecules is endocytosed via the transferrin receptor 1 (TfR1). After endocytosis the iron is released from transferrin, converted from Fe^{3+} to Fe^{2+} by the ferroreductase STEAP3, and transported to the cytosol by DMT1, where it is available mainly for the heme synthesis. Erythropoiesis has been reported to communicate with the hepatocyte by the proteins TWSG1, GDF15 and erythroferrone (ERFE) that inhibit signaling to hepcidin.[11,27,101]

E. The mitochondrion of the erythroid progenitor: in the mitochondria the heme synthesis and iron-sulfur cluster (Fe-S clusters) synthesis takes place. In the first rate-limiting step of heme synthesis, 5-aminolevulinic acid (ALA) is synthesized from glycine and succinyl-CoA by the enzyme delta-aminolevulinic acid synthase (ALAS2) in the mitochondrial matrix. The protein SLC25A38 is located in the mitochondrial membrane and is probably responsible for the import of glycine into the mitochondria and might also export ALA to the cytosol. In the heme synthesis pathway, the uroporhyrinogen III synthase (UROS*)* in the cytosol is the fourth enzyme. It is responsible for the conversion of hydroxymethylbilane (MHB) to uroporphyrinogen III, a physiologic precursor of heme. In the last step, ferrochelatase (FECH) located in the mitochondrial intermembrane space is responsible for the last step, i.e. the incorporation of Fe^{2+} in protoporhyrin IX (PPIX) to form heme. GATA binding factor I (GATA 1) is critical for normal erythropoiesis, globin gene expression and megakaryocyte development and among others regulates expression of UROS and ALAS2 in erythroblasts.

The enzyme glutaredoxin-5 (GLRX5) plays a role in the synthesis of the Fe-S clusters, which are transported to the cytoplasm, probably via the transporter ABCB7.

Figure adapted from van Rooijen *et al.*[2]

DISORDERS DUE TO LOW IRON AVAILABILITY FOR ERYTHROPOIESIS

1A. Iron refractory iron deficiency anemia (IRIDA) due to defects in *TMPRSS6*

Pathogenesis and epidemiology

TMPRSS6 (OMIM 609862) encodes matriptase 2 (a type II plasma membrane serine protease), that senses iron deficiency and blocks hepcidin transcription by cleaving hemojuvelin (HJV). Consequently, pathogenic *TMPRSS6* defects result in uninhibited hepcidin production, causing iron refractory iron deficiency anemia (IRIDA).[14-16] At the population level Genome Wide Association Studies (GWAS) show that *TMPRSS6* is polymorphic with a relatively large amount of high frequency polymorphisms of which some (particularly p.Ala736Val) are associated with significant decrease of the concentrations of iron, hemoglobin (Hb) and Mean Cellular Volume (MCV) of the red blood cell.[17,18] The prevalence of pathogenic mutations leading to IRIDA is unknown, but under-diagnosis seems likely. IRIDA patients due to a suspected homozygous or compound heterozygous *TMPRSS6* defect are described in 61 cases in 39 families. Since functional studies are not always performed, it is unclear whether all these mutations are pathogenic. A few cases with microcytic anemia, low TSAT, and low-normal ferritin are reported to be heterozygous for *TMPRSS6* defects (**Supplement 1**, **online**).

Clinical presentation and diagnosis

Most IRIDA patients present in childhood with a microcytic anemia, which tends to become less severe with increasing age,[19] in combination with a remarkably low TSAT and – if untreated- a low to normal ferritin. Most patients fail to respond to oral iron (see below), but since this feature is also observed in iron deficient anemic patients with autoimmune atrophic gastritis, *Helicobacter pylori* infection and celiac disease, these (non-genetic) disorders should be considered in the diagnostic work up.[20]

In IRIDA, serum hepcidin is inappropriately high given the low body iron status. Consequently, the hepcidin/TSAT ratio is high.[21] In the absence of inflammation, an increased hepcidin/TSAT ratio is specific for IRIDA, whereas a low hepcidin/ferritin ratio is characteristic for many genetic iron loading disorders.

In affected children, growth, development and intellectual performances are normal.[22] Although the pedigree structure of most IRIDA patients shows an autosomal recessive

inheritance, anecdotal reports suggest that heterozygous pathogenic *TMPRSS6* mutations might cause a mild IRIDA phenotype (**Supplement 1, online**). It is still unclear whether this can be explained by environmental factors, a combination with modulating polymorphisms or a low expressing allele, or whether the current Sanger sequencing strategy misses certain defects in the exons, introns of the gene or its regulatory regions, or whether defects in other genes are involved. Therefore, we conclude that IRIDA due to a *TMPRSS6* defect can only be diagnosed with certainty when the patient is homozygous or compound heterozygous for a pathogenic mutation.

Treatment

Case reports indicate that the pathogenicity of the *TMPRSS6* defect determines the response to oral iron. Severe *TMPRSS6* defects usually lead to oral iron resistance. Only few cases (partially) respond to oral iron (**Supplement 1, online**). Ascorbic acid (3mg/d) supplementation along with oral ferrous sulfate has been reported to improve Hb and iron status in an infant resistant to oral iron supplementation only.[23] Case series show that repeated administration of intravenous iron (iron sucrose or iron gluconate) increase Hb and ferritin and to a lesser extent MCV and TSAT, although complete normalization of Hb is rarely achieved. Attempting to correct the Hb level into the reference range may place the patient at risk of iron overload. We found no evidence for a threshold of circulating ferritin levels above which the iron in the reticulo-endothelial (RE) macrophages becomes toxic on the long term. However, following guidelines for iron treatment in patients with chronic kidney disease,[24] we recommend monitoring serum ferritin levels and not exceed a concentration of 500 µg/L to avoid this risk, especially in children and adolescents. The role of erythropoietin (EPO) treatment in IRIDA is controversial.[25,26] Trials with novel hepcidin lowering compounds in these patients have not been performed.[27]

1B. Ferroportin disease due defects in *SLC40A1*

Pathogenesis and epidemiology

SLC40A1 (or *ferroportin-1*, *IREG1, MTP1, SLC11A3*) (OMIM 606069) encodes the protein ferroportin,[28] the only known human cellular iron exporter (**Figure 1**).[11,29] In 2000, ferroportin was identified in the zebrafish mutant *weissherbst*, as the defect gene responsible for the hypochromic anemia in these animals was ascribed to inadequate circulatory iron levels.[6] In man only heterozygous mutations in *SLC40A1* are reported and microcytosis is not observed. The resulting hereditary hemochromatosis (HH) type

4, or ferroportin disease, is an autosomal dominant condition primarily characterized by iron overload with a heterogeneous phenotype.[30,31] In almost all case series and the available narrative reviews, mutations causing ferroportin disease are classified into two groups: loss-of-function (LOF, HH type 4A or classical HH) and gain-of-function (GOF, HH type 4B or atypical HH) mutations.[32,33] Functional studies and clinical data show that a LOF mutation typically leads to iron retention in the duodenal cell and macrophages due to reduced ferroportin activity and preserved inhibitory capacity of hepcidin. The phenotype is characterized by an elevated serum ferritin level in association with a low to normal TSAT and predominant iron deposition in macrophages with low tolerance to phlebotomy in some patients.[32] GOF mutations lead to increased iron absorption due to increased ferroportin activity, which is resistant to the inhibitory effect of hepcidin. As a consequence, the iron overload phenotype of these patients is indistinguishable from other forms of hereditary hemochromatosis,[30,31] and therefore this subtype of ferroportin disease does not fit into the category of "disorders due to low iron available for erythropoiesis".

For certain genotypes, however, the functional studies and the biochemical and histological phenotypes vary between studies and patients.[34] Therefore, the distinction between LOF and GOF mutations is not always straightforward. In order to define the type of mutation, we developed for the purpose of this guideline a classification system based on both phenotypic and functional characteristics (**Supplement 3**). Based on this classification we identified 36 different LOF mutations and 15 GOF mutations. A total of 207 patients with a LOF mutation and 73 patients with a GOF were found worldwide.

Clinical presentation and diagnosis

Symptoms are related to iron overload and are non-specific. Since the current guideline is on anemias, in our evaluation of the literature on ferroportin disease we investigated the occurrence of anemia (defined by the WHO) [35] as presenting symptom.

In 76 (27.7 %) and 24 (8.5 %) out of 280 patients, both Hb and MCV were numerically noted. Eight (10%) of the patients fulfilled the WHO criteria of anemia without additional causes of microcytic anemia (**Table 1 in Supplement 3**). Five males had an Hb between 12.0 and 12.9 g/dL and three females had Hb between 11.1 and 11.5 g/dL without microcytosis. Seven of the 8 anemic patients were annotated a LOF mutation.

We conclude that of the total of 76 patients diagnosed with iron overload due to both LOF and GOF mutations in *SLC40A1* and documented Hb levels only ≈ 10% was anemic. This anemia is described as mild and normocytic.

Treatment

Iron overload in patients with both LOF and GOF ferroportin disease is treated with phlebotomy. Both the application of phlebotomy and its effects were described for 94 patients. In 14 of the 94 patients (14.8%) a transient mild or profound anemia during phlebotomy treatment was reported. Our literature evaluation showed that the risk for the development of anemia during phlebotomy was associated with older age and a LOF mutation phenotype, e.g. a relatively low TSAT (**Table 2 in Supplement 3**). Data from case reports suggest that patients who develop anemia upon phlebotomies benefit from extension of the phlebotomy interval.

1C. Aceruloplasminemia (ACP) due to defects in *CP*

Pathogenesis and epidemiology

CP (OMIM 604290) encodes ceruloplasmin (CP), which is secreted into plasma and carries 95 % of circulating plasma copper. CP catalyzes cellular efflux of iron by oxidation of Fe^{2+} to Fe^{3+} for binding to circulating transferrin (**Figure 1**).[36] *In vitro* studies and mice studies show that CP is also required for the stability of the iron exporter ferroportin, especially in glial cells.[37] Mice studies demonstrate that the absence of CP leads to wide spread iron overload in parenchymal and reticuloendothelial organs, including the nervous system.[36]

The incidence of ACP in Japan is estimated to be approximately 1 per 2,000,000 in non-consanguineous marriages. There are no reliable data on the incidence and prevalence in Western European countries.[38] In total 35 pathogenic *CP* mutations have been described in 50 families. ACP is a rare autosomal recessive disease. However, seven patients with a clinical phenotype of ACP were heterozygous for a *CP* defect (**Supplement 1, online**).

Clinical presentation and diagnosis

Iron accumulation in ACP affects the liver, pancreas and central nervous system.[39] ACP patients develop the classical triad of i) diabetes mellitus, ii) retinal degeneration and iii) neurodegenerative disease with extrapyramidal and cerebellar symptoms in

combination with mental dysfunction. A mild normo- to microcytic anemia with low serum iron and elevated serum ferritin is a constant feature in ACP.[40] However, in none of the described patients, was anemia the presenting symptom. Disease onset typically occurs in the fourth to fifth decade of life with neurodegenerative symptoms. Most patients die in the sixth decade due to neurological complications.[39] Despite the hepatic iron overload, ACP has not been described to be associated with liver disease.

Absent or very low serum ceruloplasmin in combination with low serum copper and iron, high serum ferritin and increased hepatic iron concentration is indicative of ACP. The diagnosis is supported by characteristic findings on MRI that are compatible with iron accumulation in liver, pancreas and brain. Homozygous or compound heterozygous *CP* defects confirm the diagnosis of ACP.

Treatment

Case series describe normalization of serum ferritin and decrease of hepatic iron overload after treatment with iron chelation.[39] In six cases neurological improvement,[39-42] and in two other cases reduction of insulin demand was described on treatment with iron chelators.[43]

Oral administration of zinc sulphate in a symptomatic heterozygous ACP patient has been reported but effect on neurological symptoms remains unclear.[41,44] Three homozygous ACP patients were treated with fresh frozen plasma; their outcome was not reported.[45-47] The anemia in ACP is mild and does not need any intervention.

DISORDERS DUE TO DEFECTS IN IRON ACQUISITION BY THE ERYTHROID PRECURSORS

2A. Hypotransferrinemia due to defects in *TF*

Pathogenesis and epidemiology

Genetic hypotransferrinemia is a rare autosomal recessive disease due to a defect in *TF* (OMIM 19000).[48] Transferrin deficiency leads to low concentrations of transferrin bound iron with iron deficient erythropoiesis and high concentrations of non-transferrin bound iron with subsequent iron overload in non-hematopoietic tissues. Functional studies in a limited number of patients indicate that the intestinal iron absorption is increased with augmented plasma iron clearance and reduced red cell iron utilization.[49,50] The increased intestinal iron absorption results from the strongly reduced hepatic hepcidin production ascribed to iron deficient erythropoiesis and low transferrin concentration.[51,52]

Since 1961 at least 13 cases in 11 families with of hypotransferrinemia are reported worldwide,[49,50,53-56] Underlying molecular defects in *TF* are recognized in six patients (**Supplement 1, online**).

Clinical presentation and diagnosis

Patients present in early life with microcytic and hypochromic anemia. The anemia is characterized by low serum iron and high ferritin levels. The transferrin level varies between below limits of detection to 20 % of normal and is fully saturated. Serum hepcidin levels are low. The iron content in the bone marrow is reduced with a decreased myeloid to erythroid ratio in most cases.[49,50, 53-56]

Affected children may show a failure to thrive with occasionally retardation in the mental development. There are signs of widespread iron overload with hepatomegaly and strikingly early endocrinopathy, skin iron deposition, and sometimes fatal, cardiomyopathy. Some patients have osteoporosis.[55,56]

The diagnosis is confirmed by molecular analysis of *TF*. Heterozygous relatives have decreased transferrin concentrations, without anemia or systemic iron loading.

Treatment

Treatment consists of infusions of apo-transferrin either directly or as plasma. Data on the dosage and frequency of these infusions are limited. Monthly infusions of plasma have been reported to be sufficient to normalize Hb and serum ferritin levels. To date, only three patients have been treated with apo-transferrin on a compassionate use-basis.[50,55] The calculated elimination half-life reported varied between 4.8 and 10 days. Apo-transferrin is not on the market for clinical purposes but has an orphan drug status (EU/3/12/1027; 2012). A clinical trial in patients with hypotransferrinemia is ongoing (ClinicalTrials.gov Identifier: NCT01797055). Reluctance with repeated erythrocyte transfusion or iron substitution is advocated to avoid further iron overload.

2B. Anemia with systemic iron loading due to defects in *SLC11A2 (DMT1)*

Pathogenesis and epidemiology

SLC11A2 (OMIM 600523) encodes Divalent Metal Transporter-1 (DMT1), a cellular membrane bound iron transporter (**Figure 1**).[1,57,58]

Molecular studies indicate that *SLC11A2* defects cause defective enterocyte and erythroid iron uptake.[59] Systemic iron loading suggests the presence of an additional pathway for intestinal iron absorption, e.g. as heme.[60] Another explanation might be the low hepcidin levels, resulting in increased dietary iron absorption in case *SLC11A2* is not completely eliminated.[61] Anemia with systemic iron loading due to defects in *SLC11A2* is a rare autosomal recessive disorder: Seven patients from six families have been described due to homozygous (n=2) or compound heterozygous defects (n=5).

Clinical presentation, diagnosis

Four out of seven described patients presented at birth with microcytic anemia and increased TSAT. Serum ferritin levels varied from low to moderately increased, with some association with erythrocyte transfusions or intravenous iron supplementation. Liver iron loading was demonstrated by MRI or biopsy in five out of seven patients at ages varying between 5-27 years despite normal or only mildly increased ferritin concentrations in three of them.

Treatment

The seven described patients with severe anemia were treated with erythrocyte transfusions. Three patients received oral iron, which increased Hb and led to transfusion independency in one patient.

Three patients received EPO, resulting in an increase of Hb, but - based on the clinical course of one patient - not in prevention of liver iron loading.[62] Erythrocyte transfusions and probably also oral or intravenous iron cause additional liver iron loading. Chelation was not effective in reducing liver iron and resulted in decrease of Hb (unpublished data, Tchernia and Beaumont [1,62]).

2C. Sideroblastic anemia due to defects in *STEAP3*

Pathogenesis and epidemiology

STEAP3 (OMIM 609671) encodes a ferroreductase, responsible for the reduction of Fe^{3+} to Fe^{2+} in endosomes of erythroblasts (**Figure 1**).[63] Mice studies show that absence or reduced activity of ferroreductase results in severe microcytic anemia, which can be corrected by introduction of a functional *STEAP3*.[64] The first and so far only human *STEAP3* mutation was recently described in three siblings born to healthy, non-consanguineous parents.[65]

Clinical presentation and diagnosis

The three siblings displayed a transfusion-dependent severe hypochromic anemia with a normal to slightly decreased MCV. Serum iron and ferritin were normal to increased, while TSAT was markedly increased. A bone marrow smear in the index patient showed ring sideroblasts. Liver biopsy after multiple erythrocyte transfusions showed iron loading. All patients suffered from gonadal dysfunction, as described for *STEAP3* deficient mice.[65]A heterozygous nonsense *STEAP3* mutation was inherited from the father, while no defect was found in the mother. The authors explain the normal phenotype of the father by the STEAP3 expression of the 'healthy' allele in lymphocytes, which was significantly higher than in his affected children.

Treatment

Treatment consisted of a combination of erythrocyte transfusions and chelation while EPO increased the transfusion interval.

DISORDERS DUE TO DEFECTS IN THE HEME AND/OR IRON-SULPHUR CLUSTER SYNTHESIS

3A. Sideroblastic anemia due to defects in *SLC25A38*

Pathogenesis and epidemiology

SLC25A38 (OMIM 610819) encodes a protein on the inner membrane of the mitochondria of hematopoietic cells, especially erythroblasts, and is essential for the heme synthesis (**Figure 1**).[66] Its specific function is not known, but it has been hypothesized that *SLC25A38* facilitates 5-aminolevulinic acid (ALA) production by importing glycine into mitochondria or by exchanging glycine for ALA across the mitochondrial inner membrane.

Defects in *SLC25A38* result in severe congenital sideroblastic anemia with microcytic hypochromic erythrocytes.[66] Until now 26 patients have been reported with 20 different mutations.[66,67] The inheritance pattern is autosomal recessive; heterozygous relatives are not anemic.

Clinical presentation and diagnosis

Patients present with severe, often transfusion dependent, microcytic hypochromic anemia in childhood, which is clinically similar to thalassemia major. Bone marrow smears show ringed sideroblasts. Serum ferritin levels and TSAT are increased, even before treatment with erythrocyte transfusions.

Treatment

Symptomatic treatment consists of erythrocyte transfusions and iron chelation. Hematopoietic stem cell transplantation (HSCT) is the only curative treatment and was performed in eight out of 29 patients and resulted in disease free survival in four patients (follow up < 5 years).

3 B. X-linked sideroblastic anemia with ataxia due to defects in *ABCB7*

Pathogenesis and epidemiology

ABCB7 (OMIM 300135) on the X-chromosome encodes a protein in the inner membrane of the mitochondria, which is the putative mitochondrial exporter of Fe-S

complexes (**Figure 1**).[3,68] Defects in *ABCB7* result in disrupted iron metabolism and heme synthesis causing a mild, slightly microcytic, sideroblastic anemia but also cerebellar ataxia.[69] Sideroblastic anemia with ataxia due to defects in *ABCB7* is a rare, X-linked disease.[69] Seventeen patients with four different pathogenic *ABCB7* defects have been described in case reports, of which five were female, which might be explained by skewed X-inactivation. None of the women showed neurological defects. There is no apparent genotype-phenotype correlation.

Clinical presentation and diagnosis

The presenting symptom in all male patients was cerebellar ataxia that developed in childhood. Cerebral MRI showed cerebellar hypoplasia in four patients. Mild, slightly microcytic, sideroblastic anemia was found, usually in the second decade. In 10 patients free erythrocyte protoporphyrin IX was increased. None of the patients showed systemic iron loading.

Treatment

Treatment of the mild anemia is not reported.

3C. X- linked sideroblastic anemia due to defects in *ALAS2*

Pathophysiology and epidemiology

ALAS2 (OMIM 301300) is located on chromosome X and encodes for ALAS2, an erythroid specific isoform of the catalytic enzyme involved in heme synthesis in the mitochondria (**Figure 1**).[70]

Almost all *ALAS2* defects are missense mutations, most commonly in domains important for catalysis or pyridoxal phosphate (vitamin B6) co-factor binding.[71] Recently also defects in the binding site of the transcription factor GATA1 in the first intron of *ALAS2* have been described.[72]

ALAS2 defects result in decreased protoporphyrin synthesis and subsequent reduced iron incorporation and heme synthesis, causing microcytic anemia and erythroid mitochondrial iron loading. Mitochondrial iron loading exacerbates the anemia through decreased pyridoxine sensitivity.[73] Heme deficiency is associated with ineffective erythropoiesis, followed by increased intestinal iron uptake and tissue iron accumulation. X-linked sideroblastic anemia (XLSA) is the most common

genetic form of sideroblastic anaemia.[3] Since Cooley described the first patients with XSLA in 1945,[74] 61 different pathogenic *ALAS2* defects have been described in 120 non-related families.[75,76] As in most X-linked disorders, most female carriers of XLSA are asymptomatic. However, women with *ALAS2* mutations may be affected due to skewed X–inactivation.[77] Furthermore, physiologic age-related skewed X –inactivation in hematopoietic stem cells may play a role in developing XLSA in female carriers with increasing age.[78] Estimates on the prevalence of *ALAS2* defects are not available. Since the phenotype might be mild, under-diagnosis is likely.

Clinical presentation and diagnosis

XLSA is characterized by mild hypochromic, microcytic, sideroblastic anemia in combination with systemic iron overload.[3] Elevated Red cell Distribution Width (RDW) has been described in female carriers of the mutation and is ascribed to the presence of two erythrocyte populations.[79] Phenotypic expression of XLSA is highly variable even in patients with identical mutations.[80] Case reports indicate that affected males generally present in the first two decades of life with symptoms of anemia or later with either manifestations of anemia or those of parenchymal iron overload. Manifestation at elderly age due to an acquired pyridoxine deficiency is described.[81]

Treatment

The available evidence indicates that initial doses of oral vitamin B6 (pyridoxine) 50-200 mg/day are effective in improving anemia and iron overload in all responsive XLSA patients.[82] Occasionally high doses may be considered. Once a response is obtained evidence suggests the life-long maintenance dose may be lowered to 10-100 mg/day, since too high doses may give neurotoxicity.[83]

Since iron overload may compromise mitochondrial function and hence heme biosynthesis, XLSA patients should not be considered pyridoxine refractory until iron stores are normalized.[73] Most patients can be treated with phlebotomies for iron overload, since the anemia is mild. Hb typically increases, rather than decreases, after reversal of iron overload by phlebotomies.[73]

3D. Sideroblastic anemia due to defects in *GLRX5*

Pathogenesis and epidemiology

GLRX5 (OMIM 6095588) encodes for the mitochondrial disulfide glutaredoxin (GLRX) 5, which is highly expressed in early erythroid cells and is essential for the biosynthesis of Fe-S clusters (**Figure 1**).

In vitro data show that in GLRX5 deficient erythroblasts, Fe-S cluster production is decreased, and more active Iron Responsive Protein 1 (IRP1) is present in the cytosol. This reflects a low-iron state of the cell and causes repression of target genes, including *ALAS2*, resulting in reduced heme synthesis, in mitochondrial iron accumulation, and increased turnover of ferrochelatase.[84,85] Sideroblastic anemia due to defects in *GLRX5* is a rare autosomal recessive disease: only one male patient has been described.[86]

Clinical presentation and diagnosis

The patient born from consanguineous parents, presented at age 44 years with type 2 diabetes mellitus and at 60 years with icterus and hepatosplenomegaly. There was a progressive microcytic anemia with increased TSAT, serum ferritin and iron accumulation in both bone marrow macrophages and erythroblast mitochondria.

Treatment

Treatment with iron chelation and erythrocyte transfusion resulted in an increase of Hb and a decrease in serum ferritin. Pyridoxine supplementation was not effective.

3E. Erythropoietic ProtoPorphyria due to defects in *FECH* (EPP) and gain-of-function mutations in *ALAS2* (XLDPP)

Pathophysiology and epidemiology

Erythropoietic ProtoPorphyria comprises two variants, EPP and (X-linked dominant protoporphyria) XLDPP and belongs to the cutaneous porphyrias characterized by accumulation of free protoporphyrin IX. Autosomal recessive EPP (OMIM 177000) is caused by defects in *FECH* (OMIM 612386), encoding for ferrochelatase in the mitochondrium that is responsible for iron insertion into protoporphyrin IX to form heme (**Figure 1**).[87] The prevalence differs worldwide, and mode of inheritance is complex.

XLDPP results from gain-of-function mutations in *ALAS2* (OMIM 301300) leading to protoporphyrin IX overproduction.[88] XLDPP is found in 29 families worldwide and ≈ 2 % (UK) to ≈10% (USA) of patients with the EPP phenotype.[87]

The diminished ferrochelatase activity in the EPP variant results in reduced heme synthesis, with occasionally ringed sideroblasts, but without systemic iron overload.[89] This suggests that the iron absorption and supply in these patients match the requirement for reduced erythropoiesis.[90]

Clinical presentation and diagnosis
The variants EPP and XLDPP are clinically indistinguishable. The predominant clinical presentation is a painful photosensitivity, erythema, stinging and burning, beginning in childhood on sunlight exposed skin. In severe patients the liver is affected.[91]Twenty to 60% of the patients show a microcytic anemia, with a mean decrease in hemoglobin in adult male patients of 1.2 g/dL and reduced iron stores.[89]

Fluorescent erythrocytes can be seen in a fresh, unstained blood smear. Free protoporphyrin is found in plasma and erythrocytes. Zn protoporphyrin is typically increased in XLDPP, due to the relative iron deficiency for the amount of protoporphyrin IX.[91]

Treatment
Since anemia is mild, its treatment is not warranted.[91] The therapy of EPP is focused on minimizing the harmful effects of exposure to sunlight and on managing the hepatotoxic effects of protoporphyrin.

3F. Congenital Erytropoietic Porphyria (CEP), due to defects in *UROS* or *GATA1*

Pathogenesis and epidemiology
UROS (OMIM 606938) encodes uroporphyrinogen III synthase, the fourth enzyme of the heme biosynthetic pathway. CEP (OMIM 263700) is a rare autosomal genetic disease caused by defects in *UROS*, leading to erythroid accumulation of the non-physiological uroporphyrin I and coproporphyrin I.[91] A reduced red cell survival due to excess porphyrin in erythrocytes contributes to hemolytic anemia found in most patients with CEP.[92] To date, approximately 45 *UROS* mutations

have been described in > 200 individuals.[93] An X-linked variant of CEP (OMIM 314050) caused by a mutation in *GATA1* (OMIM 305371) on the X-chromosome has been reported for one patient.[94] GATA binding factor 1 regulates expression of *UROS* in erythroblasts.

Clinical presentation and diagnosis

CEP typically presents with passage of red urine shortly after birth. Lifelong bullous cutaneous photosensitivity to visible light starts in early infancy, leading to scarring with photomutilation.[91] Other manifestations include hypertrichosis, erythrodontia, osteoporosis and corneal ulceration with scarring. Age of onset and severity of CEP are both highly variable.[95] In a retrospective study of 29 CEP patients, 66% suffered from chronic hemolytic anemia with variable severity.[95] Iron parameters are not described in these patients.

The patient with CEP caused by the *GATA1* defect had a severe hypochromic microcytic, hemolytic anemia mimicking the phenotype of thalassemia intermedia, in combination with thrombocytopenia.[94] Biochemically, CEP patients have uro- and coprophorphyrin accumulation in erythrocytes, plasma, urine, faeces and increased UROS activity in erythrocytes.[91]

Treatment

Allogenic HSCT is the only curative option for CEP.[96] Chronic erythrocyte transfusion in severe cases to suppress erythropoiesis is described.[97] Protection of eyes and skin from sunlight is essential and minor skin trauma should be avoided.[98]

Table 1. Recommendations

Part 1: Anemia due to low iron bioavailability for erythropoiesis

1A. Iron refractory iron deficiency anemia (IRIDA) due to defects *TMPRSS6*

Clinical presentation and diagnostics

- In patients with unexplained microcytic anemia with low transferrin saturation (TSAT) and normal or reduced serum ferritin concentration, not or partially responding to oral iron and (partially) responsive to intravenous iron supplementation, Iron Refractory Iron Deficiency Anemia (IRIDA) due to a *TMPRSS6* defect should be considered. Determination of serum hepcidin is recommended in case the diagnosis IRIDA is suspected.
- Increased serum hepcidin in relation to TSAT (hepcidin/TSAT ratio > p.97.5 of local reference value) is suggestive of IRIDA. *TMPRSS6* mutation analysis is recommended.
- In case of a homozygous or compound heterozygous *TMPRSS6* defect, IRIDA due to a dysfunctional matriptase 2 protein should be diagnosed.
- No recommendation can be made on the clinical significance of heterozygous *TMRSS6* defects with or without concomitant polymorphisms, because of lack of evidence.

Treatment

- In a patient with iron deficiency anemia due to pathogenic *TMRPSS6* defects initial treatment with oral iron or oral iron combined with ascorbic acid should be considered.
- Patients, for whom this initial treatment does not result in acceptable Hb levels, should be treated with intravenous iron supplementation.
- In IRIDA patients, the choice of the chemical form of intravenous iron should be based on its registration for the specific age group OR a proven good safety profile in adults during several years of post-marketing surveillance.
- The total intravenous iron cumulative doses should be calculated based on formulas of the deficit on body iron allowing for the correction of the Hb deficit and rebuilding the iron stores. Doses should be repeated every 3-7 days until the total dose is administered. Single doses should not exceed the maximum single dose.
- Serum ferritin levels should be monitored and preferably not exceed 500 µg/L to avoid toxicity of iron overload, especially in children and adolescents.
- No recommendation can be made on the efficacy of the combination of iv supplementation and erythropoietin (EPO) treatment in IRIDA patients, because of low evidence. This combination therapy might prevent toxic iron loading in some patients.

*Family screening**

- The proband should be informed about the mostly autosomal recessive inheritance pattern of IRIDA. We recommend to screen relatives of the proband for the IRIDA phenotype: siblings and spouse in case of consanguinity and reproductive age. If the proband is diagnosed at young age, and his/her parents are of reproductive age, phenotyping of the parents is recommended. In case of a clinical IRIDA phenotype in the above-mentioned relatives, mutation analysis is recommended.
- Children of the proband should only be phenotyped and, in case of an IRIDA phenotype, genotyped in case of consanguinity of the proband and his/her spouse or in case of proven carrier ship of both proband and his/her spouse.
- Because of the complex genotype-phenotype correlation in IRIDA, we recommend referral to a clinical geneticist in case of an IRIDA phenotype and a pathogenic heterozygous *TMRSS6* defect.

Table 1. Continued

1B. Ferroportin disease due to defects in *SLC40A1*
Clinical presentation and diagnostics
• Ferroportin disease due to loss-of -function (LOF) mutations should not be considered as a cause of microcytic anemia. • When anemia occurs in a patient with primary iron overload during treatment with repeated phlebotomies the presence of LOF ferroportin disease may be considered.
Treatment
• Patients with iron overload due to LOF and gain-of-function (GOF) ferroportin disease should be treated with repeated phlebotomies. • For patients that develop anemia during phlebotomies despite elevated ferritin levels extension of the phlebotomy interval is recommended • In patients who develop anemia during phlebotomies additional treatment with EPO may be considered.
*Family screening**
• The proband should be informed about the autosomal dominant inheritance pattern of ferroportin disease. • We recommend screening the first-degree relatives (parents, siblings and children) and additional family members (via cascade screening) for the *SLC40A1* mutation identified in the proband. Mutation carriers should be screened for the ferroportin disease phenotype.
1C. Aceruloplasminemia (ACP) due to defects in *CP*
Clinical presentation and diagnostics
• In patients with the combination of insulin dependent diabetes, neurodegenerative disease, retinal degeneration and mild anemia with systemic iron loading, *CP* defects should be considered. • In patient with absent or very low ceruloplasmin in combination with low serum copper and iron, high serum ferritin, and characteristic findings on MRI that are compatible with iron accumulation in liver, pancreas and brain, ACP should be considered. • In case of homozygous or compound heterozygous *CP* defects, ACP should be diagnosed.
Treatment
• In patients with ACP anemia is mild and therefore treatment is not recommended. • Iron chelation therapy should be considered for the treatment of ACP.
*Family screening**
• The proband should be informed about the autosomal recessive inheritance pattern of ACP. Siblings of the proband may be also affected. Since *CP* defects are very rare, the chance that children of the proband are affected is negligible. • We recommend to screen for pathogenic *CP* mutations: siblings and spouse in case of consanguinity and reproductive age. In case a proband is diagnosed with ACP after the fourth decade, his or her parents are not likely to be of reproductive age, and genotyping is not recommended. • Children of the proband should only be checked for *CP* mutations in case of consanguinity of the proband and his/her spouse or in case of proven carriership of both proband and his/her spouse. • Indviduals heterozygous or compound heterozygous for *CP* mutation should be screened for the ACP disease phenotype.

Table 1. Continued

Part 2: Defects in iron acquisition by the erythroid precursors
2A. Hypotransferrinemia due to defects in *TF*
Clinical presentation and diagnostics
• In patients with unexplained hypochromic microcytic anemia, low iron binding capacity/serum transferrin concentrations and increased ferritin concentrations, hypotransferrinemia should be considered. Mutation analysis of the *TF* gene is recommended. • In case of a homozygous or compound heterozygous *TF* defect, hypotransferrinemia due to a *TF* defect should be diagnosed.
Treatment
• Transferrin supplementation by either plasma transfusion or apo-transferrin infusion is recommended in patients with hypotransferrinemia due to a *TF* defect. • Iron status should be monitored in patients with hypotransferrinemia due to a *TF* defect in order to detect toxic iron loading early. • In case of systemic iron loading, phlebotomies are recommended. If phlebotomies are not tolerated due to a decreasing Hb, chelation therapy is recommended.
Family screening*
• Recommendations are identical to those described in 1C.
2B. Anemia with systemic iron loading due to defects in *SLC11A2 (DMT1)*
Clinical presentation and diagnosis
• In patients presenting in childhood with unexplained microcytic anemia with increased TSAT, (among others) *SLC11A2* defects should be considered. Genotyping of *SLC11A2* is recommended. • In case of a homozygous or compound heterozygous *SLC11A2* defect, diagnosis of microcytic anemia due to a *SLC11A2* defect is confirmed.
Treatment
• Patients with microcytic anemia due to pathogenic *SLC11A2* defects should be treated with oral iron supplementation and/or EPO and/or erythrocyte transfusions, according to the needs of the individual patient. • In case of treatment with oral iron supplementation and/or erythrocyte transfusions, iron status should be monitored in order to detect toxic iron loading in an early stage. • Since a normal serum ferritin concentration does not exclude liver iron loading in patients with *SLC11A2* defects, MRI of the liver should be considered.
Family screening*
• Recommendations are identical to those described in 1C.

Table 1. Continued

2 C. Sideroblastic anemia due to defects in *STEAP3*
Clinical presentation and diagnosis
• In patients with unexplained hypochromic sideroblastic anemia with low or normal MCV defects in the *STEAP3* gene should be considered. • In case of the combination of hypochromic anemia and gonadal dysfunction, *STEAP3* defects should be considered.
Treatment
• Patients with hypochromic anemia due to *STEAP3* defects can be treated with erythrocyte transfusions in combination with EPO. Systemic iron loading should be treated with iron chelation.
Family screening*
• Since sideroblastic anemia due to a *STEAP3* defect has been described in only 1 family, the inheritance pattern is uncertain and the proband should be referred to a clinical geneticist.
Part 3: Defects in the heme and/or iron sulphur cluster synthesis.
3A. Sideroblastic anemia due to defects in *SLC25A38*
Clinical presentation and diagnosis
• In children with severe unexplained microcytic sideroblastic anemia defects in *SLC25A38* should be considered.
Treatment
• Hematopoietic stem cell transplantation (HSCT) is recommended since this is the only curative option. • Symptomatic treatment consists of erythrocyte transfusions and chelation therapy.
Family screening*
• Recommendations are identical to those described in 1C.

Table 1. Continued

3B. X-linked sideroblastic anemia with ataxia due to defects in *ABCB7*
Clinical presentation and diagnosis
• In male patients presenting with the combination of a mild microcytic anemia and ataxia, a defect in *ABCB7* should be considered. • Increased protoporphyrin IX concentrations in red blood cells are suggestive of this disorder.
Treatment
• Treatment of (mild) anemia is not indicated.
*Family screening**
• The proband should be informed about the X-linked inheritance pattern of anemia and ataxia due to *ABCB7* defects. Brothers of the proband may be also affected. Sons of the proband are not affected. Daughters of the proband are obligate carrier of the relevant *ABCB7*defect and have no or a mildly anemic clinical phenotype. • We recommend offering screening for the *ABCB7* mutation: the mother and sisters (for carriership) and the brothers. The spouse should only be checked in case of consanguinity and reproductive age. Daughters of the proband should offered to be checked for carrier ship.

Table 1. Continued

3C. X- linked sideroblastic anemia due to defects in *ALAS2*
Clinical presentation and diagnosis
• XSLA due to an *ALAS2* defect should be considered in patients of both gender and of all ages with pyridoxine responsive or unresponsive (mild) microcytic sideroblastic anemia with or without iron loading and in patients with unexplained iron loading. • In patients suspected for XLSA, iron parameters (ferritin, TSAT) should be checked to detect iron loading, as well as liver enzymes, and signs of liver fibrosis or hepatocellular carcinoma. • In case of elderly patients presenting with MDS-RARS or MDS-RCMD without specific cytogenetic abnormalities, the presence of *ALAS2* defects should be considered, especially if the anemia is microcytic.
Treatment
• Management of patients with XLSA should involve treatment of anemia, and prevention and treatment of iron overload. • Initial treatment with pharmacological doses pyridoxine (50-200 mg/day) is recommended. Occasionally high doses (up to 300 mg/day) in heavy, active or elderly may be considered. • In case of pyridoxine responsiveness, lifelong supplementation of pyridoxine 10-100 mg daily is recommended. • Once a response is obtained evidence suggests the life-long maintenance dose may be lowered to 10-100 mg/day, since too high doses may give neurotoxicity. • Iron loading should be treated, preferably by phlebotomies.
*Family screening**
• The proband should be informed about the X-linked inheritance pattern of anemia and/or iron overload due to *ALAS2* defects. Brothers of the proband may be also affected. Sons of the proband are not affected. Daughters of the proband are obligate carrier of the relevant *ALAS2* defect. • We recommend screening for the *ALAS2* mutation: the brothers and also the mother, sisters and daughters (for carrier ship and for the reason that women may develop a XLSA phenotype later in life). The spouse should only be checked in case of consanguinity and reproductive age. • Female carriers and male hemizygous individuals should be screened for the XLSA phenotype.

Table 1. Continued

3D. Sideroblastic anemia due to defects in *GLRX5*
Clinical presentation and diagnosis
• In patients presenting with microcytic sideroblastic anemia and iron loading (among others) defects in *GLRX5* should be considered. • In case of microcytic sideroblastic anemia without *ALAS2*, or *SLC25A38* defects a lymphoblastic culture should be considered. Decreased activity of mitochondrial acitonase and succinate dehydrogenase (complex I-IV) as a manifestation of a defective Fe-S cluster synthesis, is suggestive for a *GLRX5* defect.
Treatment
• In patients with sideroblastic anemia and iron loading due to *GLRX5* defects, monitoring of the iron status and possible complications of iron overload is recommended. Iron loading should be treated with chelation therapy. Severe anemia with blood transfusions.
*Family screening**
• Recommendations are identical to those described in 1C.
3E. Erythropoietic ProtoPorphyria due to defects in *FECH* and gain-of-function mutations in *ALAS2* (XLDPP)
Clinical presentation and diagnosis
• In patients with an unexplained mild normo- to microcytic anemia, a low to normal serum ferritin and cutaneous protoporphyria, a painful photosensitivity in childhood, EPP has to be considered, both in men and women. • The diagnosis EPP should be confirmed by fluorescent erythrocytes in an unstained smear, and/or enhanced protoporphyrin in plasma and/or erythrocytes. • To prove autosomal recessive EPP FECH activity should be measured or *FECH* mutations determined. • In case EPP is not explained by the *FECH* genotype, the *ALAS2* gene should be investigated for the presence of gain-of-function mutations.
Treatment
• In case anemia in EPP is present, it is mild, and treatment is not recommended.
*Family screening**
• Because of complex genetics, influenced by genetic background, we recommend referral to a clinical geneticist in case of EPP. Family screening for a proband with XLDPP due to and *ALAS2* gain-of-function mutation should initially include phenotyping and genotyping of all first-degree family members (including women): mother in case of a male proband, both parents in case of a female proband, siblings and children.

Table 1. Continued

3F. Congenital Erythropoietic Porphyria (CEP) due to defects in *UROS* or *GATA1*
Clinical presentation and diagnosis
• In patients with an unexplained hemolytic anemia in combination with a painful cutaneous photosensitivity autosomal recessive CEP has to be considered, both in men and women. • In cases with combined cutaneous photosensitivity and severe microcytic, hypochromic hemolytic anemia X-linked CEP should be considered. • Since disease severity and onset of first symptoms is highly variable, CEP should be considered both in children and adults in case of the above-mentioned phenotype. • Diagnosis should be based on increased urinary levels of uroporphyrin I and coproporphyrin I and confirmed by decreased URO-synthase activity in the erythrocytes or the presence of pathogenic homozygous or compound heterozygous mutations in *UROS* or in *GATA1*.
Treatment
• In patients with CEP, allogenic HSCT should be considered as the only curative treatment • Chronic erythrocyte transfusions are recommended as a symptomatic treatment, and iron chelation is recommended according to guidelines for chronic transfusions.
*Family screening**
• Recommendations for CEP due to UROS defects are identical to those described in 1C. • Recommendations are identical to XLDPP due to gain-of-function *ALAS2* mutations, see 3E.

*, Recommendations on family screening are based on Borry *et al*[99], Godard *et al*[100]

Table 2. Overview of characteristics of rare microcytic anemias due to genetic disorders of iron metabolism or heme synthesis

Pathophysiology	Low iron availability for erythropoiesis			Defect in iron acquisition of erythroid progenitor cells		
Disorder	IRIDA	Ferroportin disease*	Aceruloplasminemia	Hypotransferrinemia	Microcytic anemia with iron loading	Sideroblastic anemia
Gene	*TMPRSS6*	*SLC40A1*	*CP*	*TF*	*DMT1*	*STEAP3*
Protein	Matriptase 2	Ferroportin-1	Ceruloplasmin	Transferrin	DMT1	STEAP3
Patients' desc.	20-100	>200	20-100	5-20	5-20	< 5 (=3)
Inheritance	AR[§]	AD	AR/AD	AR	AR	AR/AD[‖]
Age at present.	child	adult	40-50 yrs	variable	child	child
Symptoms						
neurological	no	no	yes	no	no	no[#]
skin	no	no	no	no	no	no
Anemia	variable	mild in 10%[++]	mild	variable	variable	variable
MCV	micro	normo	micro/ normo	micro	micro	micro/ normo [b]
Ring sideroblasts	no	no	no	no	no	yes
Iron loading	no	yes	yes	yes	variable[c]	possible[d]
Ferritin	normal-low	high	high	high	variable[c]	high[d]
Transferrin saturation	<10%	normal/ high[++]	normal/ low	100%	high	high
Hepcidin	high[e]	variable	no data	low	normal /low	normal / increased[f]
Treatment	oral iron iv iron /EPO[g]	phlebotomy[++]	chelation	erytx apoTF plasma chelation phlebotomy	oral iron erytx EPO chelation	erytx EPO chelation

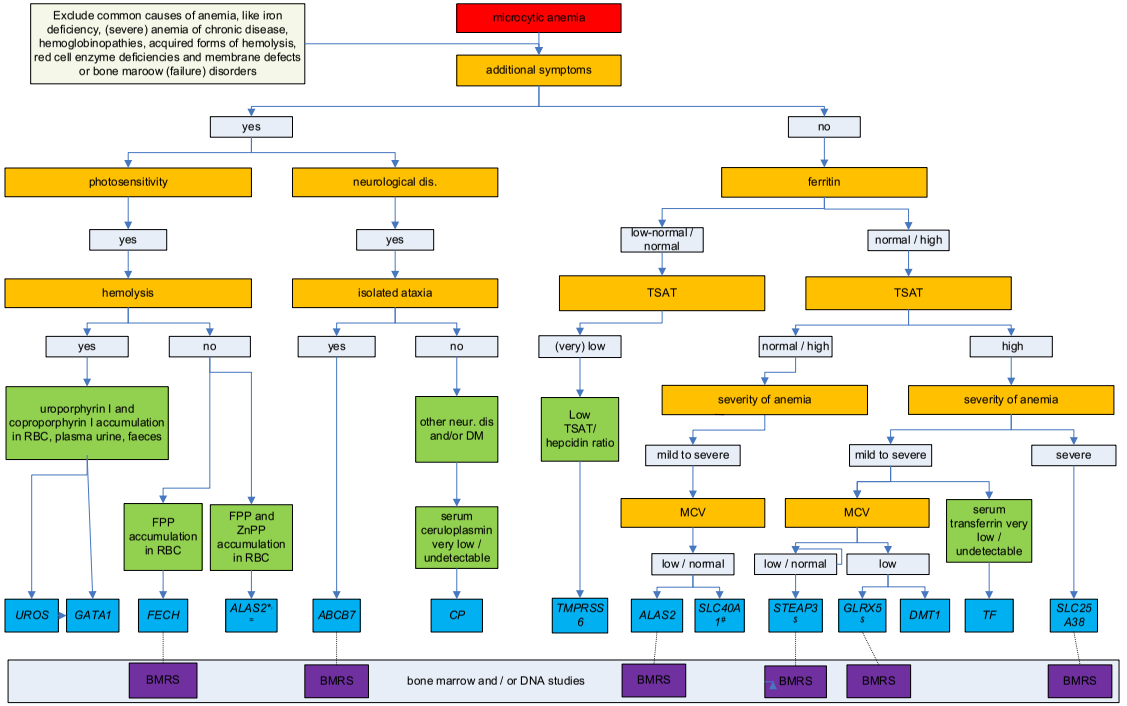
* both loss-of function and gain-of function have been described, data in column reflect those of the combined group unless stated otherwise; [+], loss-of function; [±], gain-of-function; [§], also autosomal dominant inheritance pattern described; [‖], heterozygous pathogenic mutation in combination with decreased expression of normal allele; [¶], in some families only women affected since the defect is lethal in man; [#], gonadal dysfunction; [**],neurologic symptoms manifest in childhood, anemia may develop later in life (young adolescent); [++], in case of loss-of-function mutations, anemia is more likely to occur and TSAT is lower; [a] , anemia resolves by pyridoxine treatment in most XLSA patients; [b], MCH decreased; [c],

	Defect in heme synthesis or iron-sulfur cluster biogenesis							
	Sideroblastic anemia	X-linked sideroblastic anemia with ataxia	X-linked sideroblastic anemia	Sideroblastic anemia	Erythropoietic protoporphyria		Congenital Erythropoietic Porphyria	
					EPP	XLDPP	CEP	XLCEP
	SLC25A38	*ABCB7*	*ALAS2*[+]	*GLRX5*	*FECH*	*ALAS2*[±]	*UROS*	*GATA1*
	SLC25A38	ABCB7	ALAS2	GLRX5	FECH	ALAS2	UROS	GATA1
	20-100	5-20	>100	< 5 (=1)	>100	20-100	>200	1
	AR	XL [¶]	XL	AR	most AD	XL	AR	XL
	child	child	variable	adult	neonate/ child	neonate/ child	variable fetus-adult	neonate
	no	yes [**]	no	no	no	no	no	no
	no	no	no	no	yes	yes	yes	yes
	severe	mild	mild, no anemia [a]	mild	mild, no anemia	mild, no anemia	hemolytic, severity variable	severe, hemolytic
	micro	micro	micro	micro	micro/ normo	normo	no data	micro
	yes	yes	yes	yes	yes	no data	no	No
	yes high	no normal	variable variable	yes high	no low/normal	no low/normal	no data no data	no normal
	high	normal	normal/high	high	normal/low	normal/low	no data	normal
	no data	no data	no data	no data	no data	no data	no data	no data
	HSCT transfusion chelation	not indicated	pyridoxine folate phlebotomy chelation	erytx chelation	no treatment for anemia/ avoid skin light	no treatment for anemia/ avoid skin light	HSCT erytx chelation avoid skin light	no data

liver iron loading has been described demonstrated by MRI and liver biopsy, even if serum ferritin is normal; [d], iron loading may be secondary to erythrocyte transfusion; [e], hepcidin increased in relation to iron parameters, hepcidin/ TSAT ratio > upper limit of reference range in absence of inflammation; [f], measured after treatment with transfusions; [g], only 1 human study available on EPO.

Abbreviations: EPO denotes erythropoietin; erytx, erythrocyte transfusion; TF, transferrin; HSCT, hematopoietic stem cell transplantation; AD, Autosomal Dominant; AR, Autosomal Recessive

Figure 2. Diagnostic flow chart for microcytic anemias caused by inherited defects in iron metabolism or heme synthesis



Genes are given in italics and refer to the disorders of this review (Table 2). After clinical and laboratory assessment, clinicians may proceed to perform a diagnostic workup with either bone marrow smears or gene analysis. Iron parameters should be interpreted in the context of the age of the patient and the given treatment: older patients are more likely to have developed iron overload (increased TSAT and ferritin) due to increased and ineffective erythropoiesis and iron supplementation and transfusions. Note that for some diseases, the decision tree is based on only few patients (Table 2.)

*Patients have normocytic anemia and GOF mutation; 1only 1 family described; 6patients have normocytic anemia and the majority have LOF mutations; ^in rare cases, these patients present with elevated ferritin levels

Abbreviations: BMRS denotes bone marrow ring sideroblasts; DM, diabetes mellitus; FPP, free protoporphyrin; neur. dis, neurologic disease; ZnPP, zinc protoporphyrin

Conclusions

We present a clinical guideline on orphan diseases of which many aspects are still unknown. Consequently, the level of evidence for the management of these disorders is relatively low. We therefore recommend centers of excellence with expertise with these diseases to join forces in order to identify new mechanisms, biomarkers and treatments and to optimize the management of these patients. Until more evidence is available, this guideline can be used to assist clinicians and patients in their understanding, diagnosis and management of microcytic genetic anemias of iron metabolism or heme synthesis.

Authorship

Contribution: AED, RAPR, PPTB, ND, LTV, NVAMK, RT, DWS were members of the working group, made literature and case-tables, drafted, discussed and finalized the guidelines and edited the manuscript; TvB: advisor and support in the use of the methodology for guideline development; DWS, initiated and coordinated the project and acquired funding. All authors approved the final version of the manuscript.

Conflict-of-interest disclosure

DWS is medical director of the www.hepcidinanalysis.com initiative that serves the scientific and medical community with hepcidin measurements at a fee-for service basis. She is an employee of the Radboud medical center that offers genetic testing for the genes described in the current guideline (at http://www.umcn.nl/Informatievoorverwijzers/Genoomdiagnostiek/en/Pages/default.aspx). The remaining authors declare no competing financial interests.

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REFERENCES

1. Iolascon A, De Falco L, Beaumont C. Molecular basis of inherited microcytic anemia due to defects in iron acquisition or heme synthesis. *Haematologica.* 2009;94(3):395-408.
2. van Rooijen KL, Raymakers RA, Cuijpers ML, Brons PP, Janssen MC, Swinkels DW. [New causes of microcytic anaemia: hereditary disorders of iron homeostasis]. *Nederlands tijdschrift voor geneeskunde.* 2010;154:A1039.
3. Camaschella C. Recent advances in the understanding of inherited sideroblastic anaemia. *British journal of haematology.* 2008;143(1):27-38.
4. Fleming MD. Congenital sideroblastic anemias: iron and heme lost in mitochondrial translation. *Hematology / the Education Program of the American Society of Hematology American Society of Hematology Education Program.* 2011;2011:525-531.
5. Camaschella C. Hereditary sideroblastic anemias: pathophysiology, diagnosis, and treatment. *Seminars in hematology.* 2009;46(4):371-377.
6. Donovan A, Brownlie A, Zhou Y, et al. Positional cloning of zebrafish ferroportin1 identifies a conserved vertebrate iron exporter. *Nature.* 2000;403(6771):776-781.
7. https://richtlijnendatabase.nl/over_deze_site/richtlijnontwikkeling.html. Date of Access 28 November 2019
8. Brouwers MC, Kho ME, Browman GP, et al. AGREE II: advancing guideline development, reporting and evaluation in health care. *CMAJ : Canadian Medical Association journal = journal de l'Association medicale canadienne.* 2010;182(18):E839-842.
9. Atkins D, Best D, Briss PA, et al. Grading quality of evidence and strength of recommendations. *Bmj.* 2004;328(7454):1490.
10. Guyatt GH, Oxman AD, Vist GE, et al. GRADE: an emerging consensus on rating quality of evidence and strength of recommendations. *Bmj.* 2008;336(7650):924-926.
11. Hentze MW, Muckenthaler MU, Galy B, Camaschella C. Two to tango: regulation of Mammalian iron metabolism. *Cell.* 2010;142(1):24-38.
12. Grantham-McGregor S, Ani C. A review of studies on the effect of iron deficiency on cognitive development in children. *The Journal of nutrition.* 2001;131(2s-2):649S-666S; discussion 666S-668S.
13. Fleming RE, Ponka P. Iron overload in human disease. *The New England journal of medicine.* 2012;366(4):348-359.
14. Du X, She E, Gelbart T, et al. The serine protease TMPRSS6 is required to sense iron deficiency. *Science.* 2008;320(5879):1088-1092.
15. Silvestri L, Guillem F, Pagani A, et al. Molecular mechanisms of the defective hepcidin inhibition in TMPRSS6 mutations associated with iron-refractory iron deficiency anemia. *Blood.* 2009;113(22):5605-5608.
16. Finberg KE, Heeney MM, Campagna DR, et al. Mutations in TMPRSS6 cause iron-refractory iron deficiency anemia (IRIDA). *Nature genetics.* 2008;40(5):569-571.
17. Benyamin B, Ferreira MA, Willemsen G, et al. Common variants in TMPRSS6 are associated with iron status and erythrocyte volume. *Nature genetics.* 2009;41(11):1173-1175.
18. Chambers JC, Zhang W, Li Y, et al. Genome-wide association study identifies variants in TMPRSS6 associated with hemoglobin levels. *Nature genetics.* 2009;41(11):1170-1172.
19. Melis MA, Cau M, Congiu R, et al. A mutation in the TMPRSS6 gene, encoding a transmembrane serine protease that suppresses hepcidin production, in familial iron deficiency anemia refractory to oral iron. *Haematologica.* 2008;93(10):1473-1479.
20. Hershko C, Camaschella C. How I treat unexplained refractory iron deficiency

anemia. *Blood.* 2014;123(3):326-333.
21. Heeney MM, Campagna DR, Westerman M, Fleming MD. The clinical and genetic spectrum of TMPRSS6 mutations leading to inappropriate hepcidin expression and iron refractory iron deficiency anemia (IRIDA). Paper presented at: Blood 2009.
22. Beutler E, Van Geet C, te Loo DM, et al. Polymorphisms and mutations of human TMPRSS6 in iron deficiency anemia. *Blood cells, molecules & diseases.* 2010;44(1):16-21.
23. Cau M, Galanello R, Giagu N, Melis MA. Responsiveness to oral iron and ascorbic acid in a patient with IRIDA. *Blood cells, molecules & diseases.* 2012;48(2):121-123.
24. Locatelli F, Barany P, Covic A, et al. Kidney Disease: Improving Global Outcomes guidelines on anaemia management in chronic kidney disease: a European Renal Best Practice position statement. *Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association.* 2013;28(6):1346-1359.
25. Lehmberg K, Grosse R, Muckenthaler MU, et al. Administration of recombinant erythropoietin alone does not improve the phenotype in iron refractory iron deficiency anemia patients. *Annals of hematology.* 2013;92(3):387-394.
26. Ramsay AJ, Quesada V, Sanchez M, et al. Matriptase-2 mutations in iron-refractory iron deficiency anemia patients provide new insights into protease activation mechanisms. *Human molecular genetics.* 2009;18(19):3673-3683.
27. Kroot JJ, Tjalsma H, Fleming RE, Swinkels DW. Hepcidin in human iron disorders: diagnostic implications. *Clinical chemistry.* 2011;57(12):1650-1669.
28. Wallace DF, Harris JM, Subramaniam VN. Functional analysis and theoretical modeling of ferroportin reveals clustering of mutations according to phenotype. *American journal of physiology Cell physiology.* 2010;298(1):C75-84.
29. De Domenico I, Ward DM, Langelier C, et al. The molecular mechanism of hepcidin-mediated ferroportin down-regulation. *Molecular biology of the cell.* 2007;18(7):2569-2578.
30. Pietrangelo A. The ferroportin disease. *Blood cells, molecules & diseases.* 2004;32(1):131-138.
31. Camaschella C, Poggiali E. Rare types of genetic hemochromatosis. *Acta haematologica.* 2009;122(2-3):140-145.
32. Mayr R, Janecke AR, Schranz M, et al. Ferroportin disease: a systematic meta-analysis of clinical and molecular findings. *Journal of hepatology.* 2010;53(5):941-949.
33. Nemeth E. Ferroportin mutations: a tale of two phenotypes. *Blood.* 2005;105(10):3763-3764.
34. Rice AE, Mendez MJ, Hokanson CA, Rees DC, Bjorkman PJ. Investigation of the biophysical and cell biological properties of ferroportin, a multipass integral membrane protein iron exporter. *Journal of molecular biology.* 2009;386(3):717-732.
35. Blanc B, Finch CA, Hallberg L et al. Nutritional Anemias. Report of a WHO Scientific Group. *WHO Technical Report Series.* 1968;405:1-40.
36. Harris ZL, Durley AP, Man TK, Gitlin JD. Targeted gene disruption reveals an essential role for ceruloplasmin in cellular iron efflux. *Proceedings of the National Academy of Sciences of the United States of America.* 1999;96(19):10812-10817.
37. De Domenico I, Ward DM, di Patti MC, et al. Ferroxidase activity is required for the stability of cell surface ferroportin in cells expressing GPI-ceruloplasmin. *The EMBO journal.* 2007;26(12):2823-2831.
38. Miyajima H, Kohno S, Takahashi Y, Yonekawa O, Kanno T. Estimation of the gene frequency of aceruloplasminemia in Japan. *Neurology.* 1999;53(3):617-619.
39. McNeill A, Pandolfo M, Kuhn J, Shang H, Miyajima H. The neurological presentation of ceruloplasmin gene mutations. *European neurology.* 2008;60(4):200-205.

40. Bosio S, De Gobbi M, Roetto A, et al. Anemia and iron overload due to compound heterozygosity for novel ceruloplasmin mutations. *Blood.* 2002;100(6):2246-2248.
41. Roberti Mdo R, Borges Filho HM, Goncalves CH, Lima FL. Aceruloplasminemia: a rare disease - diagnosis and treatment of two cases. *Revista brasileira de hematologia e hemoterapia.* 2011;33(5):389-392.
42. Suzuki Y, Yoshida K, Aburakawa Y, et al. Effectiveness of oral iron chelator treatment with deferasirox in an aceruloplasminemia patient with a novel ceruloplasmin gene mutation. *Internal medicine (Tokyo, Japan).* 2013;52(13):1527-1530.
43. Finkenstedt A, Wolf E, Hofner E, et al. Hepatic but not brain iron is rapidly chelated by deferasirox in aceruloplasminemia due to a novel gene mutation. *Journal of hepatology.* 2010;53(6):1101-1107.
44. Kuhn J, Bewermeyer H, Miyajima H, Takahashi Y, Kuhn KF, Hoogenraad TU. Treatment of symptomatic heterozygous aceruloplasminemia with oral zinc sulphate. *Brain & development.* 2007;29(7):450-453.
45. Skidmore FM, Drago V, Foster P, Schmalfuss IM, Heilman KM, Streiff RR. Aceruloplasminaemia with progressive atrophy without brain iron overload: treatment with oral chelation. *Journal of neurology, neurosurgery, and psychiatry.* 2008;79(4):467-470.
46. Logan JI, Harveyson KB, Wisdom GB, Hughes AE, Archbold GP. Hereditary caeruloplasmin deficiency, dementia and diabetes mellitus. *QJM : monthly journal of the Association of Physicians.* 1994;87(11):663-670.
47. Yazaki M, Yoshida K, Nakamura A, et al. A novel splicing mutation in the ceruloplasmin gene responsible for hereditary ceruloplasmin deficiency with hemosiderosis. *Journal of the neurological sciences.* 1998;156(1):30-34.
48. Beutler E, Gelbart T, Lee P, Trevino R, Fernandez MA, Fairbanks VF. Molecular characterization of a case of atransferrinemia. *Blood.* 2000;96(13):4071-4074.
49. Heilmeyer L, Keller W, Vivell O, et al. [Congenital atransferrinemia in a 7-year-old girl]. *Deutsche medizinische Wochenschrift (1946).* 1961;86:1745-1751.
50. Hayashi A, Wada Y, Suzuki T, Shimizu A. Studies on familial hypotransferrinemia: unique clinical course and molecular pathology. *American journal of human genetics.* 1993;53(1):201-213.
51. Trombini P, Coliva T, Nemeth E, et al. Effects of plasma transfusion on hepcidin production in human congenital hypotransferrinemia. *Haematologica.* 2007;92(10):1407-1410.
52. Bartnikas TB, Andrews NC, Fleming MD. Transferrin is a major determinant of hepcidin expression in hypotransferrinemic mice. *Blood.* 2011;117(2):630-637.
53. Shamsian BS, Rezaei N, Arzanian MT, Alavi S, Khojasteh O, Eghbali A. Severe hypochromic microcytic anemia in a patient with congenital atransferrinemia. *Pediatric hematology and oncology.* 2009;26(5):356-362.
54. Hamill RL, Woods JC, Cook BA. Congenital atransferrinemia. A case report and review of the literature. *American journal of clinical pathology.* 1991;96(2):215-218.
55. Hromec A, Payer Jr J, Killinger Z, Rybar I, Rovenský J. [Congenital atransferrinemia]. *Deutsche medizinische Wochenschrift (1946).* 1994;119(18):663-666.
56. Goldwurm S, Casati C, Venturi N, et al. Biochemical and genetic defects underlying human congenital hypotransferrinemia. *The hematology journal : the official journal of the European Haematology Association / EHA.* 2000;1(6):390-398.
57. Canonne-Hergaux F, Zhang AS, Ponka P, Gros P. Characterization of the iron transporter DMT1 (NRAMP2/DCT1) in red blood cells of normal and anemic mk/mk mice. *Blood.* 2001;98(13):3823-3830.
58. Fleming MD, Trenor CC, 3rd, Su MA, et al. Microcytic anaemia mice have a mutation in Nramp2, a candidate iron transporter gene. *Nature genetics.* 1997;16(4):383-386.
59. Touret N, Martin-Orozco N, Paroutis P, et al. Molecular and cellular mechanisms

underlying iron transport deficiency in microcytic anemia. *Blood.* 2004;104(5):1526-1533.
60. Priwitzerova M, Pospisilova D, Prchal JT, et al. Severe hypochromic microcytic anemia caused by a congenital defect of the iron transport pathway in erythroid cells. *Blood.* 2004;103(10):3991-3992.
61. Iolascon A, Camaschella C, Pospisilova D, Piscopo C, Tchernia G, Beaumont C. Natural history of recessive inheritance of DMT1 mutations. *The Journal of pediatrics.* 2008;152(1):136-139.
62. Iolascon A, d'Apolito M, Servedio V, Cimmino F, Piga A, Camaschella C. Microcytic anemia and hepatic iron overload in a child with compound heterozygous mutations in DMT1 (SCL11A2). *Blood.* 2006;107(1):349-354.
63. Ohgami RS, Campagna DR, Greer EL, et al. Identification of a ferrireductase required for efficient transferrin-dependent iron uptake in erythroid cells. *Nature genetics.* 2005;37(11):1264-1269.
64. Ohgami RS, Campagna DR, Antiochos B, et al. nm1054: a spontaneous, recessive, hypochromic, microcytic anemia mutation in the mouse. *Blood.* 2005;106(10):3625-3631.
65. Grandchamp B, Hetet G, Kannengiesser C, et al. A novel type of congenital hypochromic anemia associated with a nonsense mutation in the STEAP3/TSAP6 gene. *Blood.* 2011;118(25):6660-6666.
66. Guernsey DL, Jiang H, Campagna DR, et al. Mutations in mitochondrial carrier family gene SLC25A38 cause nonsyndromic autosomal recessive congenital sideroblastic anemia. *Nature genetics.* 2009;41(6):651-653.
67. Kannengiesser C, Sanchez M, Sweeney M, et al. Missense SLC25A38 variations play an important role in autosomal recessive inherited sideroblastic anemia. *Haematologica.* 2011;96(6):808-813.
68. Taketani S, Kakimoto K, Ueta H, Masaki R, Furukawa T. Involvement of ABC7 in the biosynthesis of heme in erythroid cells: interaction of ABC7 with ferrochelatase. *Blood.* 2003;101(8):3274-3280.
69. Allikmets R, Raskind WH, Hutchinson A, Schueck ND, Dean M, Koeller DM. Mutation of a putative mitochondrial iron transporter gene (ABC7) in X-linked sideroblastic anemia and ataxia (XLSA/A). *Human molecular genetics.* 1999;8(5):743-749.
70. Bottomley SS, Healy HM, Brandenburg MA, May BK. 5-Aminolevulinate synthase in sideroblastic anemias: mRNA and enzyme activity levels in bone marrow cells. *American journal of hematology.* 1992;41(2):76-83.
71. Astner I, Schulze JO, van den Heuvel J, Jahn D, Schubert WD, Heinz DW. Crystal structure of 5-aminolevulinate synthase, the first enzyme of heme biosynthesis, and its link to XLSA in humans. *The EMBO journal.* 2005;24(18):3166-3177.
72. Campagna DR, de Bie CI, Schmitz-Abe K, et al. X-linked sideroblastic anemia due to ALAS2 intron 1 enhancer element GATA-binding site mutations. *American journal of hematology.* 2014;89(3):315-319.
73. Cotter PD, May A, Li L, et al. Four new mutations in the erythroid-specific 5-aminolevulinate synthase (ALAS2) gene causing X-linked sideroblastic anemia: increased pyridoxine responsiveness after removal of iron overload by phlebotomy and coinheritance of hereditary hemochromatosis. *Blood.* 1999;93(5):1757-1769.
74. Cooley TB. A SEVERE TYPE OF HEREDITARY ANEMIA WITH ELLIPTOCYTOSIS: Interesting Sequence of Splenectomy. *The American Journal of the Medical Sciences.* 1945;209(5):561-568.
75. Bergmann AK, Campagna DR, McLoughlin EM, et al. Systematic molecular genetic analysis of congenital sideroblastic anemia: evidence for genetic heterogeneity and identification of novel mutations. *Pediatric blood & cancer.* 2010;54(2):273-278.
76. Ducamp S, Kannengiesser C, Touati M, et al. Sideroblastic anemia: molecular analysis of the ALAS2 gene in a series of 29 probands and functional studies of 10 missense

mutations. *Human mutation.* 2011;32(6):590-597.
77. Aivado M, Gattermann N, Rong A, et al. X-linked sideroblastic anemia associated with a novel ALAS2 mutation and unfortunate skewed X-chromosome inactivation patterns. *Blood cells, molecules & diseases.* 2006;37(1):40-45.
78. Cazzola M, May A, Bergamaschi G, Cerani P, Rosti V, Bishop DF. Familial-skewed X-chromosome inactivation as a predisposing factor for late-onset X-linked sideroblastic anemia in carrier females. *Blood.* 2000;96(13):4363-4365.
79. Harris JW, Danish EH, Brittenham GM, McLaren CE. Pyridoxine responsive hereditary sideroblastic erythropoiesis and iron overload: two microcytic subpopulations in the affected male, one normocytic and one microcytic subpopulation in the obligate female carrier. *American journal of hematology.* 1993;42(4):400-401.
80. Barton JC, Lee PL. Disparate phenotypic expression of ALAS2 R452H (nt 1407 G --> A) in two brothers, one with severe sideroblastic anemia and iron overload, hepatic cirrhosis, and hepatocellular carcinoma. *Blood cells, molecules & diseases.* 2006;36(3):342-346.
81. Furuyama K, Harigae H, Kinoshita C, et al. Late-onset X-linked sideroblastic anemia following hemodialysis. *Blood.* 2003;101(11):4623-4624.
82. Cotter PD, Rucknagel DL, Bishop DF. X-linked sideroblastic anemia: identification of the mutation in the erythroid-specific delta-aminolevulinate synthase gene (ALAS2) in the original family described by Cooley. *Blood.* 1994;84(11):3915-3924.
83. Camaschella C. Treating iron overload. *The New England journal of medicine.* 2013;368(24):2325-2327.
84. Ye H, Jeong SY, Ghosh MC, et al. Glutaredoxin 5 deficiency causes sideroblastic anemia by specifically impairing heme biosynthesis and depleting cytosolic iron in human erythroblasts. *The Journal of clinical investigation.* 2010;120(5):1749-1761.
85. Ye H, Rouault TA. Erythropoiesis and iron sulfur cluster biogenesis. *Advances in hematology.* 2010;2010.
86. Camaschella C, Campanella A, De Falco L, et al. The human counterpart of zebrafish shiraz shows sideroblastic-like microcytic anemia and iron overload. *Blood.* 2007;110(4):1353-1358.
87. Balwani M, Doheny D, Bishop DF, et al. Loss-of-function ferrochelatase and gain-of-function erythroid-specific 5-aminolevulinate synthase mutations causing erythropoietic protoporphyria and x-linked protoporphyria in North American patients reveal novel mutations and a high prevalence of X-linked protoporphyria. *Molecular medicine (Cambridge, Mass).* 2013;19:26-35.
88. Whatley SD, Mason NG, Holme SA, Anstey AV, Elder GH, Badminton MN. Molecular epidemiology of erythropoietic protoporphyria in the U.K. *The British journal of dermatology.* 2010;162(3):642-646.
89. Holme SA, Worwood M, Anstey AV, Elder GH, Badminton MN. Erythropoiesis and iron metabolism in dominant erythropoietic protoporphyria. *Blood.* 2007;110(12):4108-4110.
90. Delaby C, Lyoumi S, Ducamp S, et al. Excessive erythrocyte PPIX influences the hematologic status and iron metabolism in patients with dominant erythropoietic protoporphyria. *Cellular and molecular biology (Noisy-le-Grand, France).* 2009;55(1):45-52.
91. Balwani M, Desnick RJ. The porphyrias: advances in diagnosis and treatment. *Hematology / the Education Program of the American Society of Hematology American Society of Hematology Education Program.* 2012;2012:19-27.
92. Fritsch C, Bolsen K, Ruzicka T, Goerz G. Congenital erythropoietic porphyria. *Journal of the American Academy of Dermatology.* 1997;36(4):594-610.
93. Solis C, Aizencang GI, Astrin KH, Bishop DF, Desnick RJ. Uroporphyrinogen III synthase erythroid promoter mutations in adjacent

GATA1 and CP2 elements cause congenital erythropoietic porphyria. *The Journal of clinical investigation.* 2001;107(6):753-762.

94. Phillips JD, Steensma DP, Pulsipher MA, Spangrude GJ, Kushner JP. Congenital erythropoietic porphyria due to a mutation in GATA1: the first trans-acting mutation causative for a human porphyria. *Blood.* 2007;109(6):2618-2621.
95. Katugampola RP, Badminton MN, Finlay AY, et al. Congenital erythropoietic porphyria: a single-observer clinical study of 29 cases. *The British journal of dermatology.* 2012;167(4):901-913.
96. Martinez Peinado C, Diaz de Heredia C, To-Figueras J, et al. Successful treatment of congenital erythropoietic porphyria using matched unrelated hematopoietic stem cell transplantation. *Pediatric dermatology.* 2013;30(4):484-489.
97. Piomelli S, Poh-Fitzpatrick MB, Seaman C, Skolnick LM, Berdon WE. Complete suppression of the symptoms of congenital erythropoietic porphyria by long-term treatment with high-level transfusions. *The New England journal of medicine.* 1986;314(16):1029-1031.
98. Desnick RJ, Astrin KH. Congenital erythropoietic porphyria: advances in pathogenesis and treatment. *British journal of haematology.* 2002;117(4):779-795.
99. Borry P, Goffin T, Nys H, Dierickx K. Attitudes regarding predictive genetic testing in minors: a survey of European clinical geneticists. *American journal of medical genetics Part C, Seminars in medical genetics.* 2008;148c(1):78-83.
100. Godard B, Hurlimann T, Letendre M, Egalite N. Guidelines for disclosing genetic information to family members: from development to use. *Familial cancer.* 2006;5(1):103-116.
101. Kautz L, Jung G, Valore EV, Rivella S, Nemeth E, Ganz T. Identification of erythroferrone as an erythroid regulator of iron metabolism. *Nature genetics.* 2014;46(7):678-684.

SUPPLEMENTAL FILES

Supplement 1 (Case Tables) and Supplement 2 (Evidence Based Conclusions) are available in the online version of this article.
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Supplement 3: Ferroportin disease

Anemia as a presenting symptom (Table 1) and during treatment with phlebotomy (Table 2) in patients diagnosed with iron overload due to a mutation in *SLC40A1* (ferroportin).

Supplemental Table 1. Characteristics of patients with and without documented anemia, diagnosed with iron overload due to mutations in *SLC40A1* (ferroportin)

	Anemia*	Normal Hb	*p*
References	1-6	2,3, 5-19	
N	8	68	
Age (years)			
Mean	40.5 (n=8)	39.8 (n=66)	ns
Median	38	38	ns
Hb (g/dL)			
Mean	12.0 (n=8)	14.4 (n=68)	< 0.0001
Median	12.1	145	< 0.0001
MCV (fL)			
Mean	94.0 (n=5)	89.6 (n=19)	ns
Median	94.0	92.0	ns
Serum Iron (µmol/L)			
Mean	18.7 (n=6)	27.6 (n=25)	ns
Median	12	20.8	ns
Transferrin Saturation (%)			
Mean	35.6 (n=7)	46.4 (n=66)	ns
Median	25.0	38.0	ns
Ferritin (µg/L)			
Mean	2282 (n=7)	2276 (n=65)	ns
Median	1364	1540	ns

Supplemental Table 1. Continued

	Anemia*	Normal Hb	*p*
Gender (n)			
Male	5 (n=8)	38 (n=66)	
Female	3	28	ns
Type of mutation+			
Gain (n)	1 (n=8)	12 (n=68)	
Loss (n)	7	56	ns

*, according to WHO criteria[42].

The mean values were compared using the Student t-test of independent samples, the median values were compared using the Mann-Whitney test of independent samples, while the distribution of gender and type of mutation between both groups were compared using a two-tailed Fisher's exact test. A *p* value < 0.05 was considered as statistically significant.

+, 2 types of mutations can be distinguished: 1. Gain-of-function (GOF) mutations: type 4B or atypical hereditary hemochromatosis (HH); 2. Loss-of-function (LOF) mutations: type 4A or classical HH. The type of mutation was classified implementing both phenotypic and functional features using the following definitions:

a) Phenotype of a LOF mutation: a normal transferrin saturation, poor tolerance to phlebotomy and iron deposition primarily in the Kuffper cells and macrophages.
b) Phenotype of a GOF mutation: an elevated transferrin saturation, good tolerance to phlebotomy and iron deposition in the hepatocytes in addition to the Kuppfer cells and macrophages.
c) Function of a LOF mutation: an intracellular localisation of the defect in the protein, a reduced capacity of iron export and preserved sensitivity to the inhibitory effects of hepcidin (all according to functional studies).
d) Function of a GOF mutation: a membrane localisation of the defect in the protein, preserved capacity of iron export and resistance to the inhibitory effects of hepcidin (all according to functional studies).

Classification was done according to the following definition:

1. In case both the phenotypic and functional features were in accordance, the type of mutation was defined as "definite".
2. In case of absence of data regarding functional features or when the data of the phenotypic and functional features were inconsistent the type of mutation was defined as "probable" in which the type of mutation was classified according to the phenotypic features.
3. For the classification of the type of mutation in this guideline the "definite" and "probable" definition were combined.

Abbreviations: ns denotes not significant

Supplemental Table 2. Tolerance to phlebotomy in patients diagnosed with iron overload due to mutations in *SLC40A1* (ferroportin)

Tolerance to phlebotomy	Transient anemia*	Good	*p*
References	6,8,18, 20-28	4, 5,7-10,12,13,15,19,26,29-41	
N	14^	80	
Age (years)			
Mean	52.0 (n=14)	41.1 (n=79)	0.04
Median	55.0	38.0	0.02
Hb (g/dL)			
Mean	14.0 (n=3)	14.7 (n=30)	ns
Median	14.2	15.0	ns
Serum Iron (µmol/L)			
Mean	14.6 (n=3)	37.9 (n=26)	ns
Median	10.7	38.1	0.03
Transferrin Saturation (%)			
Mean	41.3 (n=13)	58.9 (n=70)	0.04
Median	32.0	59.5	ns
Ferritin (µg/L)			
Mean	3256 (n=14)	2442 (n=75)	ns
Median	2224	1600	ns
Gender (n)			
Male	8 (n=14)	49 (n=78)	
Female	6	29	ns
Type of mutation+			
Gain (n)	1 (n=14)	25 (n=80)	
Loss (n)	13	55	0.06

The mean values were compared using the Student t-test of independent samples, the median values were compared using the Mann-Whitney test of independent samples, while the distribution of gender and type of mutation between both groups were compared using a two-tailed Fisher's exact test. A *p* value < 0.05 was considered as statistically significant.

*, according to WHO criteria[42]; +, see legend of Table 1; in three patients phlebotomy could not be continued because of anemia [21,22,28] , in 7 patients weekly phlebotomy resulted in a rapid decline in Hb which was restored after increasing the interval[6,8,20,23,25,27,28] , in one patient Hb normalised despite continuation of the weekly interval[26] , in 2 patients with phlebotomy-induced anemia, the addition of erythropoietin resulted in normalisation of Hb despite continuation of the same phlebotomy regimen[24], for only one patient MCV was reported (103 fL)[27].

Abbreviations: ns denotes not significant

REFERENCES

1. Subramaniam VN, Wallace DF, Dixon JL, Fletcher LM, Crawford DH. Ferroportin disease due to the A77D mutation in Australia. *Gut.* 2005;54(7):1048-1049.
2. Corradini E, Montosi G, Ferrara F, et al. Lack of enterocyte iron accumulation in the ferroportin disease. *Blood Cells Mol Dis.* 2005;35(3):315-318.
3. Cazzola M, Cremonesi L, Papaioannou M, et al. Genetic hyperferritinaemia and reticuloendothelial iron overload associated with a three base pair deletion in the coding region of the ferroportin gene (SLC11A3). *Br J Haematol.* 2002;119(2):539-546.
4. Roetto A, Merryweather-Clarke AT, Daraio F, et al. A valine deletion of ferroportin 1: a common mutation in hemochromastosis type 4. *Blood.* 2002;100(2):733-734.
5. Lok CY, Merryweather-Clarke AT, Viprakasit V, et al. Iron overload in the Asian community. *Blood.* 2009;114(1):20-25.
6. Lee PL, Gelbart T, West C, Barton JC. SLC40A1 c.1402G-->a results in aberrant splicing, ferroportin truncation after glycine 330, and an autosomal dominant hemochromatosis phenotype. *Acta Haematol.* 2007;118(4):237-241.
7. Pelucchi S, Mariani R, Salvioni A, et al. Novel mutations of the ferroportin gene (SLC40A1): analysis of 56 consecutive patients with unexplained iron overload. *Clin Genet.* 2008;73(2):171-178.
8. Lim FL, Dooley JS, Roques AW, Grellier L, Dhillon AP, Walker AP. Hepatic iron concentration, fibrosis and response to venesection associated with the A77D and V162del “loss of function” mutations in ferroportin disease. *Blood Cells Mol Dis.* 2008;40(3):328-333.
9. Pietrangelo A, Montosi G, Totaro A, et al. Hereditary hemochromatosis in adults without pathogenic mutations in the hemochromatosis gene. *N Engl J Med.* 1999;341(10):725-732.
10. Montosi G, Donovan A, Totaro A, et al. Autosomal-dominant hemochromatosis is associated with a mutation in the ferroportin (SLC11A3) gene. *J Clin Invest.* 2001;108(4):619-623.
11. De Domenico I, McVey Ward D, Nemeth E, et al. Molecular and clinical correlates in iron overload associated with mutations in ferroportin. *Haematologica.* 2006;91(8):1092-1095.
12. Liu W, Shimomura S, Imanishi H, et al. Hemochromatosis with mutation of the ferroportin 1 (IREG1) gene. *Intern Med.* 2005;44(4):285-289.
13. Zoller H, McFarlane I, Theurl I, et al. Primary iron overload with inappropriate hepcidin expression in V162del ferroportin disease. *Hepatology.* 2005;42(2):466-472.
14. Papanikolaou G, Tzilianos M, Christakis JI, et al. Hepcidin in iron overload disorders. *Blood.* 2005;105(10):4103-4105.
15. Del-Castillo-Rueda A, Moreno-Carralero MI, Cuadrado-Grande N, et al. Mutations in the HFE, TFR2, and SLC40A1 genes in patients with hemochromatosis. *Gene.* 2012;508(1):15-20.
16. Caetano G, Relvas L, Bento C, Silveira MP, Ribeiro L. Atypical iron deficiency anemia - association of two new mutations in ferroportin and TMPRSS6 genes. *Intern Biolron Soc Meeting.* 2009:7-11.
17. Lee PL, Gaasterland T, Barton JC. Mild iron overload in an African American man with SLC40A1 D270V. *Acta Haematol.* 2012;128(1):28-32.
18. Jouanolle AM, Douabin-Gicquel V, Halimi C, et al. Novel mutation in ferroportin 1 gene is associated with autosomal dominant iron overload. *J Hepatol.* 2003;39(2):286-289.
19. Letocart E, Le Gac G, Majore S, et al. A novel missense mutation in SLC40A1 results in resistance to hepcidin and confirms the

existence of two ferroportin-associated iron overload diseases. *Br J Haematol.* 2009;147(3):379-385.
20. Mougiou A, Pietrangelo A, Caleffi A, Kourakli A, Karakantza M, Zoumbos N. G80S-linked ferroportin disease: the first clinical description in a Greek family. *Blood Cells Mol Dis.* 2008;41(1):138-139.
21. Cunat S, Giansily-Blaizot M, Bismuth M, et al. Global sequencing approach for characterizing the molecular background of hereditary iron disorders. *Clin Chem.* 2007;53(12):2060-2069.
22. Saja K, Bignell P, Robson K, Provan D. A novel missense mutation c.470 A>C (p.D157A) in the SLC40A1 gene as a cause of ferroportin disease in a family with hyperferritinaemia. Br J *Haematol.* 2010;149(6):914-916.
23. Wallace DF, Browett P, Wong P, Kua H, Ameratunga R, Subramaniam VN. Identification of ferroportin disease in the Indian subcontinent. *Gut*. 2005;54(4):567-568.
24. Devalia V, Carter K, Walker AP, et al. Autosomal dominant reticuloendothelial iron overload associated with a 3-base pair deletion in the ferroportin 1 gene (SLC11A3). *Blood.* 2002;100(2):695-697.
25. Morris TJ, Litvinova MM, Ralston D, Mattman A, Holmes D, Lockitch G. A novel ferroportin mutation in a Canadian family with autosomal dominant hemochromatosis. *Blood. Cells Mol Dis* 2005;35(3):309-314.
26. Girelli D, De Domenico I, Bozzini C, et al. Clinical, pathological, and molecular correlates in ferroportin disease: a study of two novel mutations. *J Hepatol.* 2008;49(4):664-671.
27. Wallace DF, Dixon JL, Ramm GA, Anderson GJ, Powell LW, Subramaniam VN. A novel mutation in ferroportin implicated in iron overload. *J Hepatol.* 2007;46(5):921-926.
28. Griffiths WJ, Mayr R, McFarlane I, et al. Clinical presentation and molecular pathophysiology of autosomal dominant hemochromatosis caused by a novel ferroportin mutation. *Hepatology.* 2010;51(3):788-795.
29. Wallace DF, Pedersen P, Dixon JL, et al. Novel mutation in ferroportin1 is associated with autosomal dominant hemochromatosis. *Blood.* 2002;100(2):692-694.
30. Rivard SR, Lanzara C, Grimard D, et al. Autosomal dominant reticuloendothelial iron overload (HFE type 4) due to a new missense mutation in the FERROPORTIN 1 gene (SLC11A3) in a large French-Canadian family. *Haematologica.* 2003;88(7):824-826.
31. Bach V, Remacha A, Altes A, Barcelo MJ, Molina MA, Baiget M. Autosomal dominant hereditary hemochromatosis associated with two novel Ferroportin 1 mutations in Spain. *Blood Cells Mol Dis.* 2006;36(1):41-45.
32. Njajou OT, Vaessen N, Joosse M, et al. A mutation in SLC11A3 is associated with autosomal dominant hemochromatosis. *Nat Genet.* 2001;28(3):213-214.
33. Njajou OT, de Jong G, Berghuis B, et al. Dominant hemochromatosis due to N144H mutation of SLC11A3: clinical and biological characteristics. *Blood Cells Mol Dis*. 2002;29(3):439-443.
34. Rosmorduc O, Wendum D, Arrive L, et al. Phenotypic expression of ferroportin disease in a family with the N144H mutation. *Gastroenterol Clin Biol.* 2008;32(3):321-327.
35. Wallace DF, Clark RM, Harley HA, Subramaniam VN. Autosomal dominant iron overload due to a novel mutation of ferroportin1 associated with parenchymal iron loading and cirrhosis. *J Hepatol.* 2004;40(4):710-713.
36. Speletas M, Kioumi A, Loules G, et al. Analysis of SLC40A1 gene at the mRNA level reveals rapidly the causative mutations in patients with hereditary hemochromatosis type IV. *Blood Cells Mol Dis.* 2008;40(3):353-359.
37. Cremonesi L, Forni GL, Soriani N, et al. Genetic and clinical heterogeneity of ferroportin disease. *Br J Haematol.* 2005;131(5):663-670.
38. Del-Castillo-Rueda A, Moreno-Carralero MI, Alvarez-Sala-Walther LA, et al. Two novel mutations in the SLC40A1 and HFE genes

implicated in iron overload in a Spanish man. *Eur J Haematol.* 2011;86(3):260-264.

39. Sham RL, Phatak PD, Nemeth E, Ganz T. Hereditary hemochromatosis due to resistance to hepcidin: high hepcidin concentrations in a family with C326S ferroportin mutation. *Blood.* 2009;114(2):493-494.
40. Sham RL, Phatak PD, West C, Lee P, Andrews C, Beutler E. Autosomal dominant hereditary hemochromatosis associated with a novel ferroportin mutation and unique clinical features. *Blood Cells Mol Dis.* 2005;34(2):157-161.
41. Pietrangelo A, Corradini E, Ferrara F, et al. Magnetic resonance imaging to identify classic and nonclassic forms of ferroportin disease. *Blood Cells Mol Dis.* 2006;37(3):192-196.
42. Blanc B, Finch CA, Hallberg L, et al. Nutritional anaemias. Report of a WHO Scientific Group. *WHO Tech Rep Ser.* 1968;405:1-40.

Supplement 4: Starting Questions

Subjects	Starting questions
Pathophysiology and epidemiology	What is the cause of the anemia? What is the prevalence of the anemia?
Clinical presentation and diagnosis	What is the clinical presentation of the anemia? How should the anemia be diagnosed? How should the diagnostic parameters be interpreted?
Treatment	How should the patient be treated?
Family screening	Which relatives need to be screened for the relevant phenotype and/or genotype?
Distribution of knowledge*	Where can the patients and professionals find more information? What information should be provided to the family?

*, not included in this article since largely specific for the Dutch situation.

Chapter 4

Iron Refractory Iron Deficiency Anemia (IRIDA): a Heterogeneous Disease that is not always Iron Refractory

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ABSTRACT

TMPRSS6 variants that affect protein function result in impaired matriptase 2 function and consequently uninhibited hepcidin production, leading to Iron Refractory Iron Deficiency Anemia (IRIDA). This disease is characterized by microcytic, hypochromic anemia and serum hepcidin values that are inappropriately high for body iron levels. Much is still unknown about its pathophysiology, genotype-phenotype correlation and optimal clinical management. We describe 14 different *TMPRSS6* variants, of which nine are novel, in 21 phenotypically affected IRIDA patients from 20 families living in the Netherlands. Sixteen out of 21 patients were female. In seven out of 21 cases DNA sequencing and Multiplex Ligation dependent Probe Amplification demonstrated only heterozygous *TMPRSS6* variants. Age at presentation, disease severity and response to iron supplementation were highly variable, even for patients and relatives with similar *TMPRSS6* genotypes. Mono-allelic IRIDA patients had a milder phenotype with respect to hemoglobin and MCV and presented significantly later in life with anemia than bi-allelic patients. Transferrin saturation (TSAT)/hepcidin ratios were lower in IRIDA probands than in healthy relatives. Most patients required parenteral iron. Genotype alone was not predictive for the response to oral iron.

We conclude that IRIDA is a genotypically and phenotypically heterogeneous disease. The high proportion of female patients and the discrepancy between phenotypes of probands and relatives with the same genotype, suggest a complex interplay between genetic and acquired factors in the pathogenesis of IRIDA. In the absence of inflammation, the TSAT/hepcidin ratio is a promising diagnostic tool, even after iron supplementation has been given.

Key words
iron, *TMPRSS6*, hepcidin, IRIDA

INTRODUCTION

Matriptase 2, encoded by *TMPRSS6*, plays an essential role in down-regulating hepcidin, the key regulator of iron homeostasis. Pathogenic *TMPRSS6* mutations result in uninhibited hepcidin production, causing Iron Refractory Iron Deficiency Anemia (IRIDA), a disease characterized by a microcytic, hypochromic anemia due to serum hepcidin values that are inappropriately high for the body iron levels. [1-6]

IRIDA patients typically present in childhood with microcytic anemia not responding to oral iron, in combination with remarkably low transferrin saturation (TSAT), which tends to become less severe with increasing age.[7] Serum ferritin levels are generally within the low-normal range, and increase following intravenous iron treatment. [5] Only a few patients with elevated ferritin concentrations have been described before intravenous iron treatment had been given. [8] To date, 69 different *TMPRSS6* defects have been identified in 65 IRIDA families with 94 patients of different ethnic origin. [4,5,9,10]

At the population level, Genome Wide Association Studies (GWAS) show that *TMPRSS6* is polymorphic with a relatively large amount of polymorphisms of which the non-synonymous c.2207C>T (p.Ala736Val) is associated with a significant decrease of the concentrations of iron, hemoglobin (Hb), hematocrit (Ht), Mean Corpuscular Volume (MCV), Mean Cellular Hemoglobin (MCH) and red blood cells. [11,12] These findings are corroborated by functional studies, which show that the 736Ala variant inhibits hepcidin production more efficiently. [13]

The increasing number of IRIDA cases that are being reported, generate more knowledge on the disease, but many questions remain concerning the mode of inheritance, the genotype-phenotype correlation, the diagnostic workup and the optimal treatment.

Since usually bi-allelic *TMPRSS6* variants are found in IRIDA patients, the disease is considered as recessive. However, anecdotal data are available of phenotypically affected IRIDA patients in which only a heterozygous *TMPRSS6* variant was found. [3,14-17]

Concerning the genotype-phenotype relationship, there is a tendency towards lower Hb, MCV and TSAT in patients with two nonsense mutations. [10] Much is unknown on the influence of high frequency *TMPRSS6* variants, other still unrecognized genes and environmental factors in the phenotypic expression of the disease. Furthermore, diagnosing IRIDA is challenging because of the highly variable phenotype[4,5,10] and the unclear genotype –phenotype relation. The optimal oral and/or intravenous iron treatment regimen has not been established yet.

In this paper we describe the characteristics of 21 IRIDA patients and relatives in the Netherlands, the relation between genotype and phenotype in terms of age of presentation, severity of anemia and response to iron supplementation. Our observations add to the understanding of the clinical and genetic heterogeneity of IRIDA. They moreover suggest the TSAT/hepcidin ratio as a promising tool in the diagnosis of IRIDA.

METHODS

Patients and relatives

We included 21 IRIDA patients and their relatives. All IRIDA patients were inhabitants of the Netherlands and consecutively diagnosed between 2010 and 2015.

IRIDA probands were defined as patients with both an "IRIDA phenotype" (detected after clinical presentation, microcytic anemia, TSAT < 10%, in the absence of inflammation, Hb and MCV not or partially responsive to oral iron) and an "IRIDA genotype" (a mono- or bi-allelic *TMPRSS6* variant that – probably, possibly – affects function, called "defect" hereafter). Iron deficiency anemia in these patients could not be explained (exclusively) by increased physiological needs (growth, menstrual blood loss in premenopausal women), by gastrointestinal disease like *Helicobacter Pylori* infection, celiac disease of atrophic gastritis [18] or by gynecological problems. Responsiveness to oral iron treatment was defined as a hemoglobin (Hb) increment of 2 g/dL after three weeks of oral iron supplementation. [19] A positive family history for unexplained iron deficiency anemia and anemia presenting in childhood were considered as suspect for IRIDA.

Laboratory measurements

Hb, red blood cell indices and serum iron parameters were measured in accredited Dutch hospital laboratories. Serum hepcidin measurements were performed by a combination of weak cation exchange and time-of-flight mass spectrometry (WCX-TOF MS). [20,21]The median reference level of serum hepcidin-25 is 4.5 nM for men (p 2.5-p 97.5= < 0.5-14.7 nM), 2.0 nM for premenopausal women (p 2.5-p 97.5=0.1-12.3 nM) and 4.9 nM for postmenopausal women (p 2.5-p 97.5=0.2-15.6 nM). The reference level of serum hepcidin-25 for children aged 0.5 – 3 years is 3.6 nM (p 2.5-p.97.5=0.94-12.2). [22,23] For children > 3 years no reference ranges are available until now, so for this group we recommend using those of premenopausal women. The median reference level of serum TSAT/hepcidin-25 ratio is 7.3 %/nM for men (p 2.5-p 97.5=1.7-256.3 %/nM), 13.9 %/nM for premenopausal women (p 2.5-p 97.5=2-330 %/nM) and 5.7 %/nM for postmenopausal women (p 2.5-p97.5=1.5-73.4 %/nM). [23]

Gene analysis

Genotyping was performed by PCR, DNA Sanger sequencing (until March 2014) and Ion Torrent sequencing (after March 2014) of the coding part of *TMPRSS6* (**Supplemental Table 1**). Comparative Genomic Hybridization as performed in patient 1 and her relatives, for whom PCR products of the *TMPRSS6* gene could not be obtained, using the Affymetrix CytoScan HD array platform according to the manufacturer's protocol (Affymetrix, Santa Clara, CA, USA). Multiplex Ligation-dependent Probe Amplification (MLPA) analysis was performed in patients who had an IRIDA phenotype and a mono-allelic pathogenic *TMPRSS6* variant to exclude large deletions and/or duplications in the 'healthy' allele, and also in patient 1 (**Supplemental Table 2**, The Netherlands, www.mlpa.com). [24]

The pathogenicity of genetic variants was assessed by review of the literature on previous reported cases and functional studies, association of the variant with the phenotype within a family and bio-informatic tools (SIFT, Align GVGD, Polyphen, SpliceSiteFinder-like, MaxEntScan, NNSplice, GeneSplicer and Human Splicing Find, all as part of Alamut software, Alamut). [25] Alamut was used to assess pathogenicity in case of not previously reported *TMPRSS6* variants. Haplotype analysis was performed in a search for a founder effect in families with identical pathogenic *TMPRSS6* defects by using 10 different intra-gene exon High Frequency Variants (HFV's) detected by Sanger DNA sequencing and 3 Short Tandem Repeats surrounding *TMPRSS6*. We also performed parental haplotype analysis in two sibling pairs with identical heterozygous *TMPRSS6* variants but different phenotypes, using 7 different intra-gene HFV's also detected by Sanger DNA sequencing.

RESULTS

Patient characteristics

We report 21 IRIDA patients from 20 unrelated families. IRIDA was diagnosed in case of the combination of a relevant phenotype and genotype.

After exclusion of other conditions as the (exclusive) explanation, a patient with a current or past microcytic hypochromic anemia, in absence of inflammation, not or partially responsive to oral iron with a low TSAT (< 10 %) was considered as a suspected case, after which serum hepcidin analysis was performed in 20 out of 22 patients and genotyping of *TMPRSS6* in all patients.

In our diagnostic work-up, very low to elevated ferritin levels, either before or after treatment with iron supplementation could fit the diagnosis.

Nine female and five male patients were diagnosed with a bi-allelic *TMPRSS6* defect (**Table 1 A**). In patient 1, comparative genomic hybridization was performed since no signal of *TMPRSS6* was obtained by Sanger sequencing. A homozygous deletion of 118 kb was detected in 22q12.3, with the distal breaking point in intron 2 of the *TMPRSS6* gene (deletion breakpoints at Mb positions 37,374,751 and 37,492,851), effectively knocking out exons 3 – 18 (UCSC Genome Browser on Human Feb 2009 GRCh 37/hg 19 assembly; [26] **Supplemental Figure 1**). (In the other seven patients (all female) only a mono-allelic *TMPRSS6* defect was found (**Table 1 B**). In these patients MLPA showed no large deletions and/or duplications in the 2nd allele.

For the bi-allelic affected patients, age at the time of evaluation for IRIDA ranged from 1 to 40 years; 10 out of 14 patients were in their first or second decade. Evaluation for IRIDA of the 7 heterozygous patients all occurred in the third or fourth decade.

Table 1. Characteristics of IRIDA patients

Table 1 A. Characteristics of 14 homozygous or compound heterozygous patients

Patient characteristics					Laboratory characteristics				
ID	Sex	Age years			Hb[a] g/dL	MCV[a] fL	Ferritin[a] µg/L	TSAT[a] (%)	TSAT/ Hepcidin[b] %/nM
		I[d]	II[e]	III[f]					
1	F	3	9	14	7.9	53	29	4.0	0.27
2[g]	F	2	7	12	6.8	54	53	5.2	0.48
3	F	4	21	24	7.1	65	44	np	0.59
4[h]	M	child	31	33	10	63	np	2.8	np
5	F	5	18	23	8.1	61	16	2.7	1.0
6	F	18	34	36	7.6	61	18	3.0	np
7	M	0	6	9	5.5	51	np	4.0	0.53
8	F	33	38	41	np	np	np	np	0.43
9	F	27	32	35	7.4	63	66	5.0	0.51
10	M	10	12	12	8.4	53	43	2.0	0.13
11[i]	F	0	1	2	9.8	66	16	4.0	0.78
12	M	2	3	8	6.4	52	23	4.0	0.53
13	M	8	8	11	7.9	56	9.0	1.9	0.29

Genetic characteristics	Treatment				Remarks
TMPRSS6 variant that (probably, possibly) affects function	Oral iron[c]	Iv iron[c]	Other	Duration of treatment	
Allele 1;Allele 2 deletion; deletion					
c.del of 118 kb in 22q12.3[j] c.del of 118 kb in 22q12.3 p.nonsense;p.nonsense deletion; frameshift	+	+	-	Iron iv age 9-14 yrs	
c.del promotor, exon 1-3;c.497delT p.nonsense;p.Leu166Argfs*37 frameshift; frameshift	+	-	BloodTx	BloodTx age 2-8 yrs	
c.497delT; c.497delT p.Leu166Argfs*37;p.Leu166Argfs*37	+	+		Iron iv age 4-21 yrs	
c.497delT; c.497delT p.Leu166Argfs*37;p.Leu166Argfs*37	+	+	-	Iron iv as a child	
c.1904_1905dup; c.1904_1905dup p.Lys636Alafs*17;p.Lys636Alafs*17	+	+	-	Iron iv at age 19 yrs	
c.1904_1905dup; c.1904_1905dup p.Lys636Alafs*17;p.Lys636Alafs*17 frameshift; nonsense	+	+	BloodTx	Iron iv from age 34 yrs	Pregnant at presentation
c.497delT; c.1832G>A p.Leu166Argfs*37; p.Trp611X frameshift; missense	+	-	-	Iron po from age <6 yrs	
c.497delT; c.1228T>C p. Leu166Argfs*37; p.Cys410Arg	+	-	Iron im	Iron im age 33-38 yrs	
c.497delT; c.1324G>A p.Leu166Argfs*37; p.Gly442Arg	+	+	-	Iron iv age 27-28 yrs	
c.497delT; c.1324G>A p.Leu166Argfs*37; p.Gly442Arg	+	+	-	Iron iv age 10-12 yrs	Diabetes Mellitus
c.497delT; c.2383G>A p.Leu166Argfs*37; p.Val795Ile	+	-	-	Iron po from age 1 yr	
c.1904_1905dup ;c.1229G>C p.Lys636Alafs*17; p.Cys410Ser missense; missense	+	+	-	Iron iv from age 3 yrs	
c.521T>C c.1228T>C p.(Leu174Pro); p.(Cys410Arg)	+	-	Vitamin C	Iron po + vitamin C from age 8 yrs	

Table 1. Continued

Table 1 A. Characteristics of 14 homozygous or compound heterozygous patients

Patient characteristics					Laboratory characteristics				
ID	Sex	Age years			Hb[a] g/dL	MCV[a] fL	Ferritin[a] µg/L	TSAT[a] (%)	TSAT/ Hepcidin[b] %/nM
14	F	child	44	47	9.3	66	34	3.0	np
Median (range)	M:F 5:9	4.5 (0-33)			7.9 (5.5-10)	61 (51-66)	29 (9-66)	3.5 (1.9-5.2)	0.51 (0.13-1.0)

Table 1 B. Characteristics of 7 heterozygous IRIDA patients

Patient characteristics					Laboratory characteristics				
ID	Sex	Age years			Hb g/dL	MCV fL	Ferritin µg/L	TSAT (%)	TSAT/ Hepcidin %/nM
		I[d]	II[e]	III[f]					
15[g]	F	10	40	42	5.4	76	np	np	2.7
16	F	47	48	53	12	79	130	5.0	0.70
17	F	31	32	33	10	76	32	4.0	0.30
18	F	10	47	48	12	67	np	np	1.1
19	F	39	41	46	10	78	53	m	1.0
20	F	43	43	44	7.9	62	10	2.7	np
21	F	31	34	40	9.7	68	22	4.0	2.6
Median (range)	M:F	31 (10-47)			10 (5.4-12)	76 (62-79)	32 (10-130)	4.0 (2.7-6.0)	1.1 (0.33-2.7)

Genetic characteristics	Treatment				Remarks
TMPRSS6 variant that (probably, possibly) affects function	Oral iron[c]	Iv iron[c]	Other	Duration of treatment	
c.2383G>A; c.2105G>T p.Val795Ile; p.Cys702Phe	+	+	BloodTx (child)	Iron iv age 36-41 yrs	Hypothyroidism

Genetic characteristics	Treatment				Remarks
TMPRSS6 variant that (probably, possibly) affects function	Oral iron[c]	Iv iron[c]	Other	Duration of treatment	
Allele 1;Allele 2 deletion; Wt[k]					
c.del promotor, exon 1-3; Wt p.nonsense; Wt frameshift; Wt[k]	+	+	-	Start iv iron unknown Iron iv till age of 35 yrs	
c.497delT; Wt p.Leu166Argfs*37; Wt	+	+	-	Iron iv from age 49 yrs	
c.497delT; Wt p.Leu166Argfs*37; Wt splicing; Wt[k]	+	+	Vitamin C	Iron iv from age 31 yrs	
c.230-6G>A[l]; Wt splicing; Wt	+	+	-	Iron iv when a child No treatment anymore	
c.863+1G>T[m]; Wt splicing; Wt missense; Wt[k]	+	-	-	Iron po from age 31 yrs	
c1654G>A; Wt p.(Asp552Asn); Wt	+	+	BloodTx	Iron iv from age 40 yrs	
c.2105G>T; Wt p.Cys702Phe; Wt	+	-	-	Iron po from age 31 yrs	

Table 1. Continued

Patients are ordered according to type of variant. Family studies have confirmed that variants of all compound heterozygous affected patients are located on 2 different alleles.
[a] Hb, MCV, ferritin and TSAT of the proband at time of presentation with anemia;
[b] Hepcidin, TSAT/hepcidin ratio at time of (genotypic) diagnosis of IRIDA, in absence of inflammation except for patient 14 and 20 with CRP levels of 24 and 45 mg/L respectively.
The median reference level of serum hepcidin-25 is 4.5 nM for men (p 2.5-p 97.5= < 0.5-14.7 nM), 2.0 nM for premenopausal women (p 2.5-p 97.5=0.1-12.3 nM), and 4.9 nM for postmenopausal women (p 2.5-p 97.5=0.2-15.6 nM). The reference level of serum hepcidin-25 for children aged 0.5 – 3 yrs is 3.6 nM (p 2.5-p.97.5=0.94-12.2). For children > 3 yrs no reference ranges are available at present, so we recommend for them using those of premenopausal women. The median reference level of serum TSAT/hepcidin-25 ratio is 7.3 %/nM for men (p 2.5-p 97.5=1.7-256.3 %/nM), 13.9 %/nM for premenopausal women (p 2.5-p 97.5=2.0-
330 %/nM) and 5.7 %/nM for postmenopausal women (p 2.5-p97.5=1.5-73.4 %/nM) (www.hepcidinanalysis.com) [23]
[c] +: has been given, -: has not been given
[d] I, age at presentation with anemia;
[e] II, age at assessing IRIDA and determination of hepcidin and TSAT/hepcidin ratio;
[f] III, age in 2015;
[g] Patient 2 is the daughter of patient 15. However, since both patients presented with anemia at childhood, both were considered as independent probands;

All 14 patients diagnosed with a bi-allelic *TMPRSS6* defect had been treated with oral iron at referral to our clinic. Of these patients two were responsive to oral iron with a slow, sluggish increase of Hb and MCV (ID 7, ID 11), while patient 13 responded to a combination of oral iron and vitamin C with an increase of Hb, MCV and TSAT. The other 11 bi-allelic patients were unresponsive to oral iron and partially responsive to parenteral iron or blood transfusions in terms of a moderate increase of Hb and MCV, while TSAT remained below 10% in 9 out of 11 patients (ID 8: TSAT 13%, ID 14: TSAT 15% after iron treatment). (Supplemental Table 1 A and Supplemental Table 3).

All seven patients diagnosed with a heterozygous *TMPRSS6* defect had been treated with oral iron at referral to our clinic, of which two were responsive (ID 19 and 21) with a slow, sluggish increase of Hb, MCV and TSAT. The other 5 patients received intravenous iron supplementation, which resulted in a moderate increase of Hb and MCV. TSAT remained below reference values, except in patient 15. One heterozygous patient (ID 20) received a blood transfusion. Ferritin increased above concentrations of 500 µg/L in three out of 22 patients (patient 8, 14 and 16).

[h] Patient 4 was diagnosed with anemia refractory to oral iron as a child for which he temporarily received intravenous iron. IRIDA was diagnosed after screening for this disorder because of pregnancy of his wife. No data are available on Hb and iron parameters at time of presentation of anemia;
[i] Hb and iron parameters of patient 11 at referral, after oral iron supplementation had been given;
[j] Deletion 118 kb in intron 2, knocking out exon 3-18 of *TMPRSS6* gene. Also other genes were deleted; RefSeq genes *TEX33, MPST, TST* and *KCTD17*
[k] Multiplex Ligation dependent Probe Amplification (MLPA) confirmed heterozygosity;
[l] This substitution is predicted to introduce a new and more efficient acceptor splice site 4 bases downstream from the original acceptor splice site in intron 2 leading to a frameshift in the open reading frame. Not proven on RNA or protein level. (http://www.interactive-biosoftware.com/doc/alamut-visual/2.7/splicing.html), accessed on July 14 2016.
[m] This substitution is located in the donor splice site of intron 7. The consequence of this change is not predictable, but a skip of exon 7 is very likely resulting in the loss of a classical splice site. Not proven on mRNA or protein level (http://www.interactive-biosoftware.com/doc/alamut-visual/2.7/splicing.html), accessed on July 14 2016.
Abbreviations: Hb denotes hemoglobin; MCV, mean corpuscular volume; TSAT, transferrin saturation; F, female; M, male; iv, intravenous; po, per os; im, intramuscular; BloodTx, blood transfusion; np, not provided; Wt, Wild-type

In patient 8 (ferritin 924 µg/L) MRI of the liver showed only moderate signs of iron overload (60 µmol (or 3.35 mg) Fe dry weight tissue). Since no genotyping of the known hemochromatosis genes was performed for our patients, we cannot exclude ferroportin disease due to a variant of SLC40A1 for this woman.

Dose of intravenous iron, treatment regimen and duration of treatment was highly variable in our small population, with a tendency to a reduction of intravenous iron requirements with increasing age. None of our patients was treated with erythropoietin (EPO). We conclude that only a minority of both bi-allelic and mono-allelic affected patients was responsive to oral iron and that most patients needed parenteral iron to increase their Hb levels.

Genotypic and phenotypic family screening was performed in 27 relatives of 12 out of 21 probands; in 20 out of these 27 relatives a TSAT/hepcidin ratio was available. Four wild-type relatives and 20 relatives with a mono-allelic *TMPRSS6* defect had no IRIDA phenotype. Two relatives with a bi-allelic *TMPRSS6* defect (brother of patient 9 and sister of patient 11) had no complaints since their Hb was normal (MCV and TSAT

were decreased). Only the mother of patient 17, who had the same heterozygous genotype as her daughter, had an IRIDA phenotype. She had a history of unexplained iron deficiency (**Supplemental Table 4**).

Molecular characterization

We observed 14 different defects in 21 patients (**Table 1, Supplemental Table 5**). Five out of 14 are previously described *TMPRSS6* defects: three missense variants, [3,15,27] and two frameshift variants. [3,15] Of these variants, the frameshift alteration c.497delT (p.Leu166Argfs*37) was the most prevalent defect in our population and was found in eight out of 14 bi-allelic patients and in two out of eight mono-allelic patients (12 out of 36 affected alleles = 33%) (**Table 1**). All patients with the c.497delT (p.Leu166Argfs*37) were of Dutch origin, whereas all three patients with the c.1904_1905dup (p.Lys636Alafs*17) were of Turkish origin (**Supplemental Table 6**).

For the non-synonymous missense variant c.2383G>A (p.Val795Ile) a functional test had previously been performed. This showed physiological inhibition of *HAMP1* expression (*in vitro* studies by site directed mutagenesis into murine *TMPRSS6*, [15] despite the IRIDA phenotype of the patient who harbored this variant. However, although considered as the most reliable method to predict the pathogenicity of an allele variant, the results of cell based functional testing by site directed mutagenesis should be interpreted with caution. The technique has been described as error prone and is likely to be limited in its extrapolation because the test environment may not fully reflect the *in vivo* situation. [15,28] Allele frequency of the c.2383G>A (p.Val795Ile) missense variant is < 1% (**Supplemental Table 5**), which argues against a neutral or beneficial effect of this nucleotides change. Because of the not fully reliable functional tests, the low allele frequency and the *in-silico* analysis that point towards pathogenicity, we considered this missense variant as pathogenic. [29]

Nine novel pathogenic *TMPRSS6* defects were demonstrated in our case series; two large deletions, four missense mutations, one nonsense mutation and two splice defects (**Table 2**).

Pathogenicity of the new mutations was predicted with *in-silico* software. In all seven heterozygous IRIDA patients, no deletions and/or duplications in the *TMPRSS6* gene of the 'healthy' allele were found with MLPA.

Haplotype analysis exploiting 10 intragenic HFV's with a Minor Allele Frequency in Caucasians of > 0.005 and three Short Tandem Repeats surrounding *TMPRSS6* was performed in patients with identical defects. The results were consistent with a common ancestor of the Dutch patients 3 and 4, who all carried the c.497delT defect. For the other patients with this defect the haplotype analysis might indicate relationship but was less clear. The haplotype analysis of patients with the c.1904_1905dup defect, found in three patients (5, 6 and 12) from Turkish origin, was consistent with a common ancestor of the patients 5 and 6. Patients 8 and 13 who carried the c.12282T>C defect might be related but haplotype analysis was not clearly indicative. (**Supplemental Table 7**).

We conclude that i) in the Netherlands certain defects are more prevalent than others, ii) patients who share a country of origin are likely to share specific defects and iii) common ancestry of patients with identical defects is not always obvious.

Genotype-phenotype relation

The IRIDA phenotype was highly variable in our population (**Table 1**). To improve insight in genotype-phenotype relation we categorized the probands by six different groups based on the nature of the defect (**Table 1**). Patients with large deletions either bi-allelic (ID 1) or in combination with a frameshift defect (ID 2) had the most severe IRIDA phenotype with respect to age of presentation, severity of anemia, microcytosis and duration of treatment with intravenous iron. However, patient 3 and 4, who shared the same homozygous frameshift defect, had very different phenotypes; patient 4 was only temporarily treated with intravenous iron as a child while patient 3 received intravenous iron till the age of 21 years. Only two of the 14 bi-allelic patients responded to oral iron supplementation, i.e. patient 7 and 11. Interestingly, these patients had severe genotypes. Patient 13 with two missense defects was responsive to a combination of oral iron with vitamin C.

Seven patients were diagnosed with IRIDA due to a variety of heterozygous *TMPRSS6* defects (**Table 1 B**). The large deletion in patient 15 resulted in a more severe genotype than the frameshift, aberrant splicing and missense defects in the other heterozygous patients.

Table 2. Characteristics of novel *TMPRSS6* variants

***TMPRSS6* defect**[a]		
Gene	Protein	Type of variant
c.del 118 kb intron 2- exon 3-18	Absent	Deletion
c.del promotor, exon 1-3	Absent	Deletion
c.230-6G>A	Aberrant	Splicing defect
c.[52]1T>C	p.Leu174Pro	Substitution
c.863+1G>T	del exon 7	Splicing defect
c.1228T>C	p.Cys410Arg	Substitution
c.1229G>C	p.Cys410Ser	Substitution
c.1654G>A	p.Asp552Asn	Substitution
c.1832G>A	p.Trp611X	Stop codon

[a] All novel *TMRPSS6* variants, except the defect c.del promotor, exon 1-3 in patient 15, were found in combination with a *TMPRSS6* variant that (probably, possibly) affects function, on the other allele. Therefore, the contribution of the novel variant to the clinical phenotype of the patient remains unclear.

[b] This substitution is predicted to introduce a new and more efficient acceptor splice site 4 bases downstream from the original acceptor splice site in intron 2 leading to a frameshift in the open reading frame. Not proven on RNA or protein level (http://www.interactive-biosoftware.com/doc/alamut-visual/2.7/splicing.html, accessed on July 14 2016).

[c] **Align GVGD,** web based *in silico* prediction software program that combines the biophysical characteristics of amino acids and protein multiple sequence alignments to predict where missense substitutions in genes of interest fall in a spectrum from enriched deleterious to enriched neutral. A-GVGD scores amino acid substitutions on a 7-scale scoring system, from C0 to C65. An amino acid substitution with a C0 score is considered to be neutral, amino acids with C15 and C25 scores are considered intermediate, as changes to protein structure or function are uncertain, and C35 scores or higher are considered as likely deleterious.[52]

	In silico findings	Conclusion on pathogenicity	Patient ID
TMPRSS6 domain			
Absent protein	np	Pathogenic	1
Absent protein	np	Pathogenic	2, 15
TM domain	Splice site[b]	Most probably pathogenic	18
SEA domain	Align GVGD[c]: C0 Polyphen[d]: 0.57 SIFT[e]: 0.04	Possibly pathogenic	13
CUB domain	Skip of exon 7[f]	Most probably pathogenic	19
CUB domain	Align GVGD[c]: C0 Polyphen[d]: 0.45 SIFT[e]: 0.00	Possibly pathogenic	13
CUB2 domain	Align GVGD[c]: C0 Polyphen[d]: 0.01 SIFT[e]: 0.00	Possibly pathogenic	12
LDLR domain	Align GVGD[c]: CO Polyphen[d]: 1.0 SIFT[e]: 0.00	Possibly pathogenic	20
Serine Protease domain	np	Most probably Pathogenic[g]	7

[d] **PolyPhen-2** (Polymorphism Phenotyping v2 HumVar) is a tool which predicts possible impact of an amino acid substitution on the structure and function of a human protein using straightforward physical and comparative considerations. PolyPhen scores range from $0 \leq 1 \leq X$, outcome scores of 0.00–0.15 are classified as benign, 0.15–1.0 as possibly damaging, 0.85-1.0 as more confidently predicted to be damaging. [53]

[e] **The SIFT** algorithm combines sequence homology and physical properties of amino acid substitutions to analyze whether or not amino acid substitutions are tolerated, in light of the predicted effect on the protein structure. SIFT score ranges from 0 to 1. The amino acid substitution is predicted damaging if the score is < 0.05 and tolerated if the score is > 0.5. [54]

[f] This substitution is located in the donor splice site of intron 7. The consequence of this change is not predictable, but a skip of exon 7 is very likely as *in silico* analysis resulting in the loss of a classical splice site. Not proven on mRNA or protein level (http://www.interactive-biosoftware.com/doc/alamut-visual/2.7/splicing.html, accessed on July 14 2016).

[g] Nonsense substitution in exon 15. The reading frame is interrupted by a stop codon. The mRNA produced might be targeted to nonsense-mediated decay (NMD). No formal proof.

Abbreviations: np denotes not provided

Overall, i) patients with bi-allelic, severe gene defects had the most severe phenotype, but exceptions occurred, and genotype alone was not fully predictive for the response to oral iron, ii) mono-allelic patients presented later in life with anemia (median 31 years, range 10 – 47 years) than bi-allelic patients (median 4.5 years, 0-33 years, Mann-Whitney p=0.03), were more likely to be females and had a milder phenotype with respect to severity of anemia, microcytosis and duration of intravenous iron treatment.

Since patients with identical defects were scarce, our patient series do not allow to decipher a clear influence of the presence of the high frequency *TMPRSS6* variant c.2207C>T (p.Ala736Val) on the severity of the disease (**Supplemental Table 6**). Nevertheless, our data suggest that for prediction of the phenotype, the nature of the pathogenic *TMPRSS6* defect overrides a possible influence of the c.2207C>T (p.Ala736Val) variant.

Interestingly, mono-allelic mutated relatives with the same *TMPRSS6* defect as the probands, screened at the time of diagnosis of the proband, were not affected at all, except for the mother of patient 17.

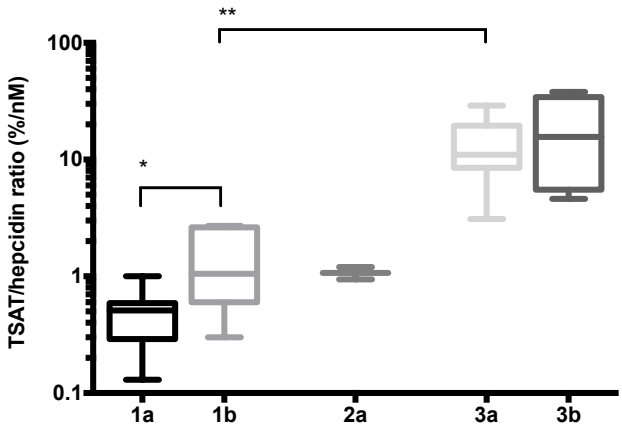
In two sibling pairs with identical heterozygous *TMPRSS6* variants but different phenotypes parental haplotype analysis was performed (**Supplemental Table 8**). In the family of patient 19, both the female proband and her unaffected sister inherited the mutant allele from their mother. However, they received different paternal alleles. Their unaffected brother received two wild type alleles from his mother and father. In the family of patient 20, genetic data were only available from the proband and her sister, not from the parents. Both the female proband and her unaffected sister shared the same *TMPRSS6* variant, but had different wild type alleles, indicating different parental haplotypes of the 'healthy' allele. Although based on analysis of only two families these data suggest that in individuals with a heterozygous *TMPRSS6* variant, differences in their 2nd allele that is designated as 'wild type' account for difference in IRIDA phenotype.

TSAT/hepcidin ratio in IRIDA patients and their unaffected relatives

We evaluated TSAT/hepcidin ratios in i) bi-allelic probands ii) mono-allelic probands, iii) bi-allelic relatives, iv) mono-allelic relatives and iv) wild-type relatives for whom the TSAT and hepcidin were determined and who had no signs of inflammation.

TSAT/hepcidin was significantly lower for bi-allelic IRIDA patients (median: 0.51 %/nM, range 0.13-1.0 %/nM, n=11) than for mono-allelic IRIDA patients (1.0 %/nM, 0.3-2.7 %/nM, n=7, unpaired t-test $p < 0.05$). Interestingly, mono-allelic IRIDA patients had lower ratios than their relatives with the same genotype but without an IRIDA phenotype (11 %/nM, 3.1-29 %/nM, n=14, $p < 0.001$). Among relatives without a phenotype, the ratios were similar for mono-allelic (11 %/nM, 3.1-29 %/nM, n=14) and wild –type subjects (16%/nM, 4.6-38 %/nM, n=4)(Figure 1).

Figure 1. TSAT/hepcidin ratio in bi-allelic (1a) and mono-allelic (1b) affected IRIDA patients and their clinically not affected relatives (2a, 3a, 3b)



TSAT/hepcidin ratio in bi-allelic (1a) and mono-allelic (1b) affected IRIDA patients and their clinically not affected relatives (2a, 3a, 3b, n=38). Patients are defined as having both an IRIDA phenotype (detected after clinical presentation, microcytic anemia, TSAT below the reference range, in the absence of inflammation, not or partially responsive to oral iron) and an IRIDA genotype (a mono- or bi-allelic pathogenic defect in the TMPRSS6 gene). 1. Patients with an IRIDA phenotype; 1a. Probands with bi-allelic TMPRSS6 defect, n=11; 1b. Probands with mono-allelic TMPRSS6 defect, n=6, and affected relative (mother of patient 17) with mono-allelic TMPRSS6 defect, n=1; 2. Relatives without an IRIDA phenotype. 2a. Relatives with bi-allelic TMPRSS6 defect, n=2; 3. Relatives without an IRIDA phenotype; 3a. Relatives with mono-allelic TMPRSS6 defect, n=14; 3b. Wild-type TMPRSS6 relatives, n=4. Patients and relatives with signs of inflammation were excluded from the analysis. Boxes indicate median and interquartile ranges; whiskers describe the range of the data (min–max). *$P < 0.05$; **$P < 0.001$ as tested by unpaired t test.

Iron oral absorption test in the diagnosis of IRIDA patients

To evaluate intestinal iron absorption, an iron oral absorption test (IOAT) was performed in two bi-allelic and three mono-allelic IRIDA patients. [30,,31] In the bi-allelic

patients no enteral iron absorption was demonstrated. In the mono-allelic patients, the quantitative results of the IOAT did not predict the severity of the phenotype and the response to oral iron (**Supplemental Files**).

Ferrokinetic studies

Prior to diagnosis of IRIDA, ferrokinetic studies with radio-active labelled iron were performed in patients 3, 14 and 15 in order to evaluate mucosal iron uptake, transfer, plasma iron clearance, red cell iron incorporation and iron retention. [32-34] The data showed a defect in intestinal iron uptake but adequate iron uptake and incorporation by erythroblasts illustrating that IRIDA is a defect of cellular iron release and that erythroblast iron uptake from the circulation and incorporation occurs fast and without restrictions (**Supplemental Table 9**).

The kinetics of the effect of intravenous iron on blood and serum parameters was studied by administration of 200 mg intravenous iron in patient 14, followed by measurements during the first day and at t= 1 week. Results demonstrated a significant but temporary increase of serum hepcidin on day 1 and a slight increase of Hb, MCV and ferritin after one week. TSAT and the TSAT/hepcidin ratio remained low (**Supplemental Table 10**).

DISCUSSION

We describe a Dutch case series of 21 IRIDA patients and their relatives. We found that patients with bi-allelic severe genotype defects had more severe IRIDA phenotypes than patients with milder *TMPRSS6* defects. This corresponds to scarce data in literature,[4,5] where a tendency to a more severe IRIDA phenotype has been described in patients with bi-allelic nonsense *TMPRSS6* defects compared to patients with missense defects. However, our data show that exceptions occur.

We observed that for as many as seven out of 21 of our patients only a heterozygous *TMPRSS6* defect was found. MLPA showed no deletions or duplications in the 'healthy' allele. We appreciate that we did not exclude mutations and deletions in the promotor region, deep intronic inversions or balanced translocations of chromosome 22. However, our findings corroborate previous reports on mono- allelic patients that present with an IRIDA phenotype, which is generally mild (reviewed in [4]). Interestingly, none of the relatives with a heterozygous *TMPRSS6* defect - except for one - were affected. We hypothesize that differences in phenotype between probands and relatives with identical genotypes might be ascribed to a different expression of the wild type *TMPRSS6* allele compared to the affected allele in patients and in unaffected individuals. [35] Indeed, Serre *et al* describe an array-based analysis of 643 genes expressed in lymphoblastoid cell lines, which shows that for a large proportion (22%) of them, including *TMPRSS6,* the two alleles are differentially expressed. This imbalance in allelic expression can at least partially be explained by epigenetic mechanisms such as lyonization in females and imprinting. [36] This hypothesis is substantiated by the results of our parental haplotype analysis performed in two families suggesting that differences in phenotype between probands and unaffected siblings with the same heterozygous *TMPRSS6* defect is attributable to differences in inherited parental wild type alleles. The finding that the TSAT/hepcidin ratio is lower for bi-allelically mutated probands, than for mono-allelically mutated probands, reflects that the degree of dysregulation of hepcidin production in IRIDA probands is a sliding scale that correlates with the severity of the genotype. These observations suggest that the mode of inheritance in IRIDA is complex. Overall, our small amount of data on the genotype-phenotype correlation in IRIDA patients and their relatives support the notion that phenotypical penetrance of *TMPRSS6* defects is influenced by other (epi)genetic and environmental factors such as growth, co-morbidity as inflammation and blood loss, corroborating some previous observations in mice and man. [37]

Up to now, 94 IRIDA patients of different ethnic origin with 69 different homozygous or compound heterozygous *TMPRSS6* defects have been described in 65 families. [4,5,9,10] Our case series of 21 patients adds nine new mutations, spread throughout the entire matriptase 2 protein and illustrates a geographical distribution of *TMPRSS6* defects with different mutations in the patients from Dutch versus Turkish descent. However, haplotype analysis could not prove common ancestry of all patients with identical defects.

Since the cardinal feature of IRIDA is a discrepantly high serum hepcidin in relation to the low iron body status, we hypothesized that the TSAT/hepcidin ratio as first mentioned by Heeney [38] could be a useful diagnostic tool. In our population, consisting of clinically presenting patients and their relatives, TSAT/hepcidin ratio was able to discriminate between bi-allelic and mono-allelic IRIDA patients, and between mono-allelic IRIDA patients and their phenotypically unaffected relatives with the same heterozygous *TMPRSS6* defect, even after iron supplementation had been given, provided that inflammation was absent. However, before its introduction as a diagnostic test in the work up of iron deficient microcytic anemic patients suspected for the presence of IRIDA, the ratio needs confirmation in phenotypically and genotypically proven IRIDA patients versus patients presenting with an iron deficient microcytic anemia because of other reasons, e.g. inadequate intake, blood loss or other forms of refractory IDA, such as celiac disease, autoimmune gastritis and Helicobacter pylori.

In five of our patients an OIAT test was performed. [31,31] Results were abnormal but since quantitative results did not correlate with the severity of the phenotype and response to oral iron therapy, we concluded that the diagnostic value of this test is limited in the work up of patients suspected or diagnosed with IRIDA.

As the acronym IRIDA implies, patients with *TMPRSS6* defects are usually unresponsive to oral iron. However, as shown by our case series and also by others in some IRIDA patients with both mono-allelic and bi-allelic *TMPRSS6* defects it is possible to increase the Hb to a clinically acceptable level with only oral iron, or with a combination of oral iron and vitamin C. [39] Nevertheless, most IRIDA patients require parenteral iron in order to correct the anemia. According to the literature and to our data, there is a tendency to a reduction of intravenous iron requirements with increasing age. To our knowledge, no studies are available on the optimal dose

and dosing intervals of intravenous iron in IRIDA patients. Kitsaki *et al* [40] investigated hepcidin and other iron parameters in hemodialysis patients, characterized by elevated hepcidin levels [41] treated with intravenous iron. They observed a rapid, small but significant elevation and subsequent decrease to baseline level of serum hepcidin after administration of intravenous iron, as also seen in patient 14 (**Supplemental Table 9**). This increase of hepcidin inhibits the efflux of iron from the macrophages to the serum, thereby decreasing the amount of iron supply to transferrin and erythroblasts. This suggests that small doses of intravenous iron with short intervals resulting in only slightly elevated hepcidin levels might be superior to large doses with large intervals with regards to increasing the serum levels of ferric-transferrin available for erythropoiesis and minimizing substantial iron sequestration of the reticulo-endothelial system (RES) (and associated elevation of ferritin levels). [40] These observations need confirmation in IRIDA patients.

Since IRIDA is a disease with inappropriately high serum hepcidin levels and a low TSAT, excess of iron due to intravenous iron treatment will be stored in the RES of especially the liver and the spleen. This predominantly RES iron storage is also characteristic for patients with loss of function variants in *SLC40A1,* but differs from observations in patients with hereditary hemochromatosis due to variants in *HFE, TfR2, HJV* and *HAMP* and gain of function variants in *SLC40A1* that is associated with iron accumulation in the parenchymal cells, such as the hepatocytes, which can be harmful and of more concern than iron stored in the RES. [42-44] However, long-term consequences of intravenous iron treatment on for instance the occurrence of infections and tissue damage have not been established yet in IRIDA patients. Therefore, to stay on the safe side and based on the case report of Cau[39] and on the clinical course of patient 13, we recommend performing a trial of oral iron in combination with vitamin C in IRIDA patients not responsive to oral iron alone, prior to starting parenteral iron supplementation, in order to use the more natural intestinal function of the intestine to prevent toxic iron loading. In addition, since the clinical phenotype of an IRIDA patient might evolve in time from unresponsiveness to partially responsiveness to oral iron, [45,46] we suggest to critically assess whether repeated administration of intravnous iron is required, and to reassess responsiveness to oral iron and vitamin C.

Five out of our 21 patients have a Mediterranean origin. In addition, 40 out of 61 bi-allelic and six out of 10 mono-allelic patients described to date are from thalassemia or malaria endemic regions. [3,4,7] It is known that especially β-thalassemia, but also

severe α –thalassemia (HbH disease) patients have relatively low serum hepcidin levels due to an ineffective erythropoiesis. [47,48] In β-thalassemia mice, *TMRPSS6* defects counteract the low serum hepcidin levels, thereby attenuating iron overload and anemia. [37,39,50] Alternatively, since *Plasmodium falciparum* has been reported to infect iron-deficient erythrocytes less efficiently, *TMPRSS6* defects may also directly protect against malaria. [51] Altogether, these data suggest a survival benefit for patients with malaria or thalassemia syndromes that also harbor a *TMPRSS6* defect.

Taken together, in the present study we further substantiate previous observations that i) mono-allelic *TMPRSS6* defect may result in a clinical phenotype of IRIDA that is generally milder than in patients with a bi-allelic *TMPRSS6* defect, ii) IRIDA is a defect of cellular iron release as shown by ferrokinetic studies, iii) despite the acronym IRIDA, some patients benefit from treatment with oral iron, iv) IRIDA due to (a) *TMPRSS6* defect(s) is a phenotypically and genotypically heterogeneous disease.

Our novel observations include: i) identification of nine not previously described *TMPRSS6* defects, ii) the presence of country/region specific *TMPRSS6* defects, iii) a relatively high number of patients (all females) were only mono-allelically affected and iv) in the absence of inflammation, a low TSAT/hepcidin ratio was associated with *TMPRSS6* defects and an IRIDA phenotype, even after iron supplementation has been given. Implications of our findings for clinical management of IRIDA patients may comprise: i) the TSAT/hepcidin ratio may prove to be a suitable parameter to detect IRIDA patients among patients presenting with unexplained microcytic anemia with a low TSAT and ii) oral iron with vitamin C treatments should be (re)considered prior to starting intravenous iron.

In time, suppression of the hepcidin pathway may become an alternative therapeutic approach, especially for severely affected patients to prevent iron overload, but to date safety and benefits remain unknown, especially for pediatric patients. Further studies are required to i) assess the value of the TSAT/hepcidin ratio in the differential diagnosis of microcytic anemia, ii) elucidate the contribution of other (epi) genetic and environmental factors in the pathophysiology and clinical penetrance of *TMPRSS6* defects and to iii) determine the optimal treatment regimen. However, in order to prevent misdiagnosis and unnecessary invasive diagnostic work up, the first challenge for the clinician remains the recognition of the disorder and differentiation from other common causes of microcytic anemia.

Authorship

Contribution: A.E.D is a pediatric hematologist and PhD candidate who collected data, analyzed results, reviewed the literature and drafted the manuscript, with help of C.C.S. and T.M.A.P.

V.M.J.N, M.F.R, A.W.R, I.M.A, A.J.V, A.B.V, M.G, B.G, M.C.H.J, A.J.M.R, F.L.V, M.R.N, L.T.V, P.P.T.B. are clinicians who diagnosed and treated the patients and provided clinical data for this paper. R.S. and B.L.P. H. are geneticists who coordinated the genotyping of patients and drafted the genetic part of the paper. D.L.B is a clinical chemist who provided data for this study. D.W.S initiated and coordinated the project. All authors approved the final version of the manuscript.

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REFERENCES

1. Du X, She E, Gelbart T, et al. The serine protease TMPRSS6 is required to sense iron deficiency. *Science.* 2008;320(5879):1088-1092.
2. Silvestri L, Guillem F, Pagani A, et al. Molecular mechanisms of the defective hepcidin inhibition in TMPRSS6 mutations associated with iron-refractory iron deficiency anemia. *Blood.* 2009;113(22):5605-5608.
3. Finberg KE, Heeney MM, Campagna DR, et al. Mutations in TMPRSS6 cause iron-refractory iron deficiency anemia (IRIDA). *Nature genetics.* 2008;40(5):569-571.
4. Donker AE, Raymakers RA, Vlasveld LT, et al. Practice guidelines for the diagnosis and management of microcytic anemias due to genetic disorders of iron metabolism or heme synthesis. *Blood.* 2014;123(25):3873-3886; quiz 4005.
5. De Falco L, Sanchez M, Silvestri L, et al. Iron refractory iron deficiency anemia. *Haematologica.* 2013;98(6):845-853.
6. Girelli D, Nemeth E, Swinkels DW. Hepcidin in the diagnosis of iron disorders. *Blood.* 2016;127(23):2809-2813.
7. Melis MA, Cau M, Congiu R, et al. A mutation in the TMPRSS6 gene, encoding a transmembrane serine protease that suppresses hepcidin production, in familial iron deficiency anemia refractory to oral iron. *Haematologica.* 2008;93(10):1473-1479.
8. Khuong-Quang DA, Schwartzentruber J, Westerman M, et al. Iron refractory iron deficiency anemia: presentation with hyperferritinemia and response to oral iron therapy. *Pediatrics.* 2013;131(2):e620-625.
9. Wang CY, Meynard D, Lin HY. The role of TMPRSS6/matriptase-2 in iron regulation and anemia. *Frontiers in pharmacology.* 2014;5:114.
10. De Falco L, Silvestri L, Kannengiesser C, et al. Functional and clinical impact of novel TMPRSS6 variants in iron-refractory iron-deficiency anemia patients and genotype-phenotype studies. *Human mutation.* 2014;35(11):1321-1329.
11. van der Harst P, Zhang W, Mateo Leach I, et al. Seventy-five genetic loci influencing the human red blood cell. *Nature.* 2012;492(7429):369-375.
12. Benyamin B, Esko T, Ried JS, et al. Novel loci affecting iron homeostasis and their effects in individuals at risk for hemochromatosis. *Nature communications.* 2014;5:4926.
13. Nai A, Pagani A, Silvestri L, et al. TMPRSS6 rs855791 modulates hepcidin transcription in vitro and serum hepcidin levels in normal individuals. *Blood.* 2011;118(16):4459-4462.
14. Kannengiesser C, Guillem F, Silvestri L. Allelic heterogeneity of TMPRSS6 mutations in IRIDA. *American journal of hematology.* 2009;84(8):E236-E375.
15. Beutler E, Van Geet C, te Loo DM, et al. Polymorphisms and mutations of human TMPRSS6 in iron deficiency anemia. *Blood cells, molecules & diseases.* 2010;44(1):16-21.
16. Parlare M, Ferrao A, Relvast L, C B. TMPRSS6 Gene - Two new nonsense mutations associated with IRIDA. *Haematologica.* 2010;95(supplement 2):1-773.
17. Pellegrino RM, Coutinho M, D'Ascola D, et al. Two novel mutations in the tmprss6 gene associated with iron-refractory iron-deficiency anaemia (irida) and partial expression in the heterozygous form. *British journal of haematology.* 2012;158(5):668-672.
18. Hershko C, Camaschella C. How I treat unexplained refractory iron deficiency anemia. *Blood.* 2014;123(3):326-333.
19. Schrier SL. So you know how to treat iron deficiency anemia. *Blood.* 2015;126(17):1971.
20. Kroot JJ, Laarakkers CM, Geurts-Moespot AJ, et al. Immunochemical and mass-spectrometry-based serum hepcidin assays for iron metabolism disorders. *Clinical chemistry.* 2010;56(10):1570-1579.

21. Laarakkers CM, Wiegerinck ET, Klaver S, et al. Improved mass spectrometry assay for plasma hepcidin: detection and characterization of a novel hepcidin isoform. *PloS one*. 2013;8(10):e75518.
22. Uijterschout L, Swinkels DW, Domellöf M, et al. Serum hepcidin measured by immunochemical and mass-spectrometric methods and their correlation with iron status indicators in healthy children aged 0.5-3 y. *Pediatr Res*. 2014;76(4):409-414.
23. www.hepcidinanalysis.com, accessed on July 14 2016
24. White SJ, Breuning MH, den Dunnen JT. Detecting copy number changes in genomic DNA: MAPH and MLPA. *Methods in cell biology*. 2004;75:751-768.
25. www.interactive-biosoftware.com/doc/alamut-visual/2.7/, accessed on July 14 2016
26. https://genome.ucsc.edu/, accessed on September 12 2016
27. Cuijpers ML, Wiegerinck ET, Brouwer R, de Witte TJ, Swinkels DW. [Iron deficiency anaemia due to a matriptase-2 mutation]. *Nederlands tijdschrift voor geneeskunde*. 2010;154:A1038.
28. Rasmussen LJ, Heinen CD, Royer-Pokora B, et al. Pathological assessment of mismatch repair gene variants in Lynch syndrome: past, present, and future. *Human mutation*. 2012;33(12):1617-1625.
29. Richards S, Aziz N, Bale S, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genetics in medicine : official journal of the American College of Medical Genetics*. 2015;17(5):405-424.
30. Kobune M, Miyanishi K, Takada K, et al. Establishment of a simple test for iron absorption from the gastrointestinal tract. *International journal of hematology*. 2011;93(6):715-719.
31. Gross SJ, Stuart MJ, Swender PT, Oski FA. Malabsorption of iron in children with iron deficiency. *The Journal of pediatrics*. 1976;88(5):795-799.
32. Marx JJ. Mucosal uptake, mucosal transfer and retention of iron, measured by whole-body counting. *Scandinavian journal of haematology*. 1979;23(4):293-302.
33. Cavill I. The preparation of 59Fe-labelled transferrin for ferrokinetic studies. *Journal of Clinical Pathology*. 1971;24(5):472-474.
34. Huff RL, Hennessy TG, Austin RE, Garcia JF, Roberts BM, Lawrence JH. Plasma and red cell iron turnover in normal subjects and in patients having various hematopoietic disorders. *The Journal of clinical investigation*. 1950;29(8):1041-1052.
35. Skelly DA, Ronald J, Akey JM. Inherited variation in gene expression. *Annual review of genomics and human genetics*. 2009;10:313-332.
36. Serre D, Gurd S, Ge B, et al. Differential Allelic Expression in the Human Genome: A Robust Approach To Identify Genetic and Epigenetic <italic>Cis</italic>-Acting Mechanisms Regulating Gene Expression. *PLoS Genet*. 2008;4(2):e1000006.
37. Nai A, Pagani A, Silvestri L, Camaschella C. Increased susceptibility to iron deficiency of Tmprss6-haploinsufficient mice. *Blood*. 2010;116(5):851-852.
38. Heeney MM, Campagna DR, Westerman M, et al. The clinical and genetic spectrum of TMPRSS6 mutations leading to inappropriate hepcidin expression and iron refractory iron deficiency anemia (IRIDA). Blood 2009. pp 260-261. (Abstract)
39. Cau M, Galanello R, Giagu N, Melis MA. Responsiveness to oral iron and ascorbic acid in a patient with IRIDA. *Blood cells, molecules & diseases*. 2012;48(2):121-123.
40. Kitsati N, Liakos D, Ermeidi E, et al. Rapid elevation of transferrin saturation and serum hepcidin concentration in hemodialysis patients after intravenous iron infusion. *Haematologica*. 2015;100(3):e80-83.
41. van der Weerd NC, Grooteman MP, Nubé MJ, ter Wee PM, Swinkels DW, Gaillard CA.

Hepcidin in chronic kidney disease: not an anaemia management tool, but promising as a cardiovascular biomarker. *The Netherlands journal of medicine*. 2015;73(3):108-118.
42. Gualdi R, Casalgrandi G, Montosi G, Ventura E, Pietrangelo A. Excess iron into hepatocytes is required for activation of collagen type I gene during experimental siderosis. *Gastroenterology*. 1994;107(4):1118-1124.
43. Pietrangelo A, Montosi G, Totaro A, et al. Hereditary hemochromatosis in adults without pathogenic mutations in the hemochromatosis gene. *The New England journal of medicine*. 1999;341(10):725-732.
44. Pietrangelo A, Caleffi A, Corradini E. Non-HFE hepatic iron overload. Seminars in liver disease 2011;31(3):302-318.
45. Yilmaz-Keskin E, Sal E, de Falco L, et al. Is the acronym IRIDA acceptable for slow responders to iron in the presence of TMPRSS6 mutations? The Turkish journal of pediatrics 2013;55(5):479-484.
46. Guillem F, Kannengiesser C, Oudin C, et al. Inactive matriptase-2 mutants found in IRIDA patients still repress hepcidin in a transfection assay despite having lost their serine protease activity. Human mutation 2012;33(9):1388-1396.
47. Origa R, Cazzola M, Mereu E, et al. Differences in the erythropoiesis-hepcidin-iron store axis between hemoglobin H disease and beta-thalassemia intermedia. Haematologica 2015;100(5):e169-171.
48. Nemeth E. Hepcidin and beta-thalassemia major. Blood 2013;122(1):3-4.
49. Gardenghi S, Ramos P, Marongiu MF, et al. Hepcidin as a therapeutic tool to limit iron overload and improve anemia in beta-thalassemic mice. The Journal of clinical investigation 2010;120(12):4466-4477.
50. Ginzburg Y, Rivella S. beta-thalassemia: a model for elucidating the dynamic regulation of ineffective erythropoiesis and iron metabolism. Blood 2011;118(16):4321-4330.
51. Clark MA, Goheen MM, Fulford A, et al. Host iron status and iron supplementation mediate susceptibility to erythrocytic stage Plasmodium falciparum. Nature communications 2014;5:4446.
52. Mathe E, Olivier M, Kato S, et al. Computational approaches for predicting the biological effect of p53 missense mutations: a comparison of three sequence analysis based methods. Nucleic acids research 2006;34(5):1317-1325.
53. Adzhubei I, Jordan DM, Sunyaev SR. Predicting functional effect of human missense mutations using PolyPhen-2. Curr Protoc Hum Genet 2013;Chapter 7:Unit7.20.
54. Flanagan SE, Patch AM, Ellard S. Using SIFT and PolyPhen to predict loss-of-function and gain-of-function mutations. Genet Test Mol Biomarkers 2010;14:533-537

SUPPLEMENTAL FILES

Supplemental Table 1. Primers used for Sanger and Ion Torrent sequencing

Exon	Forward primer (5' → 3')	Reverse primer (5' → 3')
1-2	tgtaaaacgacggccagtTGAGACCTCCGTCTGTCCTC	caggaaacagctatgaccGCTACAGTCACCCCAAGTCC
3	tgtaaaacgacggccagtACAGGAGACTTTCCCACCTG	caggaaacagctatgaccCATGTGATCAGACCCCACC
4	tgtaaaacgacggccagtGTAGGAAGTGGGCCTGTCTG	caggaaacagctatgaccCCCCTCCCATTCTTTGAATC
5	tgtaaaacgacggccagtAGCTGGGGCCAGACCTC	caggaaacagctatgaccCCCTTGGAGCCAGCCTTGTC
6	tgtaaaacgacggccagtAAGGCTGGCTCCAAGGG	caggaaacagctatgaccTGCTTGGGACACATCGCTGA
7	tgtaaaacgacggccagtGTCCCCTCCTTCTGGCTC	caggaaacagctatgaccTGACTTTCAACTCCCCCATC
8	tgtaaaacgacggccagtCCACTCCCCTCCCAGAC	caggaaacagctatgaccAAAGGTGAGCAGTGAGCCC
9	tgtaaaacgacggccagtGTGGGGTTACAAGCTGCG	caggaaacagctatgaccACCAGGGACCTGTAGTGTGC
10	tgtaaaacgacggccagtTTTGTTGTTAGGGAGGTGGG	caggaaacagctatgaccCTGAGATTGGGGACTTGGG
11	tgtaaaacgacggccagtCACGGGACCCAGGAGAC	caggaaacagctatgaccTTGGTGGTTCCAGGGATG
12	tgtaaaacgacggccagtGCTACGCATGGCCTAATGG	caggaaacagctatgaccCTCAGCTCAGAGCAGGAAAG
13	tgtaaaacgacggccagtCGTGCAATACAGCACACCTT	caggaaacagctatgaccCTTTTGCTGAAGCATGTAGCAG
14	tgtaaaacgacggccagtCTTTGGAAGGTTTCCTTGGG	caggaaacagctatgaccAGCTTCCTGCTGTGGGC
15	tgtaaaacgacggccagtTCTGTCTGCTTCTCCCCTTC	caggaaacagctatgaccGACACAGTGCACCTCCCAC
16	tgtaaaacgacggccagtCAGCTTCCTCCCACCTCAC	caggaaacagctatgaccTTCTCCAGGCCAGGTGTTAC
17	tgtaaaacgacggccagtTAGAGAACAGGGGCTCCAGG	caggaaacagctatgaccATGTGAGCAAAGGGCCAG
18	tgtaaaacgacggccagtTCCCTATGGCTCTTCACCTG	caggaaacagctatgaccTTAGGCAGCAGTGGAGGAAG

Lower case letters indicate the universal primersequences used for Sanger Sequencing, capital letters indicate the specific parts of the *TMPRSS6* exons

Multiplex Ligation-dependent Probe Amplification (MLPA)

All reagents for the MLPA reaction and subsequent PCR amplification were purchased from MRC-Holland (Amsterdam, The Netherlands), with exception of the *TMRPSS6* and control probes. The two-color MLPA was performed as described previously.

In short, in a one-tube format, combinations of two adjacently annealing oligonucleotide probes were hybridized and ligated. After ligation, the common ends of the probes served as a template for PCR amplification with one primer pair and due to the fluorescent labeling of the primer the resulting products could be separated according to size using capillary electrophoresis on the ABI3130XL (Applied Biosystems). Fragment data were analyzed in GeneScan (Applied Biosystems). Peak heights of patient's samples were compared with control probes and ratios were calculated for all fragments (originating from *TMPRSS6* exons) using an Excel spreadsheet. Thresholds for deletions and duplications were set at 0.8 and 1.2 respectively, and all samples were tested at least twice (Table 2).

Supplemental Table 2.

MLPA probes, www.mlpa.com	
	Left Probe Oligonucleotide
Names *TMRPSS6* probes and adjacent genes	
IL2RB E10	gggttccctaagggttggaGTTTCAGACCACAAGGGGCTCCACA
TMPRSS6 E1+2	gggttccctaagggttggaAGCAGAGGCTCCCTTGCAAATGCGAGGCTGTTTCC
TMPRSS6 E3	gggttccctaagggttggaGACAGGAGACTTTCCCACCTGCTTCCTCCAC
TMPRSS6 E4+5+6	gggttccctaagggttggaAGAATCCTTCCCTGGCCTCTCTCACTTCTGCCTGCCTTAGGGA
TMPRSS6 E7	gggttccctaagggttggaAAGCCGTCACCTCCTGAGCTATTCTTGCC
TMPRSS6 E8+9+10	gggttccctaagggttggaCCCTGTGTAACAGCTGCTCTC
TMPRSS6 E11+12+13	gggttccctaagggttggaCCTGTGGTTTTGCAGCCTGCCCTGGAGAGTTCCTCTGTT
TMPRSS6 E14+15+16	gggttccctaagggttggaCATCTCATCAGGCCTCATGGCCA
TMPRSS6 E17+18	gggttccctaagggttggaTCACATCAGCCTGCTGAAGCCTCCCATCCTCCCAGCAAGGT
KCTD17 E8	gggttccctaagggttggaTACTGCCTGAGGGCCTTCAGGGACTTTGCTCCT
Names general reference probes	
CP1_SDF4_ex3_80	gggttccctaagggttggaCAGGAGGCCATGGAGGAGA
CP2_VIPR2_ex13_96	gggttccctaagggttggaCGCGCCCAGTCCTTCCTGCAAACGGAG
CP3_VIPR2_ex2_116	gggttccctaagggttggaGCCTGCGGTGAACACTGTGAACGTGCAGATCGCCTTC
CP4_MRPL41_ex2_132	gggttccctaagggttggaGACCCTGACAACCTGGAAAAGTACGGCTTCGAGCCCACACAGGAG
CP5_NCAPD3_ex8_136	gggttccctaagggttggaCAGCAATTATGCCAGCCTGACCTACCTTCAGATGGCTTGAAATGGTT

Lower case letters indicate the universal MLPA PCR primer sequences, capital letters indicate the specific parts of the *TMRPSS6* exons (Left Hybridising Sequence (LHS) in the LPO and Right Hybridising Sequence (RHS) in the RPO)

	Right Probe Oligonucleotide
	CCTTTGCTGTGTGTTCTGGGGCAACtctagattggatcttgctggcac
	AACTTTGGTCTGTTTCCCTGGCAGGATGCCCGTGGtctagattggatcttgctggcac
	TAACCCTGCTGGTTCCTTCCTGGGCAGGGTAtctagattggatcttgctggcac
	GGGACCCCTCACCTGCTTCTTCTGGTTCATTCTCCAAATCCCCtctagattggatctt
	TGTTCTGTGTCTGTCTGTCCCCGCTGTCAtctagattggatcttgctggcac
	CTGGTCCAGCCTGTGAAGTGAtctagattggatcttgctggcac
	CTGTGAATGGACTCTGTGTCCCTGCCTGTGATGGGGTCAtctagattggatcttgctggcac
	TCTTTCCCATCTGCTTGGTGGGCtctagattggatcttgctggcac
	GGTGATGGCCACCCCTACTTTACAGAAGAGGAGACTGGGGCtctagattggatcttgctggcac
	CTGTGCACCCTCACAACAACCCTGTGAGGTAGGtctagattggatcttgctggcac
	GCAAGACACACTTCCGCGCtctagattggatcttgctggcac
	ACCTCGGTCATCTAGCCCCACCCCTGCtctagattggatcttgctggcac
	TCCGGTTTGACAGAGGCATGCTGGGCCATCATGCTCCtctagattggatcttgctggcac
	GGAAAGCTCTTCCAGCTCTACCCCAGGAACTTCCTGCGCTAGCTGtctagattggatcttgctggcac
	TACTACAGTCTGCATCACTATGTCTGAGACCCTTGTGTTCTCCATCCtctagattggatcttgctggcac

Supplemental Table 3. Response to enteral and parenteral iron supplementation in patients diagnosed with IRIDA

A. Homozygous/compound heterozygous *TMRPSS6* defect	age (years)	Hb (g/L)	MCV (fL)	TSAT (%)	Ferritin (µg/L)	Hepcidin (nM)	TSAT/hepcidin (%/nM)
Patient ID 1							
At presentation	3	7.9	53	4.0	29	np	np
On oral iron	3	8.7	56	3.0	17	5	0.60
On intravenous iron	10	12	64	7.0	367	25	0.27
Without treatment	14	13	82	np	315	np	np
Patient ID 2							
At presentation	2	6.8	54	5.2	53	np	np
On blood transfusions	6	8.7	58	3.4	194	7.1	0.48
Patient ID 3							
At presentation	4	7.1	65	np	44.2	np	np
On oral iron	4	no effect					
On intravenous iron	18	13	75	np	393	15	np
Patient ID 5							
On oral iron	13	8.1	61	2.7	16	np	np
On intravenous iron	18	10.8	77	4.7	249	3.6	1.0
Patient ID 6							
At diagnosis (pregnant)	34	7.6	61	3.0	18	np	np
On intravenous iron	35	9.3	70	4.0	232	np	np
Patient ID 7							
At presentation	0.3	5.5	51	np	np	np	np
On oral iron	6	10.9	68	12	311	23	0.53
Patient ID 8							
At diagnosis On intramuscular iron	38	11.6	80	8.3	934	19	0.43
On intramuscular iron	39	12.7	79	7.9	924[a]	29	0.27
No treatment	40	np	np	13	1053	26	0.50
No treatment	41	11.4	76	np	745	np	np
Patient ID 9							
At presentation	27	7.4	62	5.0	66	np	np
On intravenous iron	30	9.8	64	5.1	143	10	0.51

Supplemental Table 3. Continued

A. Homozygous/compound heterozygous *TMRPSS6* defect	age (years)	Hb (g/L)	MCV (fL)	TSAT (%)	Ferritin (µg/L)	Hepcidin (nM)	TSAT/hepcidin (%/nM)
Patient ID 10							
At presentation	10	8.4	53	2.0	43	np	np
On intravenous iron	12	11.1	69	2.0	288	16	0.13
Patient ID 11							
At presentation	1	11.9	68	6.1	32	7.8	0.78
On oral iron	1	12.1	69	5.6	21	mp	np
Patient ID 12							
At presentation	2	6.4	52	4.0	23	7.6	0.53
On intravenous iron	7	8.7	57	3.0	140	np	np
Patient ID 13							
At presentation	8	7.9	56	1.3	9	6.6	0.29
On treatment with oral iron and vitamin C	10	10.6	72	7.3	144	np	np
Patient ID 14							
At presentation	36	9.3	66	3.8	34	np	np
On intravenous iron	41	12.6	87	15	566	15	1.0
B. Heterozygous *TMPRSS6* defect							
Patient ID 15	28						
At first pregnancy	28	8.9	np	np	np	np	np
On oral iron	28	8.9					
On intravenous iron	28	increase					
No treatment	37	11.8	86	17	280	6.4	2.69
Patient ID 16							
At diagnosis	47	12.1	79	5.0	130	7.5	0.67
On intravenous iron	48	13.9	np	22	908	np	np
Patient ID 17							
At presentation	31	10.5	76	4	36	np	np
On oral iron	32	no effect	no effect	np	np	np	np
On intravenous iron	32	11.8	83	8	196	13	0.61

Supplemental Table 3. Continued

B. Heterozygous *TMPRSS6* defect

	age (years)	Hb (g/L)	MCV (fL)	TSAT (%)	Ferritin (µg/L)	Hepcidin (nM)	TSAT/hepcidin (%/nM)
Patient ID 18							
At presentation	30	12.1	67	np	np	np	np
At diagnosis, no treatment (intravenous iron in the past)	47	12.4	70	8.0	131	7.0	1,1
Patient ID 19	35	10.3	78	6	52	np	np
At 3 mnths of oral iron	35	11.4	80	np	57	np	np
At 6 mnths of oral iron	35	12.7	91	12.5	125	5.9	2.1
Patient ID 20							
At presentation	41	7.9	62	2.8	3.0	np	np
On intravenous iron	41	12.6	89	179	np	np	np
Patient ID 21							
At presentation	34	9.7	68	4	22	np	np
At 3 mnths on oral iron	34	10.6	71	np	38	np	np
At 6 mnths on oral iron	34	13.0	normal	20	293	7.7	2.6

Quantitative data of response to oral and/or iv iron were not available of all patients.

[a]In patient 8 a MRI of the liver was performed because of increased ferritin levels, which showed a Liver Iron Content (LIC) of 60 µmol (3.35 mg) Fe/g tissue (1.5 Tesla MRI, Siemens Magnetom Vison Plus, reference value <36-µmol/g tissue). [2] Normal LIC concentrations are up to 1.8 mg Fe/g dry weight tissue with levels up to 7 mg Fe/g dry weight tissue seen in carriers of genetic hemochromatosis without apparent adverse effect. Thus, the LIC in patient 8 is not suspect for severe iron overload. Moreover, the combination of elevated ferritin and relatively low TSAT indicates iron sequestration in the RES, that is considered to be less harmful compared to parenchymal iron loading. [3,4]

Supplemental Table 4. Characteristics of relatives of IRIDA probands

ID	Family relation	Sex (M/F)	Age Diagnosis IRIDA (years)	*TMPRSS6* defect Allele 1; Allele 2
A. Relatives with 2 affected *TMPRSS6* alleles				
9	proband	F	32	c.497delT; c.1324G>A p.Leu166Argfs*37; p.Gly442Arg
	brother	M	40	c.497delT; c.1324G>A p.Leu166Argfs*37; p.Gly442Arg
11	proband	F	2	c.497delT; c.2383G>A p. Leu166Argfs*37; p.Val795Ile
	sister	F	4	c.497delT; c.2383G>A p.Leu166Argfs*37; p.Val795Ile
B. Relatives with 1 or 0 affected *TMPRSS6* allele				
1	proband	F	9	c.del 118 kb intron 2- exon 3-18[1] c.del 118 kb intron 2 -exon 3-18 p.nonsense;p.nonsense
	mother	F	32	c.del 118 kb intron 2- exon 3-18; Wt p.nonsense; Wt
	father	M	38	c.del 118 kb intron 2- exon 3-18; Wt p.nonsense; Wt
	brother	M	8	c.del 118 kb intron 2- exon 3-18; Wt p.nonsense; Wt
2[d]	proband	F	7	c.del promotor, exon 1-3; c.496delT p.nonsense;p.Leu166Argfs*37
15[d]	proband/ mother	F	37	c.del promotor, exon 1-3; Wt p.nonsense; Wt
	father	M	43	c.497delT; Wt p.Leu166Argfs*37; Wt
	brother	M	9	c.del promotor, exon 1-3; Wt p.nonsense; Wt
	maternal grandfather	M	72	c.del promotor, exon 1-3; Wt p.nonsense; Wt
	sister of maternal grandfather	F	67	c.del promotor, exon 1-3; Wt p.nonsense; Wt
3	proband	F	21	c.497delT; c.497delT p.(Leu166Argfs*37); p.(Leu166Argfs*37_
	mother	F	56	c.497delT; Wt p.Leu166Argfs*37; Wt
	father	M	59	c.497delT; Wt p.Leu166Argfs*37; Wt
	brother	M	19	Wt;Wt

Hb[a] (g/dL)	MCV[a] (fL)	Ferritin[a] (µg/L)	TSAT[a] (%)	Hepcidin[b] (nmol/L)	TSAT/hepcidin[b] (%/nM)	Treatment	p.Ala 736Val[c]
7.4	62	66	5.0	10	0.51	Iron po ineffective Iron iv partially effective	1
12.6	62	101	6.2	6.6	0.94	No treatment	1
11.9	68	32	6.1	7.8	0.78	Iron po sluggish respons No iron iv needed	1
11.1	74	20	6.1	5.2	1.2	No treatment	1
12	64	367	7.0	25	0.27	Iron po ineffective Iron iv partially effective	np
13.9	86	60	13	2.3	5.8	No treatment	np
np	np	np	np	np	np	No treatment	np
13.5	82	22	17	0.60	29	No treatment	np
8.7	58	194	3.4	7.1	0.48	Blood Tx Iron po ineffective	0
11.8	86	280	17	6.4	2.7	Iron po ineffective Iron iv	0
np	np	np	np	np	np	No treatment	0
13.2	74	53	14	np	np	No treatment	0
10.6	86	151	14	1.3	11	No treatment	0
13.2	90	128	31	np	np	No treatment	np
12.6	80	472	10	17	0.60	Iron po ineffective Iron iv partially effective	2
13.2	85	77	26	1.7	15	No treatment	1
15.1	91	27	13	<0.50	19	No treatment	1
14.7	84	22	19	<0.50	>38	No treatment	np

Supplemental Table 4. Continued

ID	Family relation	Sex (M/F)	Age Diagnosis IRIDA (years)	*TMPRSS6* defect Allele 1; Allele 2
5	proband	F	5	c.1904_1905dup; c.1904_1905dup p.Lys636Alafs*17; p.Lys636Alafs*17
	mother	F	29	c.1904_1905dup; Wt p.Lys636Alafs*17;Wt
11	proband	F	2	c.497delT; c.2383G>A p.Leu166Argfs*37; p.Val795Ile
	mother	F	32	c.497delT; Wt p.Leu166Argfs*37
	father	M	35	c.2383G>A; Wt p.Val795Ile; Wt
	sister	F	6	c.2383G>A; Wt p.Val795Ile; Wt
14	proband	F	44	c.2383G>A; c.2105G>T p.Val795Ile; p.Cys702Phe
	father	M	75	c.2383G>A; Wt p.Val795Ile; Wt
	daughter	F	14	Wt; c.2105G>T Wt; p.Cys702Phe
	daughter	F	10	c.2383G>A; Wt p.Val795Ile; Wt
	brother	M	39	Wt; Wt
17	proband	F	31	c.497delT; Wt p.Leu166Argfs*37; Wt
	mother	F	58	c.497delT; Wt p.Leu166Argfs*37; Wt
19	proband	F	41	c.863+1G>T; Wt splicing; Wt
	mother	F	82	c.863+1G>T; Wt splicing; Wt
	sister	F	54	c.863+1G>T; Wt splicing; Wt
	brother	M	49	Wt; Wt

Hb[a] (g/dL)	MCV[a] (fL)	Ferritin[a] (µg/L)	TSAT[a] (%)	Hepcidin[b] (nmol/L)	TSAT/hepcidin[b] (%/nM)	Treatment	p.Ala 736Val[c]
8.1	61	16	2.7	3.8	1.0	Iron po ineffective Iron iv partially effective	0
11.4	90	14	8.8	0.8	11	No treatment	0
11.9	68	32	6.1	7.8	0.78	Iron po sluggish respons No iron iv needed	1
12.1	80	18	6,3	0.7	8.9	No treatment	1
15.3	83	240	28.3	np	np	No treatment	1
12.4	76	14	10.6	0.5	21	No treatment	2
9.3	66	34	3.0	15	0.99	Iron po ineffective Iron iv partially effective Blood Tx (child)	1
13.9	98	56	35	4.0	8.9	No treatment	1
12.1	83	20	15	0.60	22	No treatment	2
11.8	82	33	15	1.6	9.1	No treatment	1
11.0	113	463	39	4.7	8.3	No treatment	2
10.0	76	32	4.0	13	0.30	Iron po not effective Iron iv partially effective	1
12.6	86	299	13	22	0.59	Iron po not effective Iron iv partially effective	0
12.9	87	293	20	7.7	2.6	Iron po sluggish respons No iron iv needed	1
10.0	84	507	19	23 (BSE 45 mm/h)	0.80	No treatment	np
12.9	77	88	15	4.9	3.1	No treatment	np
15.0	79	217	32	7.0	4.6	No treatment	1

Supplemental Table 4. Continued

ID	Family relation	Sex (M/F)	Age Diagnosis IRIDA (years)	*TMPRSS6* defect Allele 1; Allele 2
20	proband	F	44	c.1654G>A; Wt p.Asp552Asn; Wt
	sister	F	38	c.1654G>A; Wt p.Asp552Asn; Wt
21	proband	F	34	c.2105G>T; Wt p.Cys702Phe; Wt
	mother	F	59	c.2105G>T; Wt p.Cys702Phe; Wt
	father	M	63	Wt; Wt

Relatives were screened at the time of diagnosis of IRIDA of the proband.

[a] Hb, MCV, ferritin and TSAT at time of presentation with anemia, except for patient 4,8 and 11; values after treatment with iv iron, im iron and po iron respectively;

[b] Hepcidin, TSAT/hepcidin ratio at time of diagnosis of IRIDA, in absence of inflammation, except for patient 14 (CRP 24 mg/L, auto immune hypothyroidism). Hepcidin references ranges can be found at

Hb[a] (g/dL)	MCV[a] (fL)	Ferritin[a] (µg/L)	TSAT[a] (%)	Hepcidin[b] (nmol/L)	TSAT/hepcidin[b] (%/nM)	Treatment	p.Ala 736Val[c]
8.7	56	4.0	4.0	1.4	2.9	Iron po not effective Iron iv partially effective	1
11.9	92	68	18	2.5	7.3	No treatment	np
12.4	68	125	13	7.7	2.1	Iron po sluggish respons No iron iv needed	2
14.5	95	56	21	1.7	12	No treatment	1
16.1	85	98	38	1.7	23	No treatment	1

www.hepcidinanalysis.com. [5] The lower limit of reference range of TSAT/hepcidin ratio for adults is 1.7 %/nM for men, and 2.5 %/nM for premenopausal women. For children, no reference values are available until now, so we recommend using the reference values for premenopausal women.
Abbreviations: Hb denotes hemoglobin, MCV, mean corpuscular volume, TSAT, transferrin saturation, F, female, M, male, iv, intravenous iron, po, per os, BloodTx, bloodtransfusion, np, not provided

Supplemental Table 5. Characteristics of *TMPRSS6* variants

	Allele Frequency	Functional test	Reference	*In-silico* analysis
Deletion				
c.del of 118 kb in 22q12.3; p.nonsense	new	na	na	na
c.del promotor, exon 1-3; p.nonsense	new	na	na	na
Frameshift				
c.497delT; p.Leu166Argfs*37	0.00000826[a]	na	Beutler *et al*[6]	na
c.1904_1905dup; p.Lys636Alafs*17	na	na	Finberg *et al*[7]	na
Nonsense				
c.1832G>A; p.Trp611X	new	na	na	na
Missense				
c.521T>C; p.Leu174Pro	new	na	na	Align GVGD[b]: C0 Polyphen[c]: 0.57 SIFT[d]: 0.04
c.1228T>C; p.Cys410Arg	new	na	na	Align GVGD[b]: C0 Polyphen[c]: 0.45 SIFT[d]: 0.00
c.1229G>C; p.Cys410Ser	new	na	na	Align GVGD[b]: C0 Polyphen[c]: 0.01 SIFT[d]: 0.00
c.1324G>A; p.Gly442Arg	na	na	Finberg *et al*[7]	Align GVGD[b]: C65 Polyphen[c]: 1.0 SIFT[d]: 0.00
c.1654G>A; p.Asp552Asn	new	na	na	Align GVGD[b]: CO Polyphen[c]: 1.0 SIFT[d]: 0.00
c.2105G>T; p.Cys702Phe	0.0008389[f]	na	Cuijpers *et al*[8]	Align GVGD[b]: CO Polyphen[c]: 1.0 SIFT[d]: 0.00
c.2383G>A; p.Val795Ile	0.008571[g]	Normal inhibition of *HAMP1* expression[6]	Beutler *et al*[6] Cuijpers *et al*[8]	Align GVGD[b]: CO Polyphen[c]: 1.0 SIFT[d]: 0.00
Splicing				
c.230-6G>A; splicing	new	na	na	Alamut: splice site[h]
c.863+1G>T; splicing	new	na	na	Skip of exon 7[i]

Supplemental Table 5. Continued

[a] http://exac.broadinstitute.org/variant/22-37492064-CA-C

[b] **Align GVGD,** web based in silico prediction software program that combines the biophysical characteristics of amino acids and protein multiple sequence alignments to predict where missense substitutions in genes of interest fall in a spectrum from enriched delterious to enriched neutral. A-GVGD scores amino acid substitutions on a 7-scale scoring system, from C0 to C65. An amino acid substitution with a C0 score is considered to be neutral, amino acids with C15 and C25 scores are considered intermediate, as changes to protein structure or function are uncertain, and C35 scores or higher are considered as likely deleterious. [9]

[c] **PolyPhen-2** (Polymorphism Phenotyping v2 HumVar) is a tool, which predicts possible impact of an amino acid substitution on the structure and function of a human protein using straightforward physical and comparative considerations. PolyPhen scores range from $0 \leq 1 \leq X$, outcome scores of 0.00–0.15 are classified as benign, 0.15–1.0 as possibly damaging, 0.85-1.0 as more confidently predicted to be damaging. [10]

[d] **The SIFT** algorithm combines sequence homology and physical properties of amino acid substitutions to analyze whether or not amino acid substitutions are tolerated, in light of the predicted effect on the protein structure. SIFT score ranges from 0 to 1. The amino acid substitution is predicted damaging if the score is < 0.05 and tolerated if the score is > 0.5 [11]

[e] http://exac.broadinstitute.org/variant/22-37471208-G-A

[f] http://exac.broadinstitute.org/variant/22-37465149-A-G

[g] http://exac.broadinstitute.org/variant/22-37462173-C-T

[h] This substitution is predicted to introduce a new and more efficient acceptor splice site 4 bases downstream from the original acceptor splice site in intron 2 leading to a frameshift in the open reading frame. Not proven on RNA or protein level. (http://www.interactive-biosoftware.com/doc/alamut-visual/2.7/splicing.html), accessed on July 14 2016.

[i] This substitution is located in the donor splice site of intron 7. The consequence of this change is not predictable, but a skip of exon 7 is very likely resulting in the loss of a classical splice site. Not proven on mRNA or protein level (http://www.interactive-biosoftware.com/doc/alamut-visual/2.7/splicing.html), accessed on July 14 2016.

Abbreviations: na denotes not applicable

Supplemental Table 6. Characteristics of IRIDA patients

Table 6 A. Genetics and ethnicity in 14 homozygous or compound heterozygous patients

ID	Sex	Origin	*TMPRSS6* defect Allele 1; Allele 2	p.Ala 736Val[a]
1	F	MA	c.del of 118 kb in 22q12.3[b] c.del of 118 kb in 22q12.3 p.nonsense;p.nonsense	0
2	F	NL	c.del promotor, exon 1-3;c.497delT p.nonsense;p.Leu166Argfs*37	0
3	F	NL	c.497delT; c.497delT p.Leu166Argfs*37;p.Leu166Argfs*37	2
4	M	NL	c.497delT; c.497delT p.Leu166Argfs*37;p.Leu166Argfs*37	0
5	F	TR	c.1904_1905dup; c.1904_1905dup p.Lys636Alafs*17;p.Lys636Alafs*17	0
6	F	TR	c.1904_1905dup; c.1904_1905dup p.Lys636Alafs*17;p.Lys636Alafs*17	2
7	M	NL	c.497delT; c.1832G>A p.Leu166Argfs*37; p.Trp611X	0
8	F	NL	c.497delT; c.1228T>C p. Leu166Argfs*37 p.Cys410Arg	2
9	F	NL	c.497delT; c.1324G>A p.Leu166Argfs*37; p.Gly442Arg	1[c]
10	M	NL	c.497delT; c.1324G>A p. Leu166Argfs*37; p.Gly442Arg	0
11	F	NL	c.497delT; c.2383G>A p. Leu166Argfs*37; p.Val795Ile	1[d]
12	M	TR	c.1904_1905dup ;c.1229G>C p.Lys636Alafs*17; p.Cys410Ser	0
13	M	NL	c.521T>C c.1228T>C p.Leu174Pro; p.Cys410Arg	0
14	F	NL	c.2383G>A; c.2105G>T p.Val795Ile; p.Cys702Phe	1[e]

Supplemental Table 6. Continued

Table 6 B. Genetics and ethnicity in 7 heterozygous patients

ID	Sex	Origin	*TMPRSS6* defect allele 1; allele 2	p.Ala 736Val[a]
15	F	NL	c.del promotor, exon 1-3; Wt p.nonsense; Wt	0
16	F	NL	c.497delT; Wt p.Leu166Argfs*37; Wt	np
17	F	NL	c.497delT; Wt p.Leu166Argfs*37; Wt	1[f]
18	F	NL	c.230-6G>A[g]; Wt splicing; Wt	1[c]
19	F	NL	c.863+1G>T[h]; Wt splicing; Wt	1[f]
20	F	TR	c.1654G>A; Wt p.Asp552Asn; Wt	0
21	F	NL	c.2105G>T; Wt p.Cys702Phe; Wt	2

[a] 0 : p.Ala736 on both alleles, 1: p.Val736Ala on 1 allele, 2: p.Val36Ala on both alleles

[b] Deletion 118 kb in intron 2, knocking out exon 3-18 of *TMPRSS6* gene. Also other genes were deleted; RefSeq genes *TEX33, MPST, TST* and *KCTD17*

[c] No family studies available; so whether p.Ala736Val was located on Wt allele or mutated allele was unclear

[d] Family studies showed that p.Ala736Val was located on allele c.2383G>A, p.Val795Ile;

[e] Family studies showed that p.Val736Ala was located on allele c.2105G>T, p.Cys702Phe;

[f] Family studies showed that p.Ala736Val was located on the Wt allele

[g] This substitution is predicted to introduce a new and more efficient acceptor splice site 4 bases downstream from the original acceptor splice site in intron 2 leading to a frameshift in the open reading frame. Not proven on RNA or protein level. (http://www.interactive-biosoftware.com/doc/alamut-visual/2.7/splicing.html), accessed on July 14 2016.

[h] This substitution is located in the donor splice site of intron 7. The consequence of this change is not predictable, but a skip of exon 7 is very likely resulting in the loss of a classical splice site. Not proven on mRNA or protein level (http://www.interactive-biosoftware.com/doc/alamut-visual/2.7/splicing.html), accessed on July 14 2016

Abbreviations: F denotes female; M, male; MA, Morocco; NL, Netherlands; TR; Turkey; iv, intravenous; po, per os; im; intramuscular; Wt; Wildtype; np, not provided

Supplemental Table 7. Haplotype analysis in patients sharing a *TMPRSS6* defect

		TMPRSS6 High Frequency Variants and Single Tandem Repeats				
DNA level		Down Stream[a] 21xAC	Down Stream[a] 17xGT	c.120G>A	c.113T>C	c.99G>A
rs number				5756516	5756515	11704654
HAP MAP allele frequencies[b] Caucasian population				0.425	0.483	0.248
	Origin					
TMRPSS6 defect:						
c.497delT						
ID 2:	NL					
c.497delT		**378**	**280**	-	-	-
c.del exon 1-3		384	280	-	-	-
ID 3:	NL					
c.497delT		**378**	**280**	-	-	-
c.497delT		**378**	**280**	-	-	-
ID 4:	NL					
c.497delT		**378**	**280**	-	-	-
c.497delT		**378**	**280**	-	-	-
ID 7:	NL					
c.497delT		**378**	**280**	+/-	+/-	-
c.1832G>A		384	282	+/-	+/-	-
ID 8:	NL					
c.497delT		**378**	**280**	+/-	+/-	-
c.1228T>C		384	284	+/-	+/-	-
ID 9:	NL					
c.497delT		**378**	**280**	-	-	-
c.1324G>A		380	280	-	-	-
ID 10:	NL					
c.497delT		**380**	**280**	+	+	-
c.1324G>A		378	280	+	+	-
ID 11:	NL					
c.497delT		**378**	**280**	**na**	**na**	**na**
c.2383G>A		378	280	**na**	**na**	**na**
ID 16:	NL					
c.497delT		**378**	**280**	+	+	-
Wt		384	284	+	+	-
ID 17	NL					
c.497delT		**378**	**280**	**na**	**na**	**na**
Wt		378	282	**na**	**na**	**na**

TMPRSS6 High Frequency Variants and Single Tandem Repeats							
c.564C>T	c.757A>G	c.1083G>A	c.1254C>T	c.1563C>T	c.2207C>T	c.2217C>T	Up Stream 18xGT[a]
139207981	2235324	2111833	881144	4820268	855791	2235321	
0.006[c]	0.354	0.343	0.075[d]	0.583	0.588	0.385	
+/-	+/-	-	+/-	+/-	+/-	+/-	**218**
+/-	+/-	-	+/-	+/-	+/-	+/-	222
-	+	+	-	+	+	+	**218**
-	+	+	-	+	+	+	**218**
-	+	+	-	+	+	+	**218**
-	+	+	-	+	+	+	**218**
-	+/-	+/-	+/-	+/-	+	+	**218**
-	+/-	+/-	+/-	+/-	+	+	226
-	+/-	+/-	-	+	+	+	**218**
-	+/-	+/-	-	+	+	+	218
+/-	+/-	+/-	+/-	+/-	+/-	+/-	**218**
+/-	+/-	+/-	+/-	+/-	+/-	+/-	226
-	+/-	na	+/-	na	na	na	**226**
-	+/-	na	+/-	na	na	na	226
na	na	na	na	na	+/-	na	**218**
na	na	na	na	na	+/-	na	228
-	+	+/-	-	+	+	+/-	**218**
-	+	+/-	-	+	+	+/-	222
-	+/-	na	-	na	+/-	+/-	**218**
-	+/-	na	-	na	+/-	+/-	222

Supplemental Table 7. Haplotype analysis in patients sharing a *TMPRSS6* defect

		TMPRSS6 High Frequency Variants and Single Tandem Repeats				
DNA level		Down Stream[a] 21xAC	Down Stream[a] 17xGT	c.120G>A	c.113T>C	c.99G>A
rs number				5756516	5756515	11704654
HAP MAP allele frequencies[b] Caucasian population				0.425	0.483	0.248
	Origin					
TMPRSS6 defect:						
c.1228T>C						
ID 8:	NL					
c.1228T>C		**384**	**284**	+/-	+/-	-
c.497delT		378	280	+/-	+/-	-
ID 13:	NL					
c.1228T>C		**380**	**284**	-	-	-
c.521T>C		380	284	-	-	-
TMPRSS6 defect:						
c.1904_1905dup						
ID 5:	TR					
c.1904_1905dup		**378**	**282**	+	+	-
c.1904_1905dup		**378**	**282**	+	+	-
ID 6:	TR					
c.1904_1905dup		**378**	**282**	+/-	+/-	-
c.1904_1905dup		**378**	**282**	+/-	+/-	-
ID 12	TR					
c.1904_1905dup		**378**	**282**	-	-	-
c.1229G>C		378	284	-	-	-
TMPRSS6 defect:						
c.2105G>T						
ID 14:	**NL**					
c. 2105G>T		**na**	**na**	-	-	+/-
c.2383G>A		**na**	na	-	-	+/-
ID 22:	NL					
c.2105G>T		**na**	**na**	**na**	**na**	**na**
Wt		**na**	**na**	**na**	**na**	**na**

Haplotype analysis was performed by genotyping 10 intra-genic high frequency variants (HFV's) in the *TMPRSS6* gene with a Minor Allele Frequency in Caucasians > 0.005 and 3 Short Tandem Repeats surrounding *TMPRSS6* in patients with identical defects, by Sanger sequencing.

-/- = HFV homozygous absent; +/+ = HFV homozygous present; +/- HFV = heterozygous present, unknown on which allele the HFV is located

Abbreviations: NL denotes Netherlands; TR, Turkey; na, not available

TMPRSS6 High Frequency Variants and Single Tandem Repeats							
c.564C>T	c.757A>G	c.1083G>A	c.1254C>T	c.1563C>T	c.2207C>T	c.2217C>T	Up Stream 18xGT[a]
139207981	2235324	2111833	881144	4820268	855791	2235321	
0.006[c]	0.354	0.343	0.075[d]	0.583	0.588	0.385	
-	+/-	+/-	-	+	+	+	**218**
-	+/-	+/-	-	+	+	+	218
-	+/-	+/-	+/-	+/-	+	+	**218**
-	+/-	+/-	+/-	+/-	+	+	218
-	+	+	-	+	+	+	**218**
-	+	+	-	+	+	+	**218**
-	+	+	-	+	+	+	**218**
-	+	+	-	+	+	+	**218**
-	+	-	-	+	+	+	**218**
-	+	-	-	+	+	+	218
-	+/-	+/-	+/-	+/-	+/-	+/-	**na**
-	+/-	+/-	+/-	+/-	+/-	+/-	na
na	**na**	**na**	**na**	**na**	**na**	**na**	**na**
na	**na**	**na**	**na**	**na**	**na**	**na**	**na**

[a] Number in column indicates the length of the PCR fragments surrounding the Single Tandem Repeats

[b] From http://www.ncbi.nlm.nih.gov/projects/SNP/snp.ref.cgi;

[c] No HAPMAP frequencies available, reference population ESP Cohort Population, http://www.ncbi.nlm.nih.gov/projects/SNP/snp+ref.cgi?rs=13920798

[d] No HAPMAP frequencies available, reference population ESP Cohort Population, http://www.ncbi.nlm.nih.gov/projects/SNP/snp+ref.cgi?rs=881144

Supplemental Table 8. Parental haplotype analysis in families 19 and 20

ID	Family relation	*TMPRSS6* variant	Intragenic HFV[a] and *TMPRSS6* variants[b] considered as pathogenic			
		Allele 1; Allele 2	c.26+29C>T	c.757A>G	*c.863+1G>T*[b]	c.1083G>A
19	Proband	c.(863+1G>T); Wt	na	+ -	+ -	+ -
	Sister	c.(863+1G>T); Wt	na	+ +	+ -	+ +
	Mother	c.(863+1G>T); Wt	na	+ -	+ -	+ -
	Brother	Wt; Wt	na	- +	- -	- +
20	Proband	c.(1654G>A); Wt	+ -	+ -	- -	+ -
	Sister	c.(1654G>A); Wt	+ -	+ -	- -	+ -

[a] Haplotype analysis was performed by genotyping 7 intra-genic High Frequency Variants (HFV's) in the *TMPRSS6* gene with Sanger sequencing

[b] *TMPRSS6* variants considered as pathogenic. Since no data were available from the parents of proband 20 and her sister, the inheritance pattern of the mutant allele and the wildtype alleles (paternal versus maternal) was not clear.

						Allele
c.1563C>T	*c.1654G>A*[b]	c.1699+20C>T	c.2207T>C	c.2217C>T	
+	-	-	+	+	M1
-	-	-	-	-	P2
+	-	-	+	+	M1
+	-	-	+	+	P1
+	-	-	+	+	M1
-	-	+	-	-	M2
-	-	+	-	-	M2
+	-	-	+	+	P1
	+	na	+	-	A
	-		+	+	B
	+	na	+	-	A
	-		-	-	C

Abbreviations: M1=mutant *TMPRSS6* allele inherited from mother, M2= wild type *TMPRSS6* allele inherited from mother, P1= wild type *TMPRSS6* allele inherited from father, P2 = wild type *TMPRSS6* allele complementary to P1, inherited from father. A= mutant *TMPRSS6* allele, B and C: wild type *TMPRSS6* alleles.

Oral iron absorption test

Method

In order to evaluate the intestinal iron absorption, in five patients (two bi-allelic and three mono-allelic affected) an oral iron absorption test (OIAT) was performed. Three patients (ID 17, 19, 21) received one single dose of 400 mg ferrofumarate containing 130 mg ferrous iron. Serum iron was measured just before the administration of oral iron and after 30, 60, 90 and 120 minutes. No additional food intake was allowed. The physiological increase of serum iron in healthy individuals should be at least 15 µmol/L 120-180 minutes after oral intake of the test dose. [12] A fourth patient (ID 1), a child, received a challenge of 30 mg ferrofumarate containing 10 mg ferrous iron as an OIAT according to Gross,[13] who describes an average increase of 42 µmol/l after two hours in 10 iron replete individuals (five children, five adults) after a test dose of 1mg/kg ferrous iron. Patient ID 12, also a child, received a challenge of 80 mg ferrofumarate.

Results

In the bi-allelic patients 1 and 12 the increase of serum iron respectively 120 minutes and 90 minutes after oral administration of iron was 0 µmol/L. In the heterozygous patients 17, 19, and 21, this increase was 5.7, 1.3 and 12.2 µmol/L, respectively, and thus remained below the lower reference limit of 15 µmol/l. [12] Patients 19 and 21 were clinically responsive to oral iron in some extent (**Table 1, Supplemental Table 4**), but had a very low oral iron absorption in the test while patient 17 was clinically unresponsive to oral iron and had a moderately low oral iron absorption in the test. From these experiments we conclude that the oral iron test was abnormal in all four IRIDA patients, especially in the homozygous affected patient. However, the quantitative results did not predict the severity of the phenotype and the response to oral iron in heterozygous patients.

Ferrokinetic studies

Method

Before IRIDA had been diagnosed, in three patients (ID 3, 14, 15) ferrokinetic studies were performed in order to evaluate mucosal iron uptake, transfer, plasma iron clearance, red cell iron incorporation and iron retention. [14] To assess the kinetics of oral iron absorption a double isotope technique was used with ^{59}Fe as a tracer and ^{51}Cr as a non-absorbable indicator. With a whole body counter initial mucosal iron uptake and iron retention were measured and iron mucosal transfer was calculated. [14]

Plasma iron clearance and red cell iron incorporation were determined after injection of ^{59}Fe bound to transferrin. This labeled ^{59}Fe was prepared by incubating autologous plasma with ferrous citrate after which ^{59}Fe not bound to transferrin was removed by passing the mixture through an anion exchange resin column. [15]

Results

Intestinal iron uptake and red cell incorporation studies in patient 3, 14 and 15 indicate that mucosal absorption, intestinal mucosal transport of iron into the circulation and iron retention 14 days after oral iron administration are inadequately low for the relatively low body iron status of these patients (**Supplemental Table 9**). As shown for patient 3, plasma iron clearance and red cell iron uptake after administration of iv iron coupled to transferrin are both normal, illustrating that IRIDA is a defect of cellular iron release and that erythroblast iron uptake from the circulation and incorporation occurs fast and without restrictions.

Supplemental Table 9. Ferrokinetic studies

Parameters At presentation	ID 3 (F)	ID 14 (F)	ID 15 (F)	Reference mean ± SD (range)		
Hemoglobin (g/dL)	7.1	9.3	5.4	Men	Women	Iron deficiency
MCV (fL)	65	66	76			
Ferritin (µg/L)	44	34	np			
Serum iron (µmol/l)	7	2	10			
Mucosal iron uptake (%)	37.6	np	np	38 ±17[16] (4-61)	39±15[16] (13-68)	73±19[16] (41-91)
Mucosal iron transfer (fraction)	0.41	np	np	0.54±0.18[16] (0.23-0.87)	0.65±0.14[16] (0.42-0.91)	0.98±0.04[16] (0.92-1.08)
Plasma iron T1/2 (min)	24.7	np	np	(60-120)[17]		3[8 a]
Red cell iron incorporation 14 days after iv iron (%)	90.6	np	np	(75-85)[17]	(75-85)[17]	(90-100[17]
Iron retention 14 days after oral iron (%)	15.4	14.0	23.9	20±10[16] (2-34)	25±11[16] (6-51)	71±19[16] (41-95)

mucosal iron uptake, the percentage of iron taken up by the mucosal cells from the lumen of the gut; mucosal iron transfer, the fraction of the iron taken up by the mucosal cells that passed into the blood; iron retention, the iron still present in the body 14 days after oral ingestion according to Marx [14]

[a] based on 1 patient with iron deficiency anemia, mentioned in reference. [17]

Abbreviations: np denotes not provided; F, female

Effect of intravenous iron on serum iron parameters

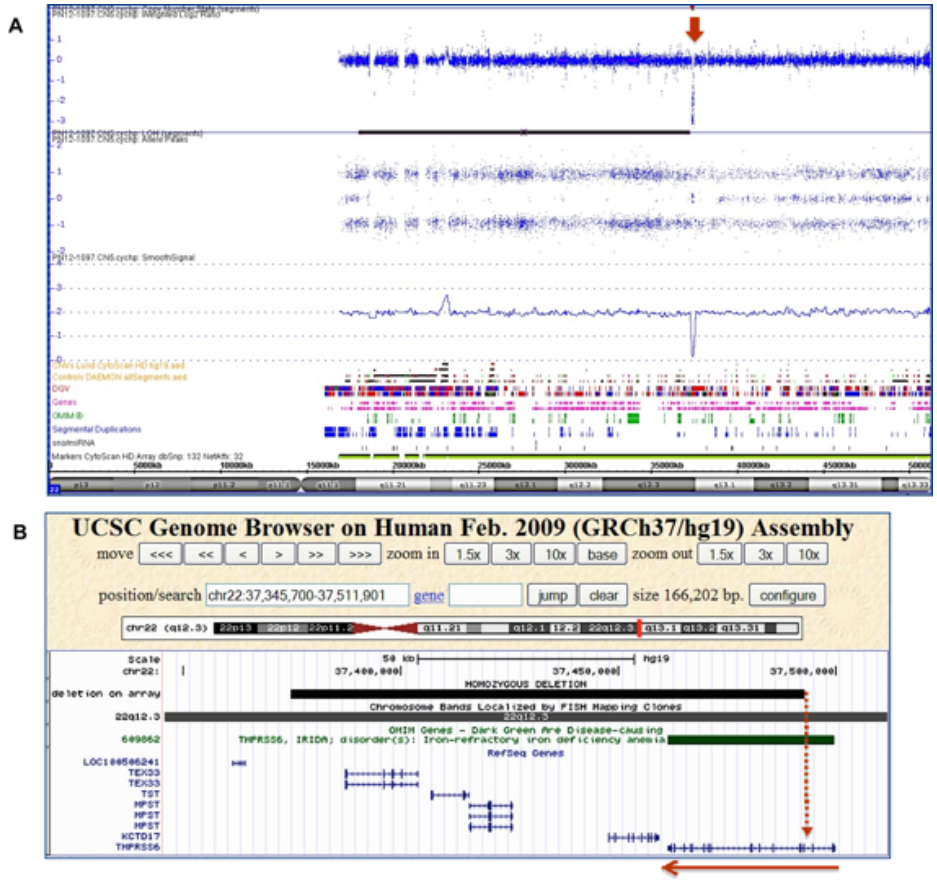
To assess the kinetics of the effect of intravenous iron on blood and serum parameters we investigated the effect of intravenous administration of 200 mg iron sucrose on serum iron parameters and hepcidin in one bi-allelic affected patient (ID 14). Serum hepcidin increased significantly from 7.3 to 8.9 nmol/L within 6 hours after administration and returned to baseline level 1 week after intravenous iron administration. Serum iron and TSAT were not different before and 1week after intravenous iron administration. Compared to baseline, one week after treatment, ferritin, Hb and MCV were (slightly) increased from 592 to 649 µg/L, 13.5 to 14.2 g/dL and 88 to 90 fL, respectively. These latter findings are in agreement with clinical observations in the other patients, demonstrating that in IRIDA patients intavenous iron leads to increase of Hb, MCV and ferritin, but not of TSAT.

Supplemental Table 10. Change in serum iron parameters in time after administration of intravenous iron in an IRIDA patient

Parameters	t=0 h	t=1h	t=3h	t=6 h	t=1 week
Serum iron (µmol/L)	11	na	na	na	9
TSAT (%)	14	na	na	na	14
Serum hepcidin (nmol/L)	7.3	7.3	7.6	8.9	7.2
TSAT/hepcidin (%/nM)	1.9	na	na	na	1.9
CRP (mg/L)	19	19	29	19	23
Serum ferritin (µg/L)	592	na	na	na	649
Hemoglobin (g/dL)	13.5	13.0	13.4	13.2	14.2
MCV (fL)	88	87	87	88	90

Data of patient 14; At t=0 h, 200 mg iron sucrose (Venofer®, Vifor Pharma) was administered intravenously
Abbreviations: na denotes not available

Supplemental Figure 1. Genomic array profile of chromosome 22 of patient 1



Genomic array profile of chromosome 22 of patient 1. Profile is obtained by CytoScanHD array of Affymetrix. **A**. The genomic array profile of the index patient, showing a 118 kb homozygous deletion characterized by 140 array probes with a lowered 2Log(Test/Reference) ratio (red arrow). This homozygous deletion is situated in a 20.4 megabase homozygous region. The proximal breakpoint of the deletion is located at 37,374,751 Mb and the distal breakpoint at 37,492,851 Mb (breakpoints are mapped on human genome version Hg19, 2009). **B**. Screenshot of the UCSC genome. Here the detected deletion is indicated by the black box marked "HOMOZYGOUS DELETION" which shows that the distal breakpoint of the deletion is located between exon 2 and 3 (bold red arrow) of the *TMPRSS6* gene, which is concordant with the failed sequencing efforts of exons 3-18. The distal breakpoint probably leads to nonsense-mediated decay of the messenger RNA corresponding with exon 1 and 2, resulting in the total absence of matriptase 2. The red arrow indicates the direction in which the gene is read. This screenshot also shows that besides *TMPRSS6,* the deletion also comprises *TEX33, TST, MPST* and *KCTD17.*

REFERENCES

1. White SJ, Breuning MH, den Dunnen JT. Detecting copy number changes in genomic DNA: MAPH and MLPA. Methods in cell biology 2004;75:751-768.
2. Hoffbrand AV, Taher A, Cappellini MD. How I treat transfusional iron overload. Blood 2012;120:3657-3669.
3. Gualdi R, Casalgrandi G, Montosi G, et al. Excess iron into hepatocytes is required for activation of collagen type I gene during experimental siderosis. Gastroenterology 1994;107:1118-1124.
4. Pietrangelo A, Montosi G, Totaro A, et al. Hereditary hemochromatosis in adults without pathogenic mutations in the hemochromatosis gene. The New England journal of medicine 1999;341:725-732.
5. www.hepcidinanalysis.com, accessed on July 14 2016
6. Beutler E, Van Geet C, te Loo DM, et al. Polymorphisms and mutations of human TMPRSS6 in iron deficiency anemia. Blood cells, molecules & diseases 2010;44:16-21.
7. Finberg KE, Heeney MM, Campagna DR, et al. Mutations in TMPRSS6 cause iron-refractory iron deficiency anemia (IRIDA). Nature genetics 2008;40:569-571.
8. Cuijpers ML, Wiegerinck ET, Brouwer R, et al. [Iron deficiency anaemia due to a matriptase-2 mutation]. Nederlands tijdschrift voor geneeskunde 2010;154:A1038.
9. Mathe E, Olivier M, Kato S, et al. Computational approaches for predicting the biological effect of p53 missense mutations: a comparison of three sequence analysis based methods. Nucleic acids research 2006;34:1317-1325.
10. Adzhubei I, Jordan DM, Sunyaev SR. Predicting functional effect of human missense mutations using PolyPhen-2. Curr Protoc Hum Genet 2013;Chapter 7:Unit7.20.
11. Flanagan SE, Patch AM, Ellard S. Using SIFT and PolyPhen to predict loss-of-function and gain-of-function mutations. Genet Test Mol Biomarkers 2010;14:533-537.
12. Kobune M, Miyanishi K, Takada K, et al. Establishment of a simple test for iron absorption from the gastrointestinal tract. International journal of hematology 2011;93:715-719.
13. Gross SJ, Stuart MJ, Swender PT, et al. Malabsorption of iron in children with iron deficiency. The Journal of pediatrics 1976;88:795-799.
14. Marx JJ. Mucosal uptake, mucosal transfer and retention of iron, measured by whole-body counting. Scandinavian journal of haematology 1979;23:293-302.
15. Cavill I. The preparation of 59Fe-labelled transferrin for ferrokinetic studies. Journal of Clinical Pathology 1971;24:472-474.
16. van Rijk PP. Nuclear Techniques in Diagnostic Medicine. 1986. Chapter 14 Radionuclear Techniques in haematology p.
17. Bothwell TH, Callender S, Mallett B, et al. The study of erythropoiesis using tracer quantities of radioactive iron. British journal of haematology 1956;2:1-16.

Chapter 5

Microcytic Anemia with Low Transferrin Saturation, Increased Serum Hepcidin and Non-synonymous *TMPRSS6* Variants: not always Iron Refractory Iron Deficiency Anemia

Albertine E. Donker, Paul P.T. Brons, Dorine W. Swinkels

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TO THE EDITOR,

With interest we read the article of Nie *et a.,*[1] which describes a 15-months old girl with a severe microcytic anemia, neither responding to oral iron nor to a combination or intravenous iron and erythropoietin. The authors suggest that the patient suffers from Iron Refractory Iron Deficiency Anemia (IRIDA) due to a tri-allelic mutation in *TMPRSS6*. We do not agree with this conclusion for several reasons.

The phenotype is not very typical of IRIDA. Since the cardinal feature of IRIDA is impaired iron availability due to a reduced intestinal iron absorption and iron release from the macrophages as a result of the disproportionally high serum hepcidin level, transferrin saturation (TS) is very low (<5%).[2-4] In the described child, the TS is only moderately decreased compared to the severity of the anemia. Furthermore, serum hepcidin levels should always be interpreted in relation to serum inflammation parameters because hepcidin production by the liver is stimulated by cytokines, especially IL6.[5] The authors do not mention the results of CRP or ESR, but the sharp decline of the serum hepcidin level after glucocorticosteroids had been given, suggests that inflammation played a role in the high serum hepcidin level and subsequent anemia, a phenomenon, which is known as Anemia of Chronic Disease (ACD). As far as we know, splenomegaly is not characteristic for IRIDA.[6]

However, our main comment concerns the genetic confirmation of the diagnosis of IRIDA. The occurrence of both the matriptase 2 changes K253E (rs2235324) and V736A (rs855791), found in the patient, depends on the ethnicity but is highly prevalent in all populations and does not correspond to the relatively low frequency of IRIDA. (CSHL-HAPMAP: HapMap HCB, describing the Chinese population: prevalence of homozygous K253E: 0.133; prevalence of heterozygous V736A: 0.044). Functional studies show that the matriptase 2 736A variant inhibits hepcidin more efficiently than 736 V variant.[7] This implicates that the presence of the 736A variant has a lower prevalence in the Chinese population than the 736V variant and protects against elevated hepcidin levels and iron deficiency!

Indeed, Genome Wide Association Studies (GWAS) have identified the 736A variant to be associated with relatively *increased* iron status biomarkers, red blood cell count, Hb and erythrocyte indices.[8-10] This variant was found to explain ± 2 % in the variation of serum iron and TS and ± 1% in the variation of Hb en MCV in an adult Australian twin cohort.[8] The 253E variant in *TMPRSS6* has not been functionally assessed but bio-informatics tools predicted this polymorphism as non-pathogenic (SIFT, Polyphen). In one GWAS the 253E variant has been found to be in modest linkage disequilibrium with V736A and to be associated with a modest decrease in serum iron markers.[10] Therefore, we argue that it is not very likely that the clinical phenotype of the child can be explained by the described genotype. We recommend investigating other causes of inherited and acquired microcytic anemia, especially anemia of chronic disease due to an underlying inflammatory condition.

REFERENCES

1. Nie N, Shi J, Shao Y, et al. A novel tri-allelic mutation of TMPRSS6 in iron-refractory iron deficiency anaemia with response to glucocorticoid. *British journal of haematology.* 2014;166(2):300-303.
2. Finberg KE, Heeney MM, Campagna DR, et al. Mutations in TMPRSS6 cause iron-refractory iron deficiency anemia (IRIDA). *Nature genetics.* 2008;40(5):569-571.
3. Guillem F, Lawson S, Kannengiesser C, Westerman M, Beaumont C, Grandchamp B. Two nonsense mutations in the TMPRSS6 gene in a patient with microcytic anemia and iron deficiency. *Blood.* 2008;112(5):2089-2091.
4. Donker AE, Raymakers RA, Vlasveld LT, et al. Practice guidelines for the diagnosis and management of microcytic anemias due to genetic disorders of iron metabolism or heme synthesis. *Blood.* 2014;123(25):3873-3886; quiz 4005.
5. Kroot JJ, Tjalsma H, Fleming RE, Swinkels DW. Hepcidin in human iron disorders: diagnostic implications. *Clinical chemistry.* 2011;57(12):1650-1669.
6. Hentze MW, Muckenthaler MU, Galy B, Camaschella C. Two to tango: regulation of Mammalian iron metabolism. *Cell.* 2010;142(1):24-38.
7. Nai A, Pagani A, Silvestri L, et al. TMPRSS6 rs855791 modulates hepcidin transcription in vitro and serum hepcidin levels in normal individuals. *Blood.* 2011;118(16):4459-4462.
8. Benyamin B, Ferreira MA, Willemsen G, et al. Common variants in TMPRSS6 are associated with iron status and erythrocyte volume. *Nature genetics.* 2009;41(11):1173-1175.
9. Chambers JC, Zhang W, Li Y, et al. Genome-wide association study identifies variants in TMPRSS6 associated with hemoglobin levels. *Nature genetics.* 2009;41(11):1170-1172.
10. Tanaka T, Roy CN, Yao W, et al. A genome-wide association analysis of serum iron concentrations. *Blood.* 2010;115(1):94-96.

Chapter 6

X-linked Sideroblastic Anemia due to *ALAS2* Mutations in the Netherlands: a Disease in Disguise

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ABSTRACT

Background: X-linked sideroblastic anemia (XLSA) is the most common inherited form of sideroblastic anemia and is associated with several mutations in the erythroid specific 5-aminolevulinate synthase gene (*ALAS2*). The disorder is characterized by mostly mild hypochromic microcytic anemia with bone marrow ring sideroblasts. Even un-transfused patients with mild or no anemia are at risk for severe systemic iron overload due to ineffective erythropoiesis.

To date, 61 different *ALAS2* mutations have been reported in 120 families with XLSA. Descriptions of molecularly confirmed case series from the Netherlands, however, are lacking.

Methods: We reviewed age of presentation, clinical and biochemical features, ALAS-2 defects and treatment characteristics of 15 Dutch patients from 11 unrelated families diagnosed with XLSA.

Results and Conclusions: In one family a novel pathogenic c.1412G>A (p.Cys471Tyr) mutation was found. All other families shared the previously described c.1355G>A (p.Arg452His) mutation. Haplotype analysis in seven probands with the p.Arg452His mutation strongly suggests that six of them were ancestrally related. Nevertheless, their phenotype was very different. Our patients illustrate the phenotypical heterogeneity in the presentation of XLSA patients, the effectiveness of treatment regimens and the various pitfalls associated with the diagnosis, follow-up and treatment of the disease. A timely diagnosis avoids unnecessary investigations and allows adequate treatment that can prevent systemic iron load with subsequent severe life-threatening complications. Therefore, we suggest considering XLSA in both male and female patients with unexplained iron overload and/or (mild) microcytic anemia, also at older age.

Key words
ALAS2, iron, sideroblastic anemia

INTRODUCTION

X-linked sideroblastic anemia (XLSA; OMIM #300751) is the most common inherited form of sideroblastic anemia (SA) and is associated with several mutations in the erythroid specific 5-aminolevulinate synthase gene (*ALAS2*), which is the first and rate limiting step of heme-biosynthesis.[1-3] The disorder is characterized by hypochromic microcytic anemia with ring sideroblasts in the bone marrow in combination with systemic iron overload due to ineffective erythropoiesis. Phenotypic expression of XLSA is highly variable even in patients with identical mutations but affected males generally present in the first decades of life with symptoms of anemia or later with manifestations of parenchymal iron overload. Occasionally patients present later in life.[4,5] As in most X-linked recessive disorders, the majority of female carriers of XLSA are spared from clinical manifestations. However, sporadically women with *ALAS2* mutations may be affected due to inactivation of the normal X-chromosome or age related skewed X-inactivation in hematopoietic cells.[6-8] Standard treatment of XLSA consists of high dose pyridoxine supplementation and iron reducing strategies like phlebotomies and iron chelation.[9] The effect of high dose pyridoxine is based on the high prevalence of mutations in the pyridoxine-binding region of the *ALAS2*-gene. The high dose enhances the half-life of *ALAS2*, however, this is not true for mutations outside this region.[10] Reduction of iron overload in XLSA improves erythropoiesis and prevents complications of chronic iron overload, especially liver cirrhosis and hepatocellular carcinoma.[11-13]

In this article we describe 14 male patients and one female patient from 11 unrelated families. All patients are from Dutch origin. These case series are illustrative for the biochemical and clinical presentation of XLSA patients, the effectiveness of treatment regimens and the various pitfalls associated with the (early) diagnosis, follow-up and treatment of the disease.

PATIENTS AND METHODS

Patients

We reviewed clinical and molecular data of 15 patients (14 male and one female) diagnosed with XLSA in the Netherlands in 2011 and 2012. These patients were diagnosed with sideroblastic anemia at the University Medical Center Utrecht, Utrecht and the Radboud University Medical Center, Nijmegen, the Netherlands. We reviewed age at presentation, biochemical and clinical features, treatment regimens and type of *ALAS2* mutations.

Genotyping

Genotyping was performed by PCR and DNA sequence analysis of the full coding part of the *ALAS2* gene. The pathogenicity of a mutation was assessed by review of the literature, association of the mutation with the phenotype in a family and bio-informatic tools. The latter tools were used to complement the genetic studies in case of a not previously reported mutation. SIFT (=Sorting Intolerant from Tolerant) and PolyPhen (Polymorphism Phenotyping) and HOPE (Have (y)Our Protein Explained) provide an *in-silico* prediction of the functional consequences of missense mutations.[14-17]

Search for a founder effect was done in 7 of the 10 families with the p.Arg452His mutation by genotyping the Short Tandem Repeats (STR's) DXS1044, DXS8032, DXS991 and DXS1190 close to the *ALAS2* gene by PCR using fluorescent primers. PCR products were pooled and analyzed on an ABI 3730 DNA sequences.

RESULTS

Overall clinical and biochemical features and treatment strategies of Dutch XLSA patients

Fifteen XSLA patients from 11 unrelated families were included in the study; all were of Dutch and Caucasian origin (Table 1). Age at the time of clinical and biochemical diagnosis in our patients ranged from 2 to 72 years. In the male patients, hemoglobin at diagnosis ranged from 3.9 to 7.8 mmol/L with mean corpuscular volume (MCV) between 56 and 71 fL. Serum ferritin at diagnosis ranged from 99 to 5040 µg/L.

All patients were treated with high dose pyridoxine (200 mg daily, except for patient 10 who received 150 mg daily), phlebotomies or chelation. Withdrawal of 500 mL blood per phlebotomy was performed, except in patient 2B who started on 200 ml per phlebotomy every 2 weeks for 2 months. Because of a stable and even increasing Hb, the phlebotomy volume was increased to 400 mL every 2 weeks until his ferritin became < 100 µg/L (Figure 1).

Also, in the other patients phlebotomies were well tolerated, even in a patient with more severe anemia (patient 3). In general, this treatment regimen resulted in a significant increase of Hb in six out of 15 patient and a decrease of ferritin levels in five out of 15 patients.

The only female proband, patient 1A, died at the age of 79 years due to the complications of diabetes mellitus and heart failure. Patient 2A died at the age of 71 years from a hepatocellular carcinoma (HCC). The other patients are still alive and in good clinical condition. None of them has developed severe complications of systemic iron overload, probably due to timely treatment.

Table 1. Hematological, biochemical and molecular data, and treatment characteristics of 15 patients from 11 unrelated families diagnosed with X-linked sideroblastic anemia

Patient characteristics				Laboratory characteristics			
ID[1]		Age (yrs)	Sex (M/F)	Hb (mmol/L)	MCV (fL)	Ferritin (µg/L)	TSAT (%)
1A	at presentation	72	F	6.0	76	nd	nd
	death	79		nd	nd	nd	nd
1B son	at presentation	25	M	5.3	65	117[3]	40[3]
	with therapy	46		7.2	69	135	nd
	with therapy	61		7.6	63	244	32
2A[4,5]	at presentation	66	M	7.8	68	346	57
	with therapy	69		7.2	70	316	48
2B grandchild	at presentation	2	M	6.8	nd	180	94
	with therapy	16		7.4	70	454	97
	with therapy			7.8	71	72	80
3	at presentation	35	M	4.3	56	5040	86[6]
	with therapy	47		8.0	64	1162	nd
	with therapy	62		8.3	65	516	67
4	at presentation	25	M	7.1	69	220	48
	with therapy	34		7.0	70	281	42
	with therapy	40		7.2	71	526	24
5A	at presentation	<21	M	3.9	59	158	35
	with therapy	54		7.8	71	260	47
5B brother	at presentation	23	M	6.2	59	1200	nd
	with therapy	53		8.4	68	259	37
6	at presentation	32	M	6.8	71	258	52
	with therapy	54		7.4	76	150	48
7	at presentation	<28	M	7.2	70	193	43
	with therapy	51		6.6	66	275	42
8	at presentation	<28	M	7.1	62	573	82[7]
	with therapy	32		7.1	62	546	82

	Genotype	Treatment characteristics		Remarks
Bone marrow	*ALAS2* mutation[2]	Pyridoxine	Chelation/ phlebotomy	
nd	p.Arg452His	yes	nd	Blood transfusion per 3-6 months; Diabetes mellitus II; Myocardial infarction; Hypercholesterolemia
ring sideroblasts +++	p.Arg452His	yes	phlebotomy	
nd	p.Arg452His	yes	phlebotomy	Heterozygosity for P.Cys282Tyr in *HFE;* death at age 71 because of hepatocellular carcinoma
ring sideroblasts 30%	p.Arg452His	yes	phlebotomy	Homozygous for p.Cys282Tyr in *HFE* gene
ring sideroblasts +++	p.Arg452His[11*]	yes	chelation phlebotomy	
nd	p.Arg452His	yes	no	Nail clubbing
nd	p.Cys471Tyr	yes	nd	
nd	p.Cys471Tyr	yes	phlebotomy	
ring sideroblasts 11%	p.Arg452His[11*]	yes	no	
nd	p.Arg452His[11*]	yes	no	
nd	p.Arg452His[11*]	yes	chelation phlebotomy	Rheumatoid arthritis IgA deficiency

Table 1. Continued

Patient characteristics				Laboratory characteristics			
ID[1]		Age (yrs)	Sex (M/F)	Hb (mmol/L)	MCV (fL)	Ferritin (µg/L)	TSAT (%)
9A	at presentation	30	M	6.8	70	610	52
	with therapy[8]	32		7.4	70	436	48
9B[8] grandfather	at presentation	nd	M	nd	nd	nd	nd
	with therapy			nd	nd	nd	nd
10	at presentation	13	M	6.8	68	99	34
	with therapy[10]	14		6.8	68	64	58
11	at presentation	18	M	7.5	70	252	56
	with therapy	18		7.6	76	191	30

[1] Numbers stand for families -probands are mentioned- for families 1, 2, 5, and 9, also a 2nd affected relative is included;

[2]hemizygous for men and heterozygous for women;

[3]At age 28 years;

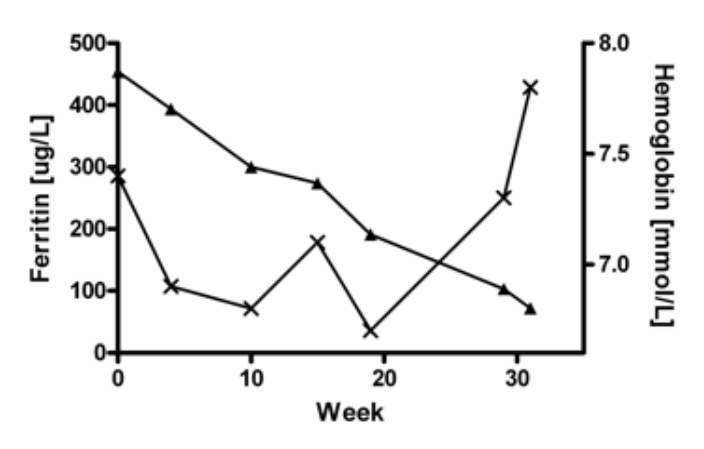
[4] patient 2 was originally diagnosed with iron overload at the age of 38 years, which later at 57 years was attributed to hereditary haemochromatosis due to heterozygosity for the p.Cys282Tyr mutation in the *HFE* gene. Treatment with phlebotomies was started. Because of low Hb levels and ferritin levels within the reference range, phlebotomies were stopped at the age of 51 years; [5]Patient previously reported in Cuijpers *et al*[18] ;

[6]At age 38 years, no earlier values available;

	Genotype	Treatment characteristics		Remarks
Bone marrow	*ALAS2* mutation[2]	Pyridoxine	Chelation/ phlebotomy	
no ring sideroblasts	p.Arg452His[11]	yes	EPO phlebotomy	Bone marrow biopsy: MDS type RCMD with iron loaded macrophages
nd	nd	nd	nd	Hereditary primary sidero-achrestic anemia[9]
nd	p.Arg452His[11*]	yes	no	Intention tremor, no ataxia, defect in *ABCB7* gene excluded
ring sideroblasts	p.Arg452His[11*]	yes	no	

[7]At age 32 years;
[8]Results of 1 year treatment with pyridoxine (200 mg per day) and phlebotomy every 4-6 weeks. EPO was stopped after diagnosis at age 30 years;
[9]Patient previously reported in thesis of Dr. Ploem[20];
[10]Low compliance; MDS type RCMD: Myelodysplastic syndrome type Refractory Cytopenia with Multilineage Dysplasia;
[11]Probands investigated by haplotype analysis, in subjects indicated by [11*] this analysis suggests a common ancestor
Abbreviations: nd denotes not determined or data not available; TSAT, transferrin saturation; NASH: non alcoholic steato-hepatitis; EPO, erythropoietin

Figure 1. Course of hemoglobin and ferritin in patient 2B diagnosed with XLSA and *HFE*-related hemochromatosis from the onset of phlebotomies



A male patient (Table 1, patient 2B) was diagnosed with sideroblastic anemia and *HFE*-related hemochromatosis at the age of 2 years. At the age of 16 years treatment was started with phlebotomies because of increasing serum ferritin levels. The patient treatment consisted of a 200 mL phlebotomy every 2 weeks during 8 weeks, followed by 400 mL blood drawings every 2 weeks for another 22 weeks. Within a 30-week time frame this treatment resulted in a significant decrease in ferritin levels and an increase in Hb. These data illustrate that reduction of systemic iron overload improves erythropoiesis in XLSA patients. X-axis indicates weeks after start of treatment with phlebotomies; X, Hb concentration; ▲, serum ferritin level.

Molecular features

Thirteen out of the 15 patients showed hemizygosity for the previously reported pathogenic c.1355G>A (p.Arg452His) mutation in exon 9 of the *ALAS2* gene. One female patient was heterozygous for the c.1355G>A (p.Arg452His) mutation (patient 1A). These 13 patients with a p.Arg452His mutation, are from 10 apparently unrelated families. Haplotype analysis of patients 3 and 6-11 showed that all patients, except for proband 9, carried the same length of the 4 STR's analyzed, suggesting that the p.Arg452His mutation arose from one common ancestor in these probands. The lengths of all 4 STR's of the patients differed from those found for proband 9. The common haplotype of patients 3, 6-8, 10 and 11 is at least 2.473 kilobase in size.

In two patients (brothers 5A and 5B) a novel mutation was found in exon 9: c.1412G>A (p.Cys471Tyr). For this mutation bio-informatic tools were not consistent in their assessment, i.e. SIFT predicted the mutation as non-pathogenic, whereas PolyPhen predicted the mutation as "probably damaging". HOPE reports: "the wild type

(cysteine) and mutant amino acids (tyrosine) differed in size. The wild type residue was buried in the core of the protein; the mutant residue was bigger and probably not fitting. The hydrophobicity of the wild type and mutant residue differed. The mutation probably caused loss of hydrophobic interactions in the core of the protein". The fact that both brothers share the same mutation and have similar phenotypes suggested the mutation to be pathogenic.

Case descriptions

Table 1 shows hematological, biochemical, molecular data and treatment characteristics of the XLSA patients. We will describe some of these patients and relatives in more detail in order to illustrate the biochemical and clinical presentation of XLSA patients, the effectiveness of treatment regimens and the various pitfalls associated with the management of this disease.

Patient 1A illustrates that women may develop a phenotype of XLSA later in life. At the age of 78 years SA was diagnosed after she presented with anemia (Hb 6.0 mmol/L). Three years earlier, her Hb was 7.7 mmol/L. *Post mortem* she was found to have the same *ALAS2* defect as her son (patient 1B).

Patient 2A was originally diagnosed with iron overload at the age of 38 years.[18] Treatment with phlebotomies was started. Because of low Hb levels and ferritin levels within the reference range, phlebotomies were stopped at the age of 51 years. After the discovery of the *HFE* gene in 1996, at the age of 57 years, the patient was tested for hereditary hemochromatosis (HH). A heterozygous p.Cys282Tyr mutation in the (hemochromatosis) *HFE* gene was found. Based on this finding, the patient's iron overload was attributed to HH. However, HH is an autosomal recessive inherited disorder and complications due to iron overload alone are extremely rare in individuals who are heterozygous for defects in the *HFE* gene.[19]

In the same period, a male grandchild (patient 2B) was diagnosed with SA. DNA analysis in this child revealed a p.Arg452His mutation in the *ALAS2* gene, responsible for XLSA. The same mutation was subsequently found in his grandfather. So, in retrospect, patient 2A suffered from XLSA with secondary systemic iron overload due to ineffective erythropoiesis. At age 70 years liver biopsy revealed an HCC with substantial iron accumulation in the hepatocytes and some steatosis. The lesion was attributed to iron overload and was not resectable. At age 71 years the patient died

of this complication. The patient had no history of liver cirrhosis.[18] Because of the family history, mutation analysis of the *HFE*-gene was also performed in his grandson, which revealed homozygosity for the p.Cys282Tyr mutation. Because of increasing ferritin levels at age 16 years treatment with phlebotomies was started. Within a 30 weeks period, this resulted in a decrease in ferritin levels from 454 µg/L to 72 µg/L and an increase of Hb from 7.4 mmol/L tot 7.8 mmol/L (**Figure 1**).

Patient 3 presented with both severe anemia (4.3 mmol/L) and very severe and systemic iron overload (ferritin of 5040 µg/L) at age 35. Despite his severe anemia, phlebotomies were well tolerated and are likely to have contributed to normalization of his iron stores and Hb in addition to treatment with pyridoxine and iron chelation.

In his teens patient 5 presented with severe anemia and ferritin within reference ranges. His younger brother was diagnosed at the age of 23 years with SA by family screening. He had no signs and symptoms of anemia. However, serum ferritin was 1200 µg/L, suggesting severe iron overload. Treatment for SA and iron overload was started, consisting of pyridoxine and phlebotomies.

Patient 9 was initially diagnosed with myelodysplastic syndrome (MDS) at the age of 30 years, subtype Refractory Cytopenia with Multilineage Dysplasia (RCMD). Interestingly, no ring sideroblasts were seen in the bone marrow and MCV was low, 70 fL. Since his grandfather had previously been described with “hereditary primary sidero-achrestic anemia” (patient 41 in ref [20]) and since the index patient presented with a hypochromic microcytic anemia in combination with iron overload, an *ALAS2* mutation was suggested and subsequently confirmed.

DISCUSSION

Our Dutch case series are illustrative for the pathophysiology, the biochemical and clinical presentation of XLSA patients, the effectiveness of treatment regimens and the various pitfalls associated with the (early) diagnosis, follow-up and treatment of this disease. In this article we add a novel mutation to the previously described 61 different *ALAS2* mutations reported in 120 families with XLSA.[21-24]

All of our 15 XLSA patients had microcytic anemia and all had a mutation in the exon 9 domain of the X-chromosome. In 10 out of 11 families (13 out of 15 patients) it concerned a p.Arg452His mutation, making this the most prevalent mutation in Dutch XLSA patients. Search for a founder effect by haplotype analysis in seven of the families with this mutation suggests that this mutation arose from a common ancestor in six of them. Worldwide the p.Arg452His is also the most frequent *ALAS2* defect in XLSA**.** In one patient a novel p.Cys471Tyr mutation was found. Bio-informatic analysis and family genotype-phenotype association study was highly suggestive for a pathogenic defect. Recently, we reported on a 12th Dutch Family with XLSA due to a g.55054634G>C mutation in the GATA transcription factor binding site located in a transcriptional enhancer element in intron 1 of the *ALAS2* gene.[24]

Age of diagnosis, degree of anemia and iron overload widely differed between these patients, illustrating heterogeneity in the clinical and biochemical penetrance of this congenital disease.

One of our patients (patient 3) illustrates that besides anemia, severe systemic iron overload can occur at early age in transfusion independent XLSA patients. Preclinical and clinical studies in ß thalassemia major and intermedia and other iron loading anemias suggest the ineffective erythropoiesis in these disorders may increase the production of humoral factors that may include growth differentiation factor 15 (GDF15), twisted gastrulation (TWSG1) and erythroferrone (ERFE)[25-27] that lead to decreased production of the iron-regulatory hepatic peptide hormone hepcidin (reviewed in[28]). Hepcidin acts by inhibiting intestinal iron absorption and macrophage recycling of iron from senescent erythrocytes. Suppression of hepcidin production by these proteins has been suggested to cause inappropriately high intestinal iron absorption and iron release from the reticulo-endothelial system (RES), despite iron overload.[25-28]

We previously reported that patient 2A indeed had elevated serum GDF15 levels that were associated with inappropriately low serum hepcidin in relation to his iron stores, as reflected by a low hepcidin/ferritin ratio.[18] We did not measure serum GDF15 and/or serum hepcidin in our other SA patients since the results have no therapeutic implications. As far as we know, no studies are available on the above-mentioned humoral factors or hepcidin in SA patients due to *ALAS2* defects.

In general, systemic iron overload develops in the third or fourth decade, also in patients without overt anemia.[1,2] This emphasizes the importance of early diagnosis, since the effects of systemic iron overload are potentially very serious, such as liver cirrhosis and HCC, especially in the presence of concurrent liver toxic conditions (alcohol *abusus* or non-alcoholic steato-hepatitis). Moreover, we suggest that first-degree relatives should be screened for the relevant mutation, because they may develop severe iron overload without any signs and symptoms of anemia.

This phenotype of iron overload with only mild anemia may lead to a false diagnosis of hereditary hemochromatosis. We suggest that *ALAS2* mutations might be the underlying cause of patients (falsely) diagnosed with unexplained forms of HH. In these cases, the low MCV should point the clinician to the presence of an iron-loading anemia such as XLSA. To the best of our knowledge the prevalence of *ALAS2* defects among patients with genetically unexplained HH is unknown.

Other genes implicated in iron metabolism and HH may also affect the phenotype of XLSA. Anecdotal data support the suggestion that coinheritance of heterozygosity of the p.Cys282Tyr mutation in the *HFE* gene is likely increased in XLSA patients with moderate to severe phenotypes.[11,29,30] It is well possible that penetrance of HH due to homozygosity for the p.Cys282Tyr mutation might be modified by *ALAS2* mutations and vice versa, as the biochemical presentation of patient 2B suggests, i.e. he developed systemic iron overload already in his teens.

The majority of female carriers of XLSA are asymptomatic, as in most X-linked recessive disorders. However, as illustrated by patient 1A, they may be affected due to the predominant inactivation of the normal X-chromosome. Furthermore, physiologic age-related skewed X –inactivation in hematopoietic cells may play a role in developing XLSA in female carriers with increasing age. So a combination of congenital and acquired skewing can result in late onset of XLSA in women.[6-8]

Because of the co-existence of normal and affected erythroblasts this anemia may be normocytic with an increased red cell distribution with (RDW) or even two separate erythrocyte populations.[31] Patient 1A also shows that even in elderly patients who present with anemia, a congenital disorder should be considered. Interestingly, Furuyma *et al* describe a male patient with chronic renal failure who developed sideroblastic anemia at the age of 81 years. This patient was found to have an *ALAS2* mutation which only became manifest by an acquired pyridoxine deficiency due to hemodialysis.[32]

Anecdotal data support the possibility of misdiagnosing XLSA for MDS-RARS (myelodysplastic syndrome-refractory anemia with ringed sideroblasts) without MDS specific cytogenetic and genetic abnormalities in elderly people. This may be attributed to the fact that the diagnosis of MDS is solely based on the morphological aspect of the bone marrow, which is often difficult.[33] Our patient 9 also was originally diagnosed with MDS (type RCMD) based on the morphologic aspect of bone marrow biopsy, despite low MCH and MCV and a grandfather, who was diagnosed with inherited primary sidero-achrestic anemia 50 years ago.[20] Even in retrospect, however, ring sideroblasts, characteristic for SA, were not seen in the bone marrow. We have no explanation for this phenotype. To the best of our knowledge, no studies are available on the prevalence of inherited *ALAS2* mutation among patients diagnosed with MDS with refractory anemia (RARS, RA and RCMD). However, in a recent study among 137 sideroblastic anemias, XLSA patients had MCV levels below the reference range, whereas MCV of patients with MDS-RARS and MDS-RCMD was within reference range.[34] This indicates that a reduced MCV is important to distinguish XLSA from MDS with refractory anemia.

As illustrated by our case series, in many patients with XLSA the anemia is to some extent, responsive to pyridoxine. Pyridoxine is metabolized to pyridoxal 5'phosphate, the cofactor for ALAS2. Pyridoxine responsive XLSA is generally based on missense mutations that reduce the affinity between ALAS2 and pyridoxal 5'phosphate, resulting in a shorter half-life of the enzyme. In these cases treatment with high dose of the cofactor pyridoxine partly enhances the stability of ALAS2.[10] *ALAS2* mutations that alter the post-translational processing resulting in diminished enzyme activity are mostly pyridoxine unresponsive.[10] Apart from the mutation, the iron status is also important for the pyridoxine responsiveness, because iron overload may compromise mitochondrial function and hence heme biosynthesis. Therefore,

XLSA patients should not be considered refractory to pyridoxine therapy until iron stores are normalized with serum ferritin and transferrin saturation in the normal range.[11] Because of this mechanism it is feasible to phlebotomize in XLSA, even in patients with severe anemia. Hb typically increases, rather than decreases, after reversal of iron overload by blood removal, as shown by patient 2B en 3. In patients who develop anemia, frequent withdrawal of a small volume is often feasible (our unpublished observations).

Although 13 out of 15 patients shared the same missense mutation, response to pyridoxine was highly variable. The reason for this remains unclear. Low compliance should be considered, as was the problem in patient 10. If patients are unresponsive to pyridoxine, it is recommended to discontinue it, since increased levels of pyridoxine are associated with peripheral neuropathy.[35,36] Peripheral neuropathy was not observed in our cases.

In conclusion, our case series describes the biochemical and clinical presentation of XLSA patients and the effectiveness of treatment regime, and it illustrates the various pitfalls associated with diagnosis, follow-up and treatment of the disease. We suspect *ALAS2* mutations to be more frequent, but not easy to diagnose. The combination of these data with previously published patient information led us to the following recommendations for the clinical management of patients with XLSA:

1. Diagnosis. Consider XLSA in:

- Men with unexplained microcytic anemia, even if the anemia is mild, since missing the diagnosis might result in severe iron overload and associated morbidity and mortality.
- Men of all ages presenting with the phenotype of MDS with refractory anemia (RA), without MDS specific cytogenetic abnormalities, and microcytosis, because patients with MDS-RA have MCV levels within the reference range.
- Women with unexplained microcytic or normocytic anemia because of the possibility of late onset XLSA due to a combination of congenital and acquired unbalanced lyonization.
- Patients with unexplained hereditary hemochromatosis and concomitant (mild) microcytic anemia.

2. Treatment
- Pyridoxine unresponsiveness in XLSA should not be diagnosed until iron overload has been treated adequately, as iron accumulation is known to reduce pyridoxine activity.
- Phlebotomies should be considered even in patients with severe anemia in order to reduce the toxic effects of iron overload and to improve erythropoiesis.

3. Family screening
- All first-degree family members should be genetically and phenotypically (Hb, MCV, iron, transferrin and ferritin) screened. Even though XLSA is an X-linked disease, women can develop the disease.

Author contributions

AED is a pediatric hemato-oncologist and PhD student who analyzed the results, reviewed the literature and drafted the manuscript; RAR, HKN and MAM are consultants in hematology who diagnosed, clinically followed and treated some of the patients (HKN retired); MJHC is a molecular biologist who designed and performed the haplotype analysis; PPB is a pediatric hemato-oncologist who clinically followed and treated some of the patients. MCJ is an internal specialist who clinically followed and treated some of the patients. DWS is a laboratory physician and expert in disorders of iron metabolism. RAR and DWS initiated and edited the manuscript and supervised the work. All authors contributed to drafting the manuscript and approved the final version.

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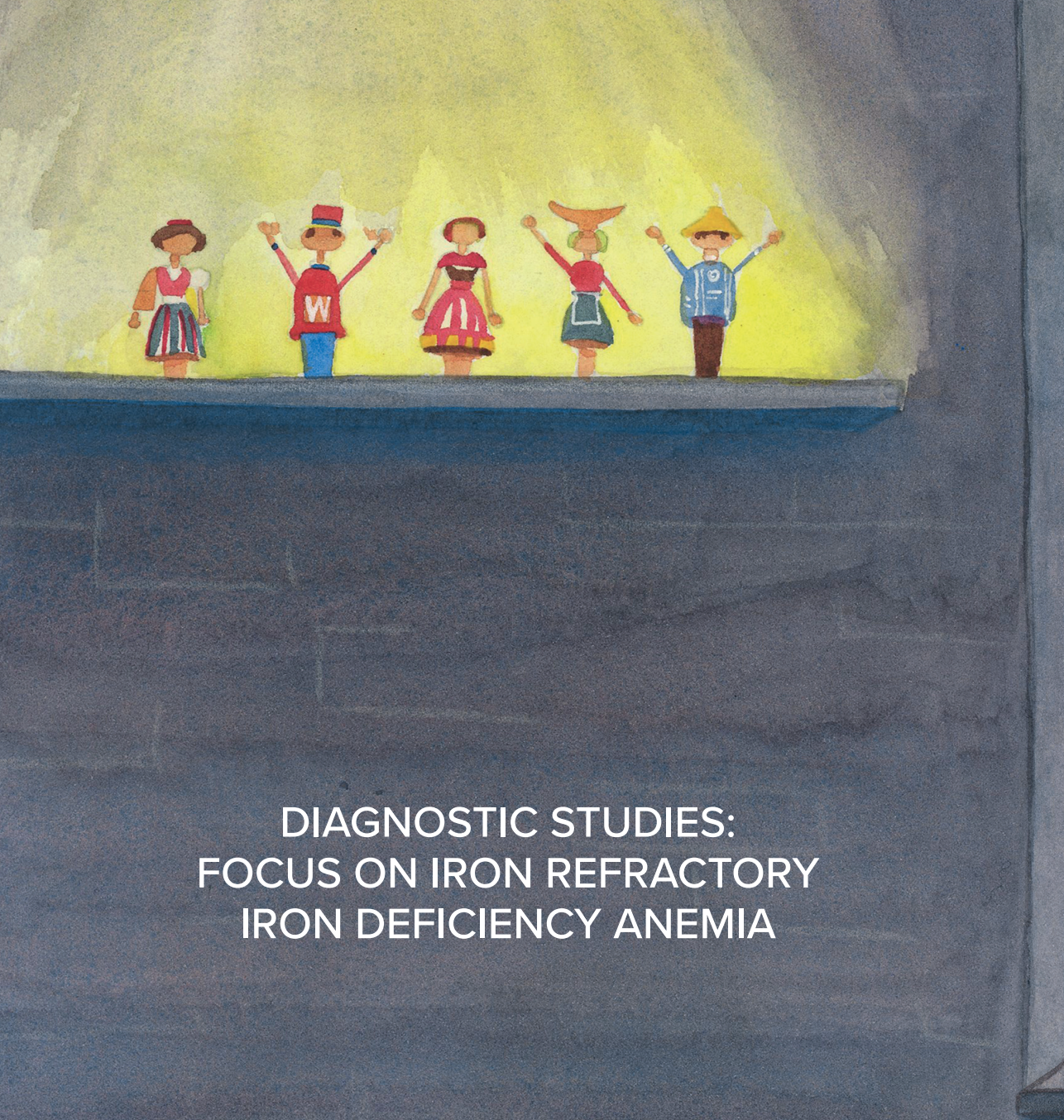
REFERENCES

1. Cox TC, Bottomley SS, Wiley JS, Bawden MJ, Matthews CS, May BK. X-linked pyridoxine-responsive sideroblastic anemia due to a Thr388-to-Ser substitution in erythroid 5-aminolevulinate synthase. *The New England journal of medicine*. 1994;330(10):675-679.
2. Cotter PD, Rucknagel DL, Bishop DF. X-linked sideroblastic anemia: identification of the mutation in the erythroid-specific delta-aminolevulinate synthase gene (ALAS2) in the original family described by Cooley. *Blood*. 1994;84(11):3915-3924.
3. Bottomley SS, Healy HM, Brandenburg MA, May BK. 5-Aminolevulinate synthase in sideroblastic anemias: mRNA and enzyme activity levels in bone marrow cells. *American journal of hematology*. 1992;41(2):76-83.
4. Cazzola M, May A, Bergamaschi G, Cerani P, Ferrillo S, Bishop DF. Absent phenotypic expression of X-linked sideroblastic anemia in one of 2 brothers with a novel ALAS2 mutation. *Blood*. 2002;100(12):4236-4238.
5. Barton JC, Lee PL. Disparate phenotypic expression of ALAS2 R452H (nt 1407 G --> A) in two brothers, one with severe sideroblastic anemia and iron overload, hepatic cirrhosis, and hepatocellular carcinoma. *Blood cells, molecules & diseases*. 2006;36(3):342-346.
6. Aivado M, Gattermann N, Rong A, et al. X-linked sideroblastic anemia associated with a novel ALAS2 mutation and unfortunate skewed X-chromosome inactivation patterns. *Blood cells, molecules & diseases*. 2006;37(1):40-45.
7. Cortesao E, Vidan J, Pereira J, Goncalves P, Ribeiro ML, Tamagnini G. Onset of X-linked sideroblastic anemia in the fourth decade. *Haematologica*. 2004;89(10):1261-1263.
8. Cazzola M, May A, Bergamaschi G, Cerani P, Rosti V, Bishop DF. Familial-skewed X-chromosome inactivation as a predisposing factor for late-onset X-linked sideroblastic anemia in carrier females. *Blood*. 2000;96(13):4363-4365.
9. Camaschella C. Hereditary sideroblastic anemias: pathophysiology, diagnosis, and treatment. *Seminars in hematology*. 2009;46(4):371-377.
10. May A, Bishop DF. The molecular biology and pyridoxine responsiveness of X-linked sideroblastic anaemia. *Haematologica*. 1998;83(1):56-70.
11. Cotter PD, May A, Li L, et al. Four new mutations in the erythroid-specific 5-aminolevulinate synthase (ALAS2) gene causing X-linked sideroblastic anemia: increased pyridoxine responsiveness after removal of iron overload by phlebotomy and coinheritance of hereditary hemochromatosis. *Blood*. 1999;93(5):1757-1769.
12. Sheftel A, Stehling O, Lill R. Iron-sulfur proteins in health and disease. *Trends in endocrinology and metabolism: TEM*. 2010;21(5):302-314.
13. Pippard MJ, Weatherall DJ. Iron absorption in non-transfused iron loading anaemias: prediction of risk for iron loading, and response to iron chelation treatment, in beta thalassaemia intermedia and congenital sideroblastic anaemias. *Haematologia*. 1984;17(1):17-24.
14. Flanagan SE, Patch AM, Ellard S. Using SIFT and PolyPhen to predict loss-of-function and gain-of-function mutations. *Genet Test Mol Biomarkers*. 2010;14(4):533-537.
15. Ng PC, Henikoff S. Predicting the effects of amino acid substitutions on protein function. *Annual review of genomics and human genetics*. 2006;7:61-80.
16. Sunyaev S, Lathe W, 3rd, Bork P. Integration of genome data and protein structures: prediction of protein folds, protein interactions and “molecular phenotypes” of single nucleotide polymorphisms. *Current opinion in structural biology*. 2001;11(1):125-

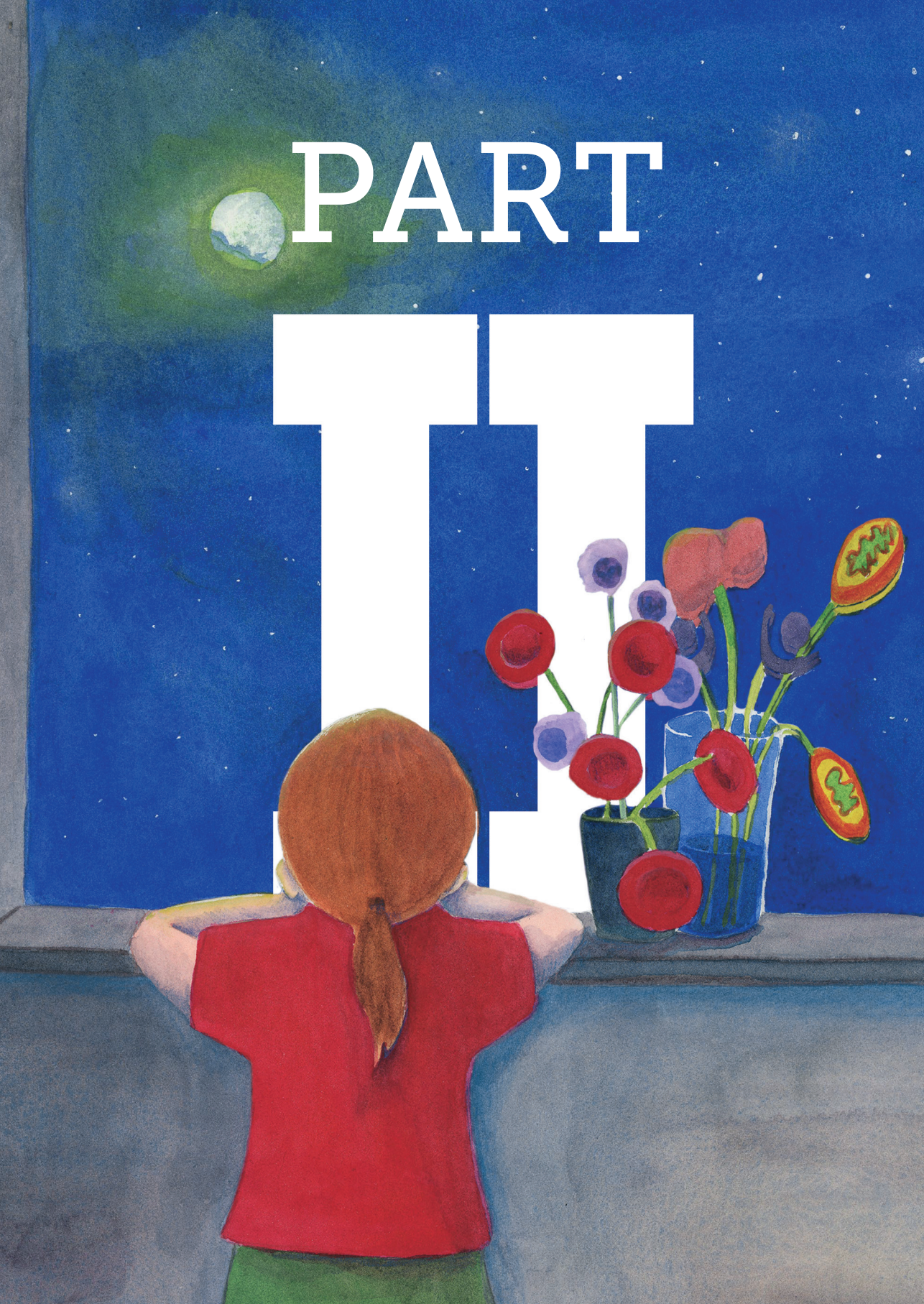
130.
17. Venselaar H, Te Beek TA, Kuipers RK, Hekkelman ML, Vriend G. Protein structure analysis of mutations causing inheritable diseases. An e-Science approach with life scientist friendly interfaces. *BMC bioinformatics.* 2010;11:548.
18. Cuijpers MLH, van Spronsen DJ, Muus P, Hamel BCJ, Swinkels DW. Need for early recognition and therapeutic guidelines of congenital sideroblastic anaemia. *International journal of hematology.* 2011;94(1):97-100.
19. Bulaj ZJ, Griffen LM, Jorde LB, Edwards CQ, Kushner JP. Clinical and biochemical abnormalities in people heterozygous for hemochromatosis. *The New England journal of medicine.* 1996;335(24):1799-1805.
20. Ploem JE. *Diagnostic test in iron overload*. Dissertation 1965, Utrecht, The Netherlands.
21. Bergmann AK, Campagna DR, McLoughlin EM, et al. Systematic molecular genetic analysis of congenital sideroblastic anemia: evidence for genetic heterogeneity and identification of novel mutations. *Pediatric blood & cancer.* 2010;54(2):273-278.
22. Ducamp S, Kannengiesser C, Touati M, et al. Sideroblastic anemia: molecular analysis of the ALAS2 gene in a series of 29 probands and functional studies of 10 missense mutations. *Human mutation.* 2011;32(6):590-597.
23. Kaneko K, Furuyama K, Fujiwara T, et al. Identification of a novel erythroid-specific enhancer for the ALAS2 gene and its loss-of-function mutation which is associated with congenital sideroblastic anemia. *Haematologica.* 2014;99(2):252-261.
24. Campagna DR, de Bie CI, Schmitz-Abe K, et al. X-linked sideroblastic anemia due to ALAS2 intron 1 enhancer element GATA-binding site mutations. *American journal of hematology.* 2014;89(3):315-319.
25. Tanno T, Porayette P, Sripichai O, et al. Identification of TWSG1 as a second novel erythroid regulator of hepcidin expression in murine and human cells. *Blood.* 2009;114(1):181-186.
26. Tanno T, Noel P, Miller JL. Growth differentiation factor 15 in erythroid health and disease. *Current opinion in hematology.* 2010;17(3):184-190.
27. Kautz L, Jung G, Valore EV, Rivella S, Nemeth E, Ganz T. Identification of erythroferrone as an erythroid regulator of iron metabolism. *Nature genetics.* 2014;46(7):678-684.
28. Kroot JJ, Tjalsma H, Fleming RE, Swinkels DW. Hepcidin in human iron disorders: diagnostic implications. *Clinical chemistry.* 2011;57(12):1650-1669.
29. Lee PL, Barton JC, Rao SV, Acton RT, Adler BK, Beutler E. Three kinships with ALAS2 P520L (c. 1559 C --> T) mutation, two in association with severe iron overload, and one with sideroblastic anemia and severe iron overload. *Blood cells, molecules & diseases.* 2006;36(2):292-297.
30. Barron R, Grace ND, Sherwood G, Powell LW. Iron overload complicating sideroblastic anemia--is the gene for hemochromatosis responsible? *Gastroenterology.* 1989;96(4):1204-1206.
31. Harris JW, Danish EH, Brittenham GM, McLaren CE. Pyridoxine responsive hereditary sideroblastic erythropoiesis and iron overload: two microcytic subpopulations in the affected male, one normocytic and one microcytic subpopulation in the obligate female carrier. *American journal of hematology.* 1993;42(4):400-401.
32. Furuyama K, Harigae H, Kinoshita C, et al. Late-onset X-linked sideroblastic anemia following hemodialysis. *Blood.* 2003;101(11):4623-4624.
33. Cotter PD, May A, Fitzsimons EJ, et al. Late-onset X-linked sideroblastic anemia. Missense mutations in the erythroid delta-aminolevulinate synthase (ALAS2) gene in two pyridoxine-responsive patients initially diagnosed with acquired refractory anemia and ringed sideroblasts. *The Journal of clinical investigation.* 1995;96(4):2090-2096.
34. Ohba R, Furuyama K, Yoshida K, et al.

Clinical and genetic characteristics of congenital sideroblastic anemia: comparison with myelodysplastic syndrome with ring sideroblast (MDS-RS). *Annals of hematology.* 2013;92(1):1-9.

35. Scott K, Zeris S, Kothari MJ. Elevated B6 levels and peripheral neuropathies. *Electromyography and clinical neurophysiology.* 2008;48(5):219-223.
36. Baumann Kreuziger LM, Wolanskyj AP, Hanson CA, Steensma DP. Lack of efficacy of pyridoxine (vitamin B6) treatment in acquired idiopathic sideroblastic anaemia, including refractory anaemia with ring sideroblasts. *European journal of haematology.* 2011;86(6):512-516.



DIAGNOSTIC STUDIES: FOCUS ON IRON REFRACTORY IRON DEFICIENCY ANEMIA


PART
II

Chapter 7

Standardized Serum Hepcidin Values in Dutch Children: Set Point relative to Body Iron changes during Childhood

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ABSTRACT

Background: Use of serum hepcidin measurements in pediatrics would benefit from standardized age- and sex-specific reference ranges in children, in order to enable the establishment of clinical decision limits that are universally applicable.

Procedure: We measured serum hepcidin-25 levels in 266 healthy Dutch children aged 0.3-17 years, using an isotope dilution mass spectrometry assay, standardized with our commutable 2nd reference material (RM), assigned by a candidate primary RM.

Results: We constructed age- and sex-specific values for serum hepcidin and its ratio with ferritin and transferrin saturation (TSAT). Serum hepcidin levels, hepcidin/ferritin and TSAT/hepcidin ratios were similar for both sexes. Serum hepcidin and hepcidin/ferritin ratio substantially declined after the age of 12 years, TSAT/hepcidin ratio gradually increased with increasing age. Serum hepcidin values for Dutch children < 12 years (n=170) and > 12 years (n=96) were 1.9 nmol/L (median); 0.1-13.1 nmol/L (p2.5-p97.5) and 0.9 nmol/l; 0.0-9.1 nmol/L, respectively. Serum ferritin was the most significant correlate of serum hepcidin in our study population, explaining 15.1 % and 7.9 % of variance in males and females, respectively. Multivariable linear regression analysis including age, blood sampling time, iron parameters, ALT, CRP and BMI as independent variables showed a statistically significant negative association between age as dichotomous variable (≤ 12 vs.>12 years) and log-transformed serum hepcidin levels in both sexes.

Conclusions: We demonstrate that serum hepcidin relative to indicators of body iron is age dependent in children, suggesting that the set point of serum hepcidin relative to stored and circulating iron changes during childhood.

Key Words
hepcidin, pediatric reference ranges, child, ferritin, transferrin saturation

INTRODUCTION

Hepcidin is a key regulatory hormone of systemic iron homeostasis by controlling iron absorption and bioavailability within the circulation.[1] In response to alterations in body iron demand, it is produced by hepatocytes and secreted into the circulation. It acts by binding the cellular iron exporter ferroportin, triggering its internalization and degradation, thereby inhibiting iron release from enterocytes and macrophages.[1]

Hepdicin production is tightly regulated to prevent both iron deficit and iron overload. Its levels are decreased by hypoxia, erythropoietic activity and reduced levels of both circulating and stored body iron.[1] In contrast, its levels are increased by inflammation and infection as a host defense mechanism. By sequestering iron within the reticulo-endothelial system, plasma iron content is reduced and consequently limits extracellular microbial growth.[1,2]

Dysregulation of hepcidin production contributes to the pathogenesis of several iron disorders.[2] Hepcidin is inappropriately low relative to stored iron in case of i) hereditary hemochromatosis[3] and ii) increased (especially ineffective) erythropoiesis[4] resulting in low hepcidin/ferritin ratios.[4] Conversely, hepcidin is inappropriately high relative to circulating transferrin bound iron in case of Iron Refractory Iron Deficiency Anemia (IRIDA) resulting in low TSAT/hepcidin ratios.[5,6]

Hepcidin levels reflect the integration of multiple key signals involved in iron regulation.[7] Therefore, its measurement is a promising clinical tool for the diagnosis and management of iron disorders involving iron deficiency, iron loading and iron maldistribution. Examples include 1) evaluation of suspected IRIDA[5,6,8]; 2) diagnosis of concomitant iron deficiency in patients with anemia of inflammation[9]; 3) prediction of responsiveness to oral iron therapy and guiding iron treatment under conditions of competing signals (anemia, iron deficiency, inflammation)[10-12]; 4) evaluation of suspected iron overload disorders[13]; 5) evaluation of iron deficiency (ID), before the occurrence of iron deficiency anemia (IDA), for example in pregnant women[14,15] in children[16,17] and in children with cystic fibrosis.[18]

Apart from diagnostic applications, multiple agents targeting the hepcidin/ferroportin axis are under development as novel therapeutics for iron disorders in adults.[19]

In order to use hepcidin as a diagnostic tool and a therapeutic target, reference values of the healthy population are crucial. For adults, reference ranges of serum hepcidin (and its ratio to ferritin and TSAT) are available for our assay.[20,21] They have been constructed based on data from the general adult population (N~3,000), and have recently been converted to standardized reference ranges.[21,22] These data have revealed that in both men and women serum hepcidin concentrations are strongly associated with serum ferritin[20,23] and only marginally with circulating iron (TSAT).[20] Serum hepcidin concentrations in men are stable over age while in women concentrations are lower in premenopausal than in postmenopausal women. This is in agreement with the observation that serum ferritin concentrations tend to increase as women progress through menopause.[20]

Implementation of serum hepcidin levels in clinical pediatrics is hampered by the lack of standardized reference values of healthy children from different age groups, relative to iron status.[24-26] Available studies concern either small series with limited age groups or series including children with (anemia of) inflammation.[16,27-31]

As a first step to the implementation of serum hepcidin measurements in pediatric clinical practice, we established age- and sex-specific standardized serum hepcidin values relative to body iron indicators in healthy Dutch children aged 0.3-17 years. Our observations suggest a changing set point of serum hepcidin relative to body iron indicators during childhood.

METHODS

Study population

Participants were consecutively enrolled at the Department of Pediatrics while attending Máxima MC, Veldhoven, the Netherlands (MMC) for either minor surgical interventions (e.g. correction of orchidopexy, removal of osteosynthesis material) or diagnostic procedures (e.g. MRI under sedation).

Inclusion criteria were age 0 -<18 years, need for a venipuncture or intravenous drip placement for the purpose of general anesthesia or sedation and both oral and written informed consent for inclusion in the study. Exclusion criteria were known iron deficiency anemia, any systemic underlying disease (malignancy, asthma, diabetes, congenital heart disease, kidney failure, congenital immunodeficiency, etc.), acute infection, trauma or operation <48 hours ago, inflammation, infection (CRP> 5 mg/L), ALT> 40 IU/L or treatment with iron preparations. Children admitted for ear tube insertion, adenotomy or adenotonsillectomy because of recurrent infections of the upper airways were not included in the study. All children that were enrolled in the study visited the hospital for elective surgery or diagnostic procedures. Therefore, none of the children had fever since this is a contra-indication for receiving general anesthesia or sedation.

Between July 2016 and April 2018, we obtained informed consent for participation in the study for 317 children. Forty-one children were excluded because of failed blood withdrawal (n=23), CRP > 5 mg/L (n=14), underlying disorders (n=2), double inclusion (n=2), ID (n=4) or IDA (n=6), according to WHO definition: ID, ferritin < 12-15 µg/L; IDA, Hb < 11-13 g/dL in combination with ferritin < 12-15 µg/L; specific cutoffs dependent on age.[32-34]

This resulted in the inclusion of 266 healthy children (157 males, 109 females) aged 0.3 -17 years (Table 1).

Blood sampling was performed (0.5 mL EDTA tube, 3.5 mL serum tube) before the surgical or diagnostic procedure, between 7:30 AM and 6 PM, after 4-6 hours of fasting because of perioperative fasting guidelines. Participants or their representatives filled out a questionnaire on medical history, general health and medication use as part of the anesthesia screening procedure.

Since we aimed to cover the different key periods of human growth and development for both sexes, we divided our study population in subgroups for age and sex; infancy and toddler stage (0- <2 years), early childhood (2-<6 years), middle childhood (6 -<12 years) and adolescence (12-17 years).[35] Because of limited numbers, infants and toddlers were merged into one group.

Body mass index was determined and interpreted according to international standards, which describe age-dependent cut off points for underweight, normal weight, overweight and obesity.[36]

The study was conducted according to the principles of the Declaration of Helsinki and approved by the Local Ethics Committee of the Máxima Medical Center, Veldhoven, the Netherlands (MMC). For all participants oral and written informed consent was obtained.

Laboratory Methods

We measured levels of the bioactive form of hepcidin: (hepcidin-25[2]) by weak cation exchange chromatography followed by time of flight mass spectrometry (WCX -MS-TOF) as described before.[37] Our hepcidin assay was recently standardized using 2nd reference material (RM) that was value assigned by a provisional primary RM.[22] For additional information on the laboratory methods see the Supplemental Files.

Statistical Analysis

Descriptive statistics were reported as medians, 2.5th -97.5th percentiles and/or ranges for continuous variables and as absolute frequencies and percentages for categorical variables, using original untransformed values.

Normality of distributions of the continuous variables was visually checked using histograms. A logarithmic transformation was applied to normalize the non-normal distributions of serum hepcidin, ferritin, CRP and hepcidin/ferritin ratio and TSAT/ hepcidin ratio. For hepcidin values below the lower detection limit of 0.5 nM, imputation was performed with a random value between 0 and 0.5 nM.

Table 1. Demographic, clinical and laboratory characteristics of the study population (N=266)

	Males (n=157)	Females (n=109)	*p*
Demographic and clinical characteristics			
Age (range)	9 (0-17)	11.5 (1-17)	0.003
Ethnicity			
Western-European	144 (92)	105 (96)	0.131
Mediterranean	13 (8)	4(4)	0.131
BMI[a]			
Underweight	3 (2)	6 (5)	0.111
Normal weight	131 (83)	91 (84)	0.922
Overweight	18 (12)	10 (9)	0.549
Obese	5 (3)	2 (2)	0.499
Time of blood sampling			
Between 7.30AM and-12 PM	76 (48)	50 (46)	0.684
Between 12 PM and 3 PM	58 (37)	44 (40)	0.572
Between 3 PM and 6 PM	23 (15)	15 (14)	0.839
Laboratory characteristics			
Hemoglobulin, g/dL	12.6 (10.4-15.5)	12.6 (10.5-15.1)	0.837
Ht, L/L	0.4 (0.3-0.5)	0.4 (0.3-0.5)	0.706
Reticulocytes, x10^9/L	47.5 (26.9- 78.4)	52.0 (25.0-106.8)	0.209
MCV, fL	82.0 (72.0-90.0)	84.0 (76.8-94.8)	0.000
MCH, fmol	1.8 (1.5-1.9)	1.8 (1.6-2.0)	0.301
Ferritin, µg/L	41.0 (15.9-98.1)	40.0 (16.5-112.8)	0.937
Iron, µmol/L	16.0 (3.0-26.2)	15.5 (4.0-29.3)	0.903
TIBC, µmol/L	64.5 (50.0-83.0)	66.0 (47.0-86.5)	0.194
TSAT, %	24.0 (9.5-45.0)	23.0 (6.6-45.5)	0.978
sTfR, mg/L	1.3 (0.9-2.0)	1.2 (0.8-1.9)	0.082
ALT, IU/L	18.0 (11.0-42.3)	17.0 (9.8-31.2)	0.229
CRP[b], mg/L	0.2 (0.0-3.7)	0.3 (0.0-4.4)	0.209

Data are given as median for continuous variables and as n for categorical variables. For the continuous variables values in parenthesis refer to ranges (age) or p2.5-p97.5, for the categorical variables values in parenthesis refer to percentages.

p values for continuous variables were calculated with independent sample median test, *p* values for categorical values were calculated with Chi Square test.

[a] BMI was assessed according to international standards established by Cole *et al*[36]

[b] In a 3 year old male, CRP was missing. Hepcidin was low (0.58 nM) in this child, arguing against inflammation. Therefore this case was not excluded although CRP>5 mg/L was an exclusion criterion for the study.

Abbreviations: BMI denotes Body Mass Index

Ranges for (untransformed) serum hepcidin concentration, stratified by age groups and sex, were constructed using the median and p25-p75. Univariable and multivariable linear regression analyses were used to evaluate the associations between (log-transformed) serum hepcidin concentrations, log-transformed hepcidin/ferritin ratio, TSAT/hepcidin ratio and selected (biochemical) variables, unadjusted and adjusted for age and time of blood sampling. The assumption of linearity between serum hepcidin concentrations and independent variables was confirmed using graphic methods. Resulting regression coefficients (ß) express the change in log-transformed serum hepcidin that are associated with a 1-unit change in the independent variable. Some of the independent variables were log-transformed as well; the interpretation of the regression coefficients for these variables is as follows: a 1% change in the independent variable corresponds to a β % change in serum hepcidin. Explained variances (R^2) were obtained to indicate the amount of variance in hepcidin concentration that was explained by the included variables.

Analyses were stratified by sex because of sex-specific differences in hepcidin levels and iron homeostasis that have been described earlier.[20,26,27,30,38,39]

SPSS version 22 was used for data analysis.

Data Sharing Statement

For original data, please contact dorine.swinkels@radboudumc.nl.

RESULTS

Characteristics of the study population

Characteristics of the study population are described in Table 1. Fifty-nine percent (n=157) of the total study population was male. Median age of males was 9 years (range 4 months – 17 years); median age of females 11 years (range 1 – 17 years).

Hb, CRP and iron indicators for the different age groups (0 - < 2 years, 2 - < 6 years, 6 - < 12 years, 12-<18 years) are presented in Supplemental Table 1. Data on blood sampling time of hepcidin and BMI of the participants are presented and discussed in Supplemental Figure 1 and Supplemental Figure 2.

Age- and sex-specific reference ranges for serum hepcidin concentrations

Reference ranges (p25- p75) for serum hepcidin remained constant until the age of 12 years (independent median sample test, males *p* 0.886, females *p* 0.712) but were substantially lower after the age of 12 years in both males and females (independent sample median test, males *p* 0.003; females *p* 0.016) (Table 2 and Supplemental Table 2).

These results were confirmed with unadjusted univariable linear regression analysis for <12 years versus >12 years (males: β= -0.49; 95 % confidence interval [CI] -0.66– -0.31, females: β= -0.26; 95% CI -0.46– -0.05) (Supplemental Table 3).

Hepcidin concentration was below the detection limit of 0.5 nmol/l in 26 out of 157 males (17%) and 19 out of 109 females (17%) (Table 2). The wide reference ranges reflect the substantial inter-individual variation of serum hepcidin concentrations.

Because of the earlier described sex-specific differences in both adults and children[20,26,27,30,39] regarding serum hepcidin levels, all analyses were stratified for sex. As shown in Table 2 and Supplemental Table 2, we did not observe a difference in serum hepcidin levels between males and females. Multivariate analysis including age, sampling time, BMI, sex, iron parameters, CRP and ALT confirmed that sex was not an independent correlate of serum hepcidin (*p* 0.127).

Table 2. Reference ranges for serum hepcidin, ferritin/hepcidin ratio and TSAT/hepcidin ratio per age group and sex

		Males		
Hepcidin[a] (nmol/L)				
Age (years)	**n(%)**	**Median**	**p25-p75**	**Min-Max**
0 - < 2	11 (7)	1.8	1.1-7.3	0.9-20.9
2 - < 6	29 (19)	2.0	1.0-3.3	0.1-7.0
6 - < 12	71 (45)	1.7	0.9-3.4	0.1-15.5
12 - < 18	46 (29)	0.7	0.3-1.8	0.0-9.3
Total	157 (100)	1.5	0.7-3.0	0.0-20.9
Hepcidin/ferritin (pmol/µg)				
Age (years)				
0 - < 2	11 (7)	49.7	34.5-124.0	11.4-302.6
2 - < 6	28 ((18)	54.0	36.9-94.8	4.6-216.5
6 - < 12	71 (46)	43.7	23.8-82.6	2.9-249.6
12 - < 18	46 (29)	18.2	5.6-31.3	0.5-106.0
Total	156 (100)	35.0	18.8-67.4	0.5-302.6
TSAT/hepcidin (%/nmol/L)				
Age (years)				
0 - < 2	11 (7)	11.4	2.5-13.8	0.2-22.4
2 - < 6	28 (18)	10.9	6.6-24.3	2.5-230.9
6 - < 12	71 (46)	14.0	7.4-25.9	0.5-377.6
12 - < 18	45 (29)	32.8	14.5-77.5	1.6-1855.2
Total	155 (100)	15.2	8.2-31.6	0.2-1855.2

[a] Hepcidin was below the detection limit of 0.5 nM in 26 out of 157 males (17%): 0-<2 years: 0 out of 11 (0%), 2-<6 years: 2 out of 29 (7%), 6-<12 years: 6 out of 71 (8%), 12-17 years: 18 out of 46 (39%) Hepcidin was below the detection limit in 19 out of 109 females (17%): (0-<2 years: 1 out of 6 (2%), 2-<6 years: 7 0 out of 18 (0%),, 6-<12 years: 6 out of 35 (17%), 12-17 years: 12 out of 50 (24%)

Abbreviations: Min-Max denotes minimum-maximum

Females				
n(%)	**Median**	**p25-p75**	**Min-Max**	*p*
6 (5)	1.8	0.9-7.5	0.2-14.1	1,00
18 (17)	2.0	1.1-3.8	0.7-9.5	0.853
35(32)	2.0	0.9-3.9	0.0-18.6	0.680
50(46)	1.0	0.5-2.2	0.0-11.4	0.409
109 (100)	1.5	0.8-2.9	0.0-18.6	1.000
6 (6)	59.7	29.2-203.4	7.8-261.7	1.000
18 (17)	71.6	38.9-143.9	19.8-399.1	0.365
35 (32)	42.5	24.9-87.6	0.5-166.9	1.000
49 (45)	22.8	12,8-40.0	0.6-151.9	0.354
108	35.7	19.0-73.2	0.5-399.1	0.900
6 (6)	9.3	3.6-13.0	2.8-37.8	0.620
17 (16)	13.2	4.6-20.3	2.9-44.4	0.908
33 (32)	13.2	6.7-27.9	1.2-869.5	1.000
48 (46)	24.9	14.3-44.4	0.5-765.2	0.606
104 (100)	15.6	7.5-34.2	0.4-869.5	0.666

Biochemical correlates of serum hepcidin concentrations

We performed also regression analyses adjusted for age and sampling time because of the demonstrated correlation with age and the earlier described circadian rhythm of serum hepcidin levels.[20,40] In these adjusted analyses, ferritin was most strongly associated with serum hepcidin concentration (ß= 1.31; 95 % CI 0.95–1.68 and ß= 0.92; 95% CI 0.47–1.77 for males and females respectively) and was responsible for the largest amount of explained variance (adjusted R^2 38.8 % and 19.9 %, R^2 for age and sampling time alone 18.9 % and 7.8 % in males and females respectively)(Table 3).

These results indicate that a 1% change in serum ferritin in micrograms per liter was associated with a 1.31% and 0.92% change in serum hepcidin concentration (nmol/l) in males and females respectively.

We observed a negative association between Total Iron Binding Capacity (TIBC) and serum hepcidin concentration for both sexes. CRP demonstrated a positive association with serum hepcidin in both males and females.

A sex-specific multivariable regression model was constructed including age as a dichotomous variable (<12 years vs > 12 years), time of blood sampling and those variables that were significantly ($p < 0.1$) associated with serum hepcidin concentrations after adjustment for age and time of blood sampling. For males these were ferritin, TIBC, ALT, CRP and BMI; for females these were ferritin, TIBC and CRP. In males, independent correlates ($p<0.1$) were age, sampling time, ferritin, underweight BMI and CRP; in females age, sampling time > 3 PM, ferritin. In males, the model explained 40.9 % of the serum hepcidin variation, in females 16.9% (Table 4).

Age- and sex-specific reference ranges for hepcidin/ferritin and TSAT/hepcidin ratios

Reference ranges (p.25- p75), minimum and maximum values of hepcidin/ferritin and TSAT/hepcidin ratios are given per age group and sex in Table 2. We chose the TSAT/hepcidin ratio instead of other earlier reported variants, e.g. TSAT/(log)hepcidin ratio[6] since this ratio was superior in discriminating IRIDA patients from IDA non-IRIDA patients in a study we recently performed in adults in our institution (unpublished results, Netherlands Trial Register, Trial NL 6845).[41]

Univariable linear regression analysis (Supplemental Table 4, Supplemental Table 5) showed that hepcidin/ferritin decreased and TSAT/hepcidin increased with aged in both sexes.

Both effects were stronger in males compared to females.

Biochemical correlates of hepcidin/ferritin and TSAT/hepcidin ratios
Regression analyses for the hepcidin/ferritin and TSAT/hepcidin ratios adjusted for age and time of blood sampling are presented in Table 5 and Table 6, respectively. R^2 for age as a continuous variable and sampling time as a categorical variable alone was 26.8 % and 13.6% and 21.3% and 6.5% for the log-transformed hepcidin/ferritin and TSAT/hepcidin ratio in males and females respectively.

Sex-specific multivariable models were constructed for both ratios including age (as a categorical variable; age groups) and time of blood sampling and those variables that were significantly associated with these ratios after adjustment for age and time of blood sampling.

The hepcidin/ferritin ratio was significantly correlated with CRP and underweight BMI in males and with CRP in females, after correction for age and time of blood sampling (Table 5). The sex-specific model with the relevant correlates explained 32.9 % of the hepcidin/ferritin ratio variation in males and 14.3 % in females (Supplemental Table 6).

The TSAT/hepcidin ratio was statistically significantly correlated with multiple biochemical correlates in both males and females after adjustment for age and time of blood sampling (Table 6). The sex-specific multivariable models including these parameters explained 46.0 % and 22.8 % of the TSAT/hepcidin ratio variation in males and females respectively (Supplemental Table 7).

Table 3. Results of linear regression models for serum hepcidin concentrations (nmol/L) adjusted for age and time of blood sampling and stratified by sex

	Males				
		95% CI			
Variable	**Beta[a]**	**Lower limit**	**Upper limit**	**R^2, %[b]**	*p*
Hb, g/dL	0.018	-0.086	0.121	18.4	0.737
Reticulocytes, x10^9/L	0.001	-0.005	0.007	18.7	0.668
MCV, fL	0.002	-0.022	0.026	18.4	0.879
Ferritin, µg/L[d]	1.313	0.946	1.679	38.8	0.000
Iron, µmol/L	0.005	-0.011	0.021	17.6	0.517
TIBC, µmol/L	-0.009	-0.018	0.001	20.3	0.071
TSAT, %	0.006	-0.004	0.015	18.2	0.233
sTfR, mg/L	-0.216	-0.526	0.094	19.3	0.170
ALT, IU/Ll[d]	0.522	-0.024	1.067	20.2	0.061
CRP, mg/L[d,e]	0.229	0.106	0.352	25.3	0.000
BMI[f]					
Normal weight	ref	ref	ref		
Underweight	0.484	-0.087	1.056	20.5	0.096
Overweight	0.196	-0.050	0.442		0.118
Adipose	0.254	-0.194	0.702		0.264

Adjustment for age was done with age as a continuous variable; adjustment for sampling time was done with sampling time as a categorical variable (7.30 AM-12 PM, 12-3 PM, 3-6 PM).

[a] Beta expresses the change in the dependent variable -log-transformed serum hepcidin- that is associated with a 1-unit change in the independent variable. Independent variables marked with [d] were log-transformed as well; the interpretation of the regression coefficients for these variables is as follows: a 1% change in the independent variable corresponds to a beta % change in serum hepcidin

[b] For males multivariate linear regression model with log-transformed hepcidin as dependent variable and age (continuous variable) and sampling time as independent variables showed: R^2 18.9 %, with ß: -0.048, CI -0.065– -0.030 (*p* 0.000) ß: 0.254, CI 0.081– 0.426 (*p* 0.004), ß: 0.496, CI 0.255–0.737

Females				
	95% CI			
Beta[a]	Lower limit	Upper limit	R^2, %[c]	*p*
0.046	-0.056	0.148	7.7	0.374
-0.001	-0.007	0.005	7.0	0.816
-0.016	-0.047	0.014	7.9	0.294
0.919	0.468	1.371	19.9	0.000
0.008	-0.010	0.026	7.0	0.402
-0.015	-0.026	-0.004	13.2	0.007
0.009	-0.002	0.021	8.6	0.121
0.211	-0.237	0.660	7.7	0.352
-0.234	-1.031	0.563	7.5	0.561
0.148	-0.001	0.297	10.3	0.052
ref	ref	ref		
-0.022	-0.482	0.457	5.5	0.923
0.102	-0.259	0.461		0.575
-0.102	-0.865	0.661		0.791

(*p* 0.000***)*** for age, sampling time 12-3 PM and sampling time 3-6 PM respectively; [c] For females, multivariate linear regression model with log-transformed hepcidin as dependent variable and age (continuous variable) and sampling time as independent variables showed: R^2 7.8 %, with ß: -0.033, CI -0.055– -0.010 (*p* 0.004), ß: 0.196, CI -0.023– 0.415 (*p* 0.079), ß: 0.246, CI -0.064–0.556 (*p* 0.119) for age, sampling time 12-3 PM and sampling time 3-6 PM respectively

[e] CRP levels of children were between > 0.1 and < 5 mg/L

[f] BMI was assessed according to international standards established by Cole *et al*[36]

Abbreviations: ref denotes reference category

Table 4. Results of multivariable linear regression analyses for serum hepcidin concentrations (nmol/L) stratified by sex

		Males			
			95% CI		
Variable		**Beta[a]**	**Lower limit**	**Upper limit**	*p*
Age, years	<12 12-<18	-0.579	-0.736	-0.423	0.000
Time Blood sampling	7.30 AM-12 PM	ref	ref	ref	
	12 -3 PM	0.143	-0.008	0.295	0.063
	3- 6 PM	0.369	0.158	0.580	0.001
Ferritin, µg/L[b]		1.014	0.604	1.425	0.000
TIBC, µmol/L		0.000	-0.009	0.010	0.950
ALT, IU/L[b]		0.091	-0.440	0.622	0.736
CRP, mg/L[b, c]		0.107	-0.017	0.230	0.091
BMI[d]					
Normal weight		ref	ref	ref	
Underweight		0.538	0.027	1.048	0.039
Overweight		0.106	-0.125	0.337	0.366
Adipose		0.081	-0.335	0.497	0.702

[a] Beta expresses the change in the dependent variable -log-transformed hepcidin- that is associated with a 1-unit change in the independent variable. Independent variables marked with [b]were log-transformed as well; the interpretation of the regression coefficients for these variables is as follows: a 1% change in the independent variable corresponds to a beta % change in serum hepcidin

[c] CRP levels of children were between > 0.1 and < 5 mg/L

[d] BMI was assessed according to international standards established by Cole *et al* [36]

Abbreviations: na denotes not applicable; ref, reference category

Females			
	95% CI		
Beta[a]	Lower limit	Upper limit	*p*
-0.240	-0.441	-0.040	0.019
ref	ref	ref	
0.157	-0.052	0.367	0.140
0.305	0.003	0.607	0.048
0.604	0.110	1.099	0.017
-0.010	-0.021	0.002	0.107
na	na	na	na
0.095	-0.050	0.240	0.197
ref	ref	ref	
na	na	na	na
na	na	na	na
na	na	na	na

Table 5. Results of linear regression models for hepcidin/ferritin ratio (pmol/µg) adjusted for age and time of blood sampling and stratified by sex

	Males				
		95% CI			
Variable	Beta[a]	Lower limit	Upper limit	R^2, %[b]	*p*
Hb, g/dL	-0.007	-0.098	0.084	26.3	0.885
Reticulocytes, x10^9/L	0.000	-0.005	0.005	26.3	0.927
MCV, fL	-0.007	-0.029	0.015	26.5	0.515
Iron, µmol/L	0.009	-0.005	0.023	26.0	0.216
TIBC, µmol/L	-0.003	-0.012	0.005	26.5	0.469
TSAT, %	0.006	-0.002	0.014	26.2	0.165
sTfR, mg/L	-0.119	-0.408	0.170	26.3	0.417
ALT, IU/L[d]	0.162	-0.321	0.645	26.5	0.508
CRP, mg/L[d,e]	0.134	0.023	0.244	29.0	0.018
BMI[f]					
Normal weight	ref	ref	ref		
Underweight	0.610	0.112	1.108	29.0	0.017
Overweight	0.169	-0.045	0.383		0.121
Adipose	0.019	-0.370	0.409		0.922

Adjustment for age was done with age as a continuous variable; adjustment for sampling time was done with sampling time as a categorical variable (7.30 AM-12 PM, 12-3 PM, 3-6 PM).

[a]Beta expresses the change in the dependent variable -log-transformed serum hepcidin/ferritin ratio- that is associated with a 1-unit change in the independent variable. Independent variables marked with [d]were log-transformed as well; the interpretation of the regression coefficients for these variables are as follows: a 1% change in the independent variable corresponds to a beta % change in hepcidin/ferritin ratio

[b] For males multivariate linear regression model with log-transformed hepcidin/ferritin ratio as dependent variable and age (continuous variable) and sampling time as independent variables showed: R^2 26.8%, with ß -0.057, CI -0.072- -0.041 (*p* 0.000), ß 0.175, CI 0.023-0.326 (*p* 0.024), ß 0.432, CI 0.221-0.643 (*p* 0.000)\

Females				
		95% CI		
Beta[a]	Lower limit	Upper limit	R^2, %[c]	*p*
0.014	-0.082	0.109	12.9	0.775
-0.003	-0.009	0.003	13.6	0.339
-0.008	-0.037	0.021	13.0	0.593
0.000	-0.016	0.017	11.7	0.958
-0.005	-0.016	0.005	13.6	0.331
0.002	-0.009	0.013	11.9	0.741
0.219	-0.203	0.641	13.7	0.306
-0.505	-1.241	0.230	14.3	0.176
0.119	-0.021	0.259	15.1	0.094
ref	ref	ref		
-0.006	-0.434	0.422	11.6	0.979
0.135	-0.201	0.471		0.427
-0.015	-0.726	0.696		0.967

for age, sampling time 12-3 PM and sampling time 3-6 PM respectively; [c] for females, multivariate linear regression model with log-transformed hepcidin/ferritin ratio as dependent variable and age (continuous variable) and sampling time as independent variables showed: R^2 13.6 %, with ß -0.042, CI -0.063- -0.021 (*p* 0.000), ß 0.179, CI 0.026-0.384 (*p* 0.087), ß 0.250, CI -0.039-0.539 (*p* 0.089) for age, sampling time 12-3 PM and sampling time 3-6 PM respectively

[e] CRP levels of children were between > 0.1 and < 5 mg/L

[f] BMI was assessed according to international standards established by Cole *et al*[36]

Abbreviations: ref denotes reference category

Table 6. Results of linear regression models for TSAT/hepcidin ratio (%/nmol/L) adjusted for age and time of blood sampling and stratified by sex

	Males				
		95% CI			
Variable	**Beta[a]**	**Lower limit**	**Upper limit**	**R^2, %[b]**	*p*
Hb, g/dL	-0.002	-0.112	0.108	22.9	0.971
Reticulocytes, x10^9/L	-0.004	-0.010	0.002	21.7	0.184
MCV, fL	0.012	-0.014	0.039	21.2	0.354
Ferritin, µg/L[d]	-1.325	-1.716	-0.934	39.0	0.000
Iron, µmol/L	0.024	0.007	0.040	24.9	0.005
TIBC, µmol/L	0.004	-0.006	0.014	21.1	0.420
sTfR, mg/L	-0.050	-0.398	0.297	20.8	0.775
ALT, IU/L[d]	-0.726	-1.295	-0.158	24.0	0.013
CRP, mg/L[d,e]	-0.338	-0.462	-0.213	33.5	0.000
BMI[f]					
Normal weight	ref	ref	ref		
Underweight	-0.674	-1.266	-0.082	25.1	0.026
Overweight	-0.280	-0.535	-0.025		0.031
Adipose	-0.305	-0.768	0.158		0.195

Adjustment for age was done with age as a continuous variable; adjustment for sampling time was done with sampling time as a categorical variable (7.30 AM-12 PM, 12-3 PM, 3-6 PM).

[a] Beta expresses the change in the dependent variable – log transformed TSAT/hepcidin ratio- that is associated with a 1-unit change in the independent variable. Independent variables marked with [d] were log-transformed; the interpretation of the regression coefficients for these variables is as follows: a 1% change in the independent variable corresponds to a beta % change in the TSAT/hepcidin ratio

[b] For males multivariate linear regression model with log transformed TSAT/hepcidin as dependent variable and age (continuous variable) and sampling time as independent variables showed: R^2 21.3 %, with ß: 0.059, CI 0.040– 0.077 (*p* 0.000), ß: -0.168, CI -0.350– 0.015 (*p* 0.071), ß: -0.484, CI -0.738–

Females				
	95% CI			
Beta[a]	Lower limit	Upper limit	R^2, %[c]	*p*
-0.008	-0.119	0.103	5.5	0.885
-0.003	-0.004	0.010	6.1	0.413
0.027	-0.005	0.060	8.0	0.102
-0.703	-1.195	-0.211	12.6	0.006
0.019	0.000	0.037	9.2	0.048
0.009	-0.003	0.021	7.4	0.162
-0.452	-0.926	0.023	8.8	0.062
0.466	-0.392	1.324	6.6	0.284
-0.217	-0.374	-0.060	12.1	0.007
ref	ref	ref		
-0.131	-0.390	0.652	4.5	0.620
-0.159	-0.539	0.220		0.407
-0.023	-0.824	0.788		0.955

-0.231 (*p* 0.000) for age, sampling time 12-3 PM and sampling time 3-6 PM respectively; [c] For females, multivariate linear regression model with log transformed TSAT/hepcidin as dependent variable and age (continuous variable) and sampling time as independent variables showed: R^2 6.5 %, with ß: 0.037, CI -0.013– 0.061 (*P* 0.003), ß: -0.103, CI -0.340– 0.133 (*p* 0.388), ß: -0.152, CI -0.487–0.183 (*p* 0.370) for age, sampling time 12-3 PM and sampling time 3-6 PM respectively

[e] CRP levels of children were between > 0.1 and < 5 mg/L

[f] BMI was assessed according to international standards established by Cole *et al*[36]

Abbreviations: ref denotes reference category

DISCUSSION

Our data provide age- and sex-specific values for hepcidin-25 and its ratios to parameters reflecting circulating and stored iron for healthy Dutch children, as assessed by a standardized assay. These values can be used as for any other hepcidin assay worldwide that is standardized using the same 2nd RM for calibration.[22]

We found no difference between serum hepcidin levels between males and females. Serum ferritin as a reflection of iron stores was the most important correlate of serum hepcidin, however the variance in serum hepcidin levels that could be explained by serum ferritin levels was considerably lower (R^2 15.1 for males, 7.9% for females) than for adults (R^2 56 for men, 60% for women).[20] Although mice studies suggest that circulating iron also influences serum hepcidin concentration,[42,43] TSAT was only marginally associated with serum hepcidin levels, as seen in adults.[20]

We obtained the serum hepcidin/ferritin ratio and the TSAT/hepcidin ratio to get insights in the set off of the hepcidin regulatory pathway relative to stored iron (ferritin) and circulating iron (TSAT) with aging, respectively, as reported before.[6]

Interestingly, serum hepcidin relative to ferritin and TSAT was lower for older compared to younger children and also compared to adults.[21] This was reflected in a lower hepcidin/ferritin ratio and an increased TSAT/ hepcidin ratio, which implies that the extent of induction of the different regulatory pathways of hepcidin by both iron stores and circulating iron in healthy children and adolescents are dependent on age.[42,43]

These age-specific differences in hepcidin set points might be an expression of the two opposite operating forces considering iron metabolism. Iron is indispensable for the production of heme proteins and for other vital functions requiring a persistent flow of iron into the blood stream, especially during periods of rapid growth during infancy. A recent study of Armitage *et al* indeed observed that antecedent weight gain was negatively associated with serum hepcidin levels in Gambian infants.[39] However, the virulence of infectious organisms depends on their ability to assimilate iron from their host. Therefore, humans and other mammals have to cope with piracy of iron by pathogens in order to battle against infections.[44] Since hepcidin has the

ability to shut down ferroportin and limit the export of iron from the intestines and the macrophages into the circulation, it has an important role in the innate immune system.[45]

We hypothesize that the high hepcidin levels in young children, relative to adolescents might result in a survival advantage during a critical period of high vulnerability for serious infections.[44,45]

We observed a positive correlation of CRP levels (between 0.1 and 5 mg/L) with serum hepcidin levels, suggesting that, as described earlier,[46] even minor infections and/or inflammation induce hepcidin production in children, consistent with the suggested protective antimicrobial activity of hepcidin.[2] Importantly, the relatively high hepcidin levels for ferritin in young children as compared to adolescents did not result in IDA in these subjects, despite the enormous growth during infancy that is accompanied by a considerable increase of circulating blood volume and other tissues requiring iron. Studies in suckling mice suggest that enterocyte ferroportin is hyporesponsive to hepcidin during infancy.[47] Alterations to ferroportin that prevent hepcidin binding during suckling may allow iron absorption to remain sufficient regardless of hepcidin expression levels, reducing the likelihood of iron deficiency during development.[47] Whether in young children ferroportin is still functional in the presence of increased hepcidin levels, as seen in infant mice, remains to be investigated.

In both males and females hepcidin levels were considerably lower after the age of 12 years, dropping below normal adult levels[21], suggesting a different regulation of hepcidin production during adolescence. We suggest that the decrease in hepcidin levels in older children can be attributed to the direct influence of the gonadal hormones testosterone, [48] estrogens, [49] and also to the indirect influence of these hormones since both testosterone and estrogen stimulate growth hormone/IGF-1 secretion, which in turn inhibits hepcidin production.[50] The relatively low hepcidin levels in relation to body iron status in post-pubertal compared to pre-pubertal children might reflect an adaptation in order to guarantee sufficient iron in this period of rapid development and maturation.

Compared with adults, young children have relatively high and children > 12 years relatively low hepcidin/ferritin ratios.[21] TSAT/hepcidin ratios in our population are comparable to those in adults until the age of 12 years. Thereafter we observe

a significant increase.[21] Altogether, our data suggest that the set point of serum hepcidin relative to indicators of stored and circulating iron changes during human growth and development.

Until now, lack of harmonization and standardization of hepcidin measurements[51] has hampered comparison with other hepcidin reference studies in children.[24,26,27]To some extent, comparison is possible with the data obtained by Uijterschout *et al* [25] from children aged 0.5-3 years (n=400). They used the same assay as we did, before standardization. However, standardization only slightly altered its values, i.e. standardized results were found to be a factor 1.054351 higher compared to historic results obtained without standardization. In their study median hepcidin was slightly higher (3.6 nmol/L; p2.5-p97.5 0.6-13.9 nmol/L) than in our age group of 0-<2 years (n=17) (1.8 nmol/L; p2.5-p75 0.2-6.3 nmol/L), with overlapping ranges. The finding of ferritin as the most important indicator of serum hepcidin concentration after adjustment for sampling time, and age was consistent between the study of Uijterschout *et al* and our study.

The strength of our study is the unique and well-defined population (e.g. no underlying disease, CRP< 5 mg/L), covering the range from infancy to adolescence. We established hepcidin values relative to indicators of iron status, described in ratios, enabling diagnosis of iron loading and iron deficiency disorders. Moreover, we used an assay that was standardized using a recently validated and value assigned 2nd RM.[22] Therefore, the here-defined values can be used by all other analytically validated hepcidin assays that are standardized using the same RM.

Our study has several limitations. First, young children, children of non-Western European descent and children with under- and overweight were underrepresented. Second, we used age > 12 years as a proxy for the discrimination between prepuberal and postpuberal stage which might not be accurate in all children. Furthermore, our study population might contain children with undiagnosed iron related disorders like IRIDA, or hereditary hemochromatosis, since we performed no genotyping of *TMPRSS6* and *HFE* in the subjects. However, given the low prevalence of these disorders,[52,53] we expect only a few of these cases, if any, among our participants.

In conclusion we provide age- and sex specific serum hepcidin values in healthy Dutch children as a first step to better elucidating the clinical utility of hepcidin in children with iron disorders. Our data suggest that the serum hepcidin set point relative to indicators of stored and circulating iron changes during human growth and development. Since we used a standardized hepcidin assay the age and sex specific values of our assay can be applied to all validated assays that are standardized using the same RM. This paves the way for future studies needed to better elucidate the clinical utility of hepcidin in children with iron disorders.

Authorship

Contribution: A.E.D. D.L.B. and D.W.S. designed the research. A.E.D. and D.L.B. coordinated the data collection. S. M.K. and C.L. performed the hepcidin analyses. A.E.D., D.L.B. T.E.G. and D.W.S. analyzed the data and interpreted the results. A.E.D. wrote the manuscript.

Conflict-of-interest disclosure

S.M.K, C.L, and D.W.S. are employees of Radboudumc that offers hepcidin assays and hepcidin reference material to the research, clinical and pharmaceutical community at a fee for service via the Hepcidinanalysis initiative (www.hepcidinanalysis.com).[21]

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REFERENCES

1. Muckenthaler MU, Rivella S, Hentze MW, Galy B. A Red Carpet for Iron Metabolism. *Cell.* 2017;168(3):344-361.
2. Girelli D, Nemeth E, Swinkels DW. Hepcidin in the diagnosis of iron disorders. *Blood.* 2016;127(23):2809-2813.
3. Papanikolaou G, Tzilianos M, Christakis JI, et al. Hepcidin in iron overload disorders. *Blood.* 2005;105(10):4103-4105.
4. Origa R, Cazzola M, Mereu E, et al. Differences in the erythropoiesis-hepcidin-iron store axis between hemoglobin H disease and beta-thalassemia intermedia. *Haematologica.* 2015;100(5):e169-171.
5. De Falco L, Silvestri L, Kannengiesser C, et al. Functional and clinical impact of novel TMPRSS6 variants in iron-refractory iron-deficiency anemia patients and genotype-phenotype studies. *Human mutation.* 2014;35(11):1321-1329.
6. Heeney MM, Guo D, De Falco L, et al. Normalizing hepcidin predicts TMPRSS6 mutation status in patients with chronic iron deficiency. *Blood.* 2018;132(4):448-452.
7. Kemna EH, Kartikasari AE, van Tits LJ, Pickkers P, Tjalsma H, Swinkels DW. Regulation of hepcidin: insights from biochemical analyses on human serum samples. *Blood cells, molecules & diseases.* 2008;40(3):339-346.
8. Donker AE, Schaap CC, Novotny VM, et al. Iron Refractory Iron Deficiency Anemia (IRIDA): A heterogeneous disease that is not always iron refractory. *American journal of hematology.* 2016.
9. van Santen S, van Dongen-Lases EC, de Vegt F, et al. Hepcidin and hemoglobin content parameters in the diagnosis of iron deficiency in rheumatoid arthritis patients with anemia. *Arthritis and rheumatism.* 2011;63(12):3672-3680.
10. Bregman DB, Morris D, Koch TA, He A, Goodnough LT. Hepcidin levels predict nonresponsiveness to oral iron therapy in patients with iron deficiency anemia. *American journal of hematology.* 2013;88(2):97-101.
11. Prentice AM, Doherty CP, Abrams SA, et al. Hepcidin is the major predictor of erythrocyte iron incorporation in anemic African children. *Blood.* 2012;119(8):1922-1928.
12. Zimmermann MB, Troesch B, Biebinger R, Egli I, Zeder C, Hurrell RF. Plasma hepcidin is a modest predictor of dietary iron bioavailability in humans, whereas oral iron loading, measured by stable-isotope appearance curves, increases plasma hepcidin. *The American journal of clinical nutrition.* 2009;90(5):1280-1287.
13. Nemeth E, Ganz T. Hepcidin and iron-loading anemias. *Haematologica.* 2006;91(6):727-732.
14. van Santen S, Kroot JJ, Zijderveld G, Wiegerinck ET, Spaanderman ME, Swinkels DW. The iron regulatory hormone hepcidin is decreased in pregnancy: a prospective longitudinal study. *Clin Chem Lab Med.* 2013;51(7):1395-1401.
15. Rehu M, Punnonen K, Ostland V, et al. Maternal serum hepcidin is low at term and independent of cord blood iron status. *European journal of haematology.* 2010;85(4):345-352.
16. Ambroszkiewicz J, Klemarczyk W, Mazur J, et al. Serum Hepcidin and Soluble Transferrin Receptor in the Assessment of Iron Metabolism in Children on a Vegetarian Diet. *Biol Trace Elem Res.* 2017;180(2):182-190.
17. Pasricha SR, Atkinson SH, Armitage AE, et al. Expression of the iron hormone hepcidin distinguishes different types of anemia in African children. *Science translational medicine.* 2014;6(235):235re233.
18. Uijterschout L, Swinkels DW, Akkermans MD, et al. The value of soluble transferrin receptor and hepcidin in the assessment of iron status in children with cystic fibrosis. *J Cyst Fibros.*

2014;13(6):639-644.
19. Crielaard BJ, Lammers T, Rivella S. Targeting iron metabolism in drug discovery and delivery. *Nature reviews Drug discovery.* 2017;16(6):400-423.
20. Galesloot TE, Vermeulen SH, Geurts-Moespot AJ, et al. Serum hepcidin: reference ranges and biochemical correlates in the general population. *Blood.* 2011;117(25):e218-225.
21 www.hepcidinanalysis.com. Accessed 28 May 2019
22. Diepeveen LE, Laarakkers CMM, Martos G, et al. Provisional standardization of hepcidin assays: creating a traceability chain with a primary reference material, candidate reference method and a commutable secondary reference material. *Clin Chem Lab Med.* 2018.
23. Traglia M, Girelli D, Biino G, et al. Association of HFE and TMPRSS6 genetic variants with iron and erythrocyte parameters is only in part dependent on serum hepcidin concentrations. *J Med Genet.* 2011;48(9):629-634.
24. Sdogou T, Tsentidis C, Gourgiotis D, et al. Immunoassay-based serum hepcidin reference range measurements in healthy children: differences among age groups. *J Clin Lab Anal.* 2015;29(1):10-14.
25. Uijterschout L, Swinkels DW, Domellöf M, et al. Serum hepcidin measured by immunochemical and mass-spectrometric methods and their correlation with iron status indicators in healthy children aged 0.5-3 y. *Pediatr Res.* 2014;76(4):409-414.
26. Mupfudze TG, Stoltzfus RJ, Rukobo S, et al. Hepcidin decreases over the first year of life in healthy African infants. *British journal of haematology.* 2014;164(1):150-153.
27. Mupfudze TG, Stoltzfus RJ, Rukobo S, et al. Plasma Concentrations of Hepcidin in Anemic Zimbabwean Infants. *PloS one.* 2015;10(8):e0135227.
28. Mahajan G, Sharma S, Chandra J, Nangia A. Hepcidin and iron parameters in children with anemia of chronic disease and iron deficiency anemia. *Blood Res.* 2017;52(3):212-217.
29. Choi HS, Song SH, Lee JH, Kim HJ, Yang HR. Serum hepcidin levels and iron parameters in children with iron deficiency. *Korean J Hematol.* 2012;47(4):286-292.
30. Jaeggi T, Moretti D, Kvalsvig J, et al. Iron status and systemic inflammation, but not gut inflammation, strongly predict gender-specific concentrations of serum hepcidin in infants in rural Kenya. *PloS one.* 2013;8(2):e57513.
31. Sanad M, Osman M, Gharib A. Obesity modulate serum hepcidin and treatment outcome of iron deficiency anemia in children: a case control study. *Ital J Pediatr.* 2011;37:34.
32. World Health Organisation. Incidence of Anemia. http://www.who.int/vmnis/anaemia/prevalence/summary/anaemia_data_status_t2/en/. Accessed 28 May 2019
33. World Health Organisation. Asessment of Iron Status. http://www.who.int/nutrition/publications/en/ida_assessment_prevention_control.pdf. Accessed 28 May 2019
34. World Health Organization. Serum ferritin concentrations for the assessment of iron status and iron deficiency in populations. Vitamin and Mineral Nutrition Information System. http://www.who.int/vmnis/indicators/serum_ferritin.pdf. Accessed 28 May 2019
35. Williams K, Thomson D, Seto I, et al. Standard 6: age groups for pediatric trials. *Pediatrics.* 2012;129 Suppl 3:S153-160.
36. Cole TJ, Lobstein T. Extended international (IOTF) body mass index cut-offs for thinness, overweight and obesity. *Pediatr Obes.* 2012;7(4):284-294.
37. Laarakkers CM, Wiegerinck ET, Klaver S, et al. Improved mass spectrometry assay for plasma hepcidin: detection and characterization of a novel hepcidin isoform. *PloS one.* 2013;8(10):e75518.
38. Domellof M, Lonnerdal B, Dewey KG, Cohen RJ, Rivera LL, Hernell O. Sex differences in iron status during infancy. *Pediatrics.* 2002;110(3):545-552.

39. Armitage AE, Agbla SC, Betts M, et al. Rapid growth is a dominant predictor of hepcidin suppression and declining ferritin in Gambian infants. *Haematologica.* 2019.
40. Schaap CC, Hendriks JC, Kortman GA, et al. Diurnal rhythm rather than dietary iron mediates daily hepcidin variations. *Clinical chemistry.* 2013;59(3):527-535.
41. Netherlands Trial Register.
42. Mirciov CSG, Wilkins SJ, Hung GCC, Helman SL, Anderson GJ, Frazer DM. Circulating iron levels influence the regulation of hepcidin following stimulated erythropoiesis. *Haematologica.* 2018.
43. Ramos E, Kautz L, Rodriguez R, et al. Evidence for distinct pathways of hepcidin regulation by acute and chronic iron loading in mice. *Hepatology (Baltimore, Md).* 2011;53(4):1333-1341.
44. Pietrangelo A. Pathogens, Metabolic Adaptation, and Human Diseases--An Iron-Thrifty Genetic Model. *Gastroenterology.* 2015;149(4):834-838.
45. Weinberg ED. Iron availability and infection. *Biochimica et biophysica acta.* 2009;1790(7):600-605.
46. Prentice AM, Bah A, Jallow MW, et al. Respiratory infections drive hepcidin-mediated blockade of iron absorption leading to iron deficiency anemia in African children. *Science advances.* 2019;5(3):eaav9020.
47. Frazer DM, Wilkins SJ, Darshan D, Mirciov CSG, Dunn LA, Anderson GJ. Ferroportin Is Essential for Iron Absorption During Suckling, But Is Hyporesponsive to the Regulatory Hormone Hepcidin. *Cellular and molecular gastroenterology and hepatology.* 2017;3(3):410-421.
48. Bachman E, Feng R, Travison T, et al. Testosterone suppresses hepcidin in men: a potential mechanism for testosterone-induced erythrocytosis. *J Clin Endocrinol Metab.* 2010;95(10):4743-4747.
49. Lehtihet M, Bonde Y, Beckman L, et al. Circulating Hepcidin-25 Is Reduced by Endogenous Estrogen in Humans. *PloS one.* 2016;11(2):e0148802.
50. Troutt JS, Rudling M, Persson L, et al. Circulating human hepcidin-25 concentrations display a diurnal rhythm, increase with prolonged fasting, and are reduced by growth hormone administration. *Clinical chemistry.* 2012;58(8):1225-1232.
51. van der Vorm LN, Hendriks JC, Laarakkers CM, et al. Toward Worldwide Hepcidin Assay Harmonization: Identification of a Commutable Secondary Reference Material. *Clinical chemistry.* 2016;62(7):993-1001.
52. Swinkels DW, Janssen MC, Bergmans J, Marx JJ. Hereditary hemochromatosis: genetic complexity and new diagnostic approaches. *Clinical chemistry.* 2006;52(6):950-968.
53. Donker AE, Raymakers RA, Vlasveld LT, et al. Practice guidelines for the diagnosis and management of microcytic anemias due to genetic disorders of iron metabolism or heme synthesis. *Blood.* 2014;123(25):3873-3886; quiz 4005.

SUPPLEMENTAL FILES

Laboratory Methods

After arrival in the laboratory, samples were aliquoted. Serum hemoglobin (Hb), red cell indices, CRP, ALT, iron, transferrin and ferritin were measured in freshly obtained sample aliquots. Left- over aliquots were stored at -80 C° until use.

Hb and red cell indices were measured on a Sysmex XN automated hematology analyzer (Sysmex Netherlands, Etten-Leur, the Netherlands). CRP (lower limit < 0.1 mg/L), ALT, iron and transferrin were all measured on a Cobas 8000 C502 chemistry analyzer with kit reagents form Roche. Ferritin was measured on a Cobas 8000 C602 (e-Module) immunochemistry module (Roche Diagnostics Nederland B.V., Transistorstraat 41, 1322 CK, Almere). TIBC (µmol/L) and TSAT (%) were calculated using transferrin (in g/l, MW 79.550 KDa) and serum iron concentrations (µmol/l).

Determination of hepcidin and soluble Transferrin Receptor (sTfR) was performed in batches in freshly thawed samples in April 2018. We measured levels of the bioactive form of hepcidin: (hepcidin-25[1]) by weak cation exchange chromatography followed by time of flight mass spectrometry (WCX -MS-TOF) as described before.[2] This method was recently standardized using 2nd reference material (RM) that was value assigned by a provisional primary RM.[3] Serum concentration of sTfR was measured by immunonephelometry (Behring Nephelometer II Analyzer; BNII, Dade Behring Marburg GmbH, Marburg, Germany).

Supplemental Table 1. Hemoglobin, iron status indicators, hepcidin and CRP for different age groups and sex

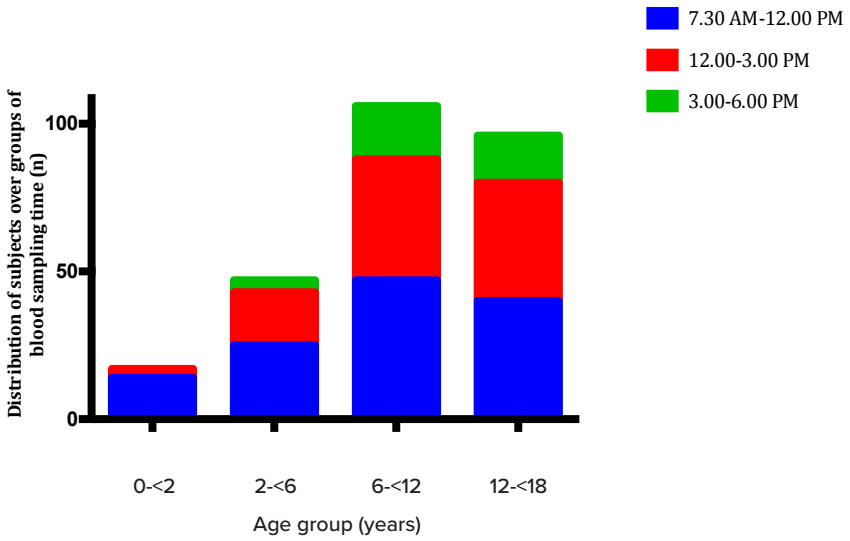
Laboratory variables			Males	
Age 0-<2 years	**n (%)**	**median**	**p25-p75**	**Min-Max**
Hemoglobin, g/dL	11(7)	11.0	10.7-11.7	10.1-12.5
Ferritin, µg/L	11 (7)	48.0	30.0-69.0	20.0-97.0
Iron, µmol/L	11 (7)	11.0	9.0-13.0	3.0-15.0
TIBC, µmol/L	11 (7)	63.0	61.0-67.0	56.0-72.0
TSAT, %	11 (7)	16.0	13.0-20.0	5.0-21.0
sTfR, mg/L	11 (7)	1.6	1.4-1.7	1.4-1.8
hepcidin, nmol/L	11 (7)	1.8	1.1-7.3	0.9-20.9
CRP, mg/L	11(7)	0.3	0.1-0.9	0.1-4.6
Age 2-<6 years	**n (%)**	**median**	**p25-p75**	
Hemoglobin, g/dL	29 (18)	11.7	11.3-12.3	10.4-14.1
Ferritin, µg/L	28 (18)	31.5	24.0-44.8	13.0-97.0
Iron, µmol/L	28 (18)	15.5	10.3-18.0	2.0-22.0
TIBC, µmol/L	28 (18)	66.0	59.2-71.8	50.0-83.0
TSAT, %	28 (18)	23.5	17.0-27.8	10.0-32.0
sTfR, mg/L	29 (18)	1.5	1.0-1.6	1.0-2.5
hepcidin, nmol/L	29 (18)	2.0	1.0-3.3	0.1-7.0
CRP, mg/L	28 (18)[a]	0.2	0.1-0.5	0.0-1.6
Age 6-<12 years	**n (%)**	**median**	**p2.5-p97.5**	
Hemoglobin, g/dL	71 (45)	12.3	10.8-14.0	10.6-14.2
Ferritin, µg/L	71 (45)	42.0	17.4-98.4	15.0-120.0
Iron, µmol/L	71 (45)	16.0	7.0-26.4	3.0-28.0
TIBC, µmol/l	71 (45)	63.0	44.6-82.0	35.0-90.0
TSAT, %	71 (45)	27.0	10.6-43.4	5.0-45.0
sTfR, mg/L	70 (44)	1.2	0.9-1.7	0.9-1.8
hepcidin, nmol/L	71 (45)	1.7	0.1-11.5	0.1-15.5
CRP, mg/L	71 (45)	0.2	0.0-3.5	0.0-3.7
Age 12-< 18 years	**n (%)**	**median**	**p2.5-p97.5**	
Hemoglobin, g/dL	46 (29)	14.2	12.1-16.0	12.0-16.0
Ferritin, µg/L	46 (29)	45.0	19.2-99.8	19.0-100.0
Iron, µmol/L	45 (28)	17.0	7.2-33.6	7.0-34.0
TIBC, µmol/L	46 (29)	67.0	53.2-85.5	53.0-86.0
TSAT, %	45 (28)	25.0	10.3-53.1	10.0-54.0
sTfR, mg/L	46 (29)	1.2	0.8-2.3	0.8-2.3
hepcidin, nmol/L	46 (29)	0.7	0.0-9.3	0.0-9.3
CRP, mg/L	46 (29)	0.3	0.0-4.7	0.0-4.9
Age 0-< 18 years	**157 (100)**			

[a] In a 3 year old male, CRP was missing. Hepcidin was low (0.58 nM) in this child, arguing against inflammation. Therefore this case was not excluded although CRP>5 mg/L was an exclusion criterion for the study.

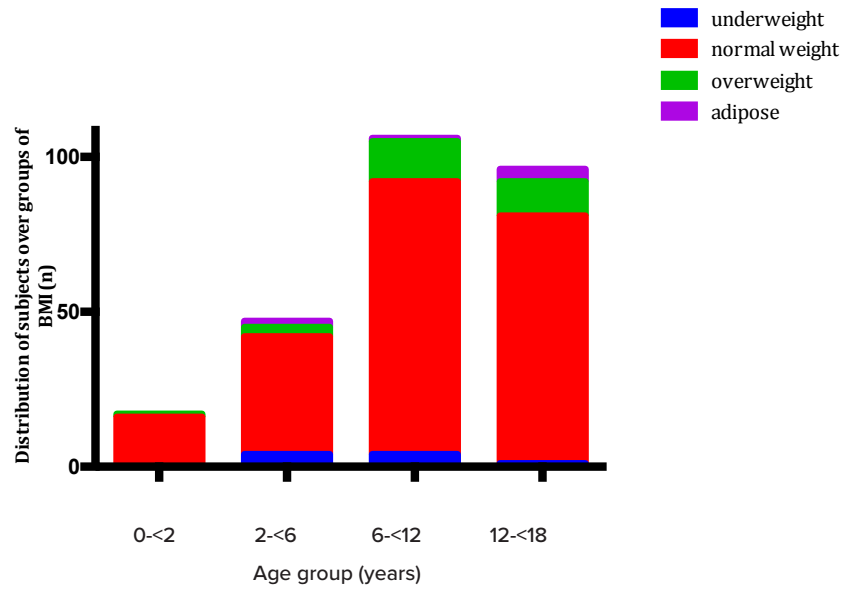
		Females		
n (%)	**median**	**p25-p75**	**Min-Max**	*p*
6 (5)	10.9	10.7-11.6	10.7-11.8	0.901
6 (5)	27.0	22.8-53.3	22.0-54.0	0.620
6 (5)	10.5	7.8-13.3	4.0-14.0	0.600
6 (5)	65.0	63.8-68.0	63.0-71.0	0.335
6 (5)	16.5	12.0-20.0	6.0-20.0	1.000
6 (5)	1.5	1.4-1.8	1.1-1.9	0.620
6 (5)	1.8	0.9-7.5	0.2-14.1	1.000
6 (5)	0.2	0.1-0.6	0.0-0.7	1.000
	median	**p25-p75**		
18 (17)	11.7	11.4-12.6	10.2-13.4	0.964
18 (17)	30.0	21.0-37.8	15.0-72.0	0.763
17 (17)	18.0	12.5-19.5	4.0-25.0	0.229
18 (17)	64.0	61.8-69.3	46.0-73.0	0.763
17 (17)	26.0	18.0-32.3	7.0-40.0	0.229
18 (17)	1.3	1.2-1.5	0.9-1.9	0.853
18 (17)	2.0	1.1-3.8	0.7-9.5	0.853
18(18)	0.2	0.0-0.9	0.0-4.8	0.763
	median	**p25-p75**		
35 (32)	13.0	11.7-13.8	10.2-14.7	0.099
35 (32)	44.0	34.0-59.0	20.0-210.0	0.336
33 (32)	14.0	12.5-19.0	8.0-29.0	0.642
35 (32)	66.0	60.0-72.0	48.0-85.0	0.039
33 (32)	23.0	18.5-27.5	14.0-48.0	0.319
35 (32)	1.3	1.2-1.5	1.0-1.3	0.147
35 (32)	2.0	0.9-3.9	0.0-18.6	0.680
35(32)	0.3	0.2-0.7	0.0-4.2	0.028
	median	**p2.5-p97.5**		
50 (46)	13.0	11.2-15.3	11.2-15.4	0.001
49 (45)	42.0	15.5-117.5	15.0-120.0	0.482
48 (44)	16.0	4.9-40.9	4.0-44.0	0.899
50 (46)	67.5	47.0-96.8	47.0-99.0	0.993
48 (46)	24.5	6.5-54.1	6.0-57.0	0.899
50 (46)	1.1	0.7-2.3	0.7-3.0	0.042
50 (46)	1.0	0.1-9.4	0.0-11.4	0.409
50 (46)	0.3	0.0-4.8	0.0-4.9	0.838
109 (100)				

Abbreviations: Min-Max denotes minimum, maximum

Supplemental Figure 1. Sampling time stratified for different age groups



Supplemental Figure 2. Body Mass Index (BMI) stratified for different age groups



Effect of blood sampling time on serum hepcidin levels, hepcidin/ferritin ratios and TSAT/hepcidin ratios

Since hepcidin concentrations in serum have been reported to follow a clear circadian rhythm in adults[1,4] and also in children,[5] we assessed the possible influence of time of blood sampling, by dividing sampling time in three categories: between 7:30 AM and 12 PM, between 12 and 3 PM and between 3 and 6 PM. For 47% of subjects, blood sampling was performed between 7:30 AM and 12 PM, for 38% between 12 and 3 PM, and for 14% between 3 PM and 6 PM. Blood sampling time was dissimilar over the age groups; children > 6 years underwent sampling more often later during the day (Chi Square *p* 0.057) (Supplemental Figure 1). Multivariate analyses including age, sampling time and other clinical and biochemical parameters showed increasing hepcidin concentrations during the day in both males and females, especially after 3 PM (males: β= 0.37; 95 % confidence interval [CI] 0.16– 0.58 (*p* 0.001), females: β= 0.31; 95% CI 0.00– 0.61 (*p* 0.048)) (Table 4). This corroborates with the above-mentioned data on hepcidin in children.[5] The time of blood sampling was also an independent correlate for both the hepcidin/ferritin and TSAT/hepcidin ratios (Supplemental Table 6, Supplemental Table 7).

The hepcidin/ferritin ratio significantly increased after 3 PM in both males and females (males: β= 0.42; 95 % confidence interval [CI] 0.21– 0.63 (*p* 0.000), females: β= 0.32; 95% CI 0.02– 0.62 (*p* 0.034)). Conversely, the TSAT/hepcidin ratio significantly decreased in males and tended to decrease in females after 3 PM (males: β= -0.41; 95 % CI -0.63– -0.19 (*p* 0.000), females: β= -0.26; 95% CI -0.58– 0.05 (*p* 0.103)). The variable effect of sampling time on the TSAT/hepcidin ratio might be explained by the fact that both TSAT and hepcidin increase during the day.[6] One might argue that the above-mentioned correlations between sampling time and serum hepcidin levels might be overestimated because of the hepcidin-increasing effect of fasting.[7] However, since only prolonged fasting of up to 66 hour[7] has been described to result in an increment of hepcidin production, we expect that the effect of the pre-operative fasting period of 4-6 hours on the hepcidin data is negligible in our study subjects. This is consistent with our previously reported observations that demonstrate ferritin sets the basal hepcidin concentration and suggest innate diurnal rhythm rather than dietary intake mediates the daily hepcidin variations.[4]

Effect of body mass index (BMI) on serum hepcidin levels, hepcidin/ferritin ratios and TSAT/hepcidin ratios

Since serum hepcidin levels have been reported to be influenced by BMI in both adults[8] and children[9], and also in mice studies[10], we assessed and interpreted the BMI in all subjects according to international standards, which describe age-dependent cut off points for underweight, normal weight, overweight and obesity.[11]The majority of children (84%) had a normal BMI according to age-adjusted reference values.[11] Distribution of BMI was similar for the different age groups (Chi Square *p* 0.310) (Supplemental Figure 2). Multivariate analyses including age, sampling time, BMI and other biochemical parameters showed that underweight was associated with increased hepcidin levels in males (β= 0.16; 95% CI 0.03– 1.05 (*p* 0.039)), while other BMI categories did not influence hepcidin levels. In females, no significant effect of any BMI category on serum hepcidin levels was found (Table 3, Table 4).

The hepcidin/ferritin ratio significantly increased while the TSAT/hepcidin ratio significantly decreased in underweight males (β= 0.51; 95% CI 0.01– 1.01 (*p* 0.046) and β= -0.64; 95% CI -1.16– -0.11 (*p* 0.018) respectively). Again, in females no significant effect of any BMI category on the hepcidin/ferritin ratio and TSAT/hepcidin ratio was observed. (Supplemental Table 6, Supplemental Table 7). Since our study population contained only a few children with underweight, overweight or adiposity (Table 1), our data lack the power to draw conclusions on the effect of BMI on serum hepcidin levels (relative to iron indicators).

Supplemental Table 2. Serum hepcidin values (nmol/L) stratified for sex and age < 12 years versus age > 12 years

	Males				Females				
Age (years)	**n (%)**	**Median (nmol/L)**	**p 2.5 (nmol/L)**	**p 97.5 (nmol/L)**	**n (%)**	**median (nmo/L)**	**p 2.5 (nmol/L)**	**p 97.5 (nmol/L)**	*p* [a]
0-<12	111 (71)	1.8	0.1	11.4	59 (54)	1.9	0.0	16.4	0.747
12-<18	46 (29)	0.7	0.0	9.3	50 (46)	1.0	0.1	9.4	0.409
p [b]		0.003				0.016			
Total	**157 (100)**	**1.5**	**0.1**	**9.4**	**109 (100)**	**1.5**	**0.0**	**12.1**	

[a] Independent sample median test confirmed that median hepcidin did not differ between males and females

[b] Independent sample median test confirmed that median hepcidin significantly dropped after the age of 12 years in both males and females

Supplemental Table 3. Univariable linear regression for serum hepcidin concentrations (nmol/L)

	Males			
		95% CI		*p*
Variable	Beta[a]	Lower limit	Upper limit	
Hb, g/dL	-0.102	-0.170	-0.034	0.003
Reticulocytes, x10^9/L	-0.001	-0.008	0.005	0.656
MCV, fL	-0.028	-0.049	-0.007	0.009
Ferritin, µg/L[b]	1.118	0.704	1.531	0.000
Iron, µmol/L	-0.006	-0.021	0.010	0.479
TIBC, µmol/l	-0.009	-0.020	0.001	0.089
TSAT, %	0.00	-0.010	0.010	0.979
sTfR, µg/mL	-0.000	-0.329	0.328	0.998
ALT, IU/L[b]	0.636	0.033	1.239	0.039
CRP, mg/L[b]	0.234	0.099	0.369	0.001
Age, years	-0.038	-0.056	-0.021	0.000
Age group, years				
0-< 2	ref	ref	ref	
2-<6	-0.204	-0.559	0.152	0.259
6-<12	-0.187	-0.512	0.138	0.258
12-<18	-0.660	-0.997	-0.323	0.000
Age, years				
<12	-0.487	-0.663	-0.312	0.000
12-< 18				
BMI[c]				
Normal weight	ref	ref	ref	
Underweight	0.560	-0.073	1.192	0.082
Overweight	0.144	-0.129	0.416	0.299
Adipose	0.320	-0.174	0.813	0.202
Time blood sampling				
8-12 PM	ref	ref	ref	
12-3 PM	0.196	0.009	0.382	0.040
3- 6 PM	0.341	0.087	0.595	0.009

[a]Beta expresses the change in the dependent variable -log-transformed serum hepcidin- that is associated with a 1-unit change in the independent variable. Independent variables marked with [b] were log-transformed as well; the interpretation of the regression coefficients for these variables is as follows: a 1% change in the independent variable corresponds to a beta % change in serum hepcidin.
[c]BMI was assessed according to international standards established by Cole *et al*[11]
Abbreviations: ref denotes reference category

Females					
		95% CI		*p*	
R^2, %	Beta*	Lower limit	Upper limit		R^2, %
4.8	-0.025	-0.117	0.067	0.585	0.3
0.1	-0.002	-0.008	0.004	0.600	0.3
3.7	-0.033	-0.058	-0.008	0.010	5.2
15.1	0.766	0.291	1.242	0.002	7.9
0.3	0.006	-0.012	0.024	0.519	0.4
1.2	-0.016	-0.027	-0.005	0.004	6.6
0.0	-0.009	-0.003	0.021	0.131	1.3
0.0	0.446	0.048	0.844	0.028	3.5
2.1	0.154	-0.644	0.952	0.703	0.1
6.5	0.113	-0.042	0.267	0.151	0.5
10.4	-0.031	-0.053	-0.009	0.006	5.9
	ref	ref	ref		
15.4	0.059	-0.477	0.566	0.816	4.0
	-0.128	-0.602	0.347	0.595	
	-0.313	-0.777	0.151	0.184	
15.7	-0.256	-0.461	-0.050	0.015	4.5
	ref	ref	ref		
1.5	0.088	-0.351	0.595	0.711	0.2
	-0.001	-0.470	0.173	0.997	
	-0.118	-0.887	0.720	0.769	
	ref	ref	ref		
4.1	0.158	-0.068	0.383	0.168	1.3
	0.263	-0.058	0.583	0.107	

Supplemental Table 4. Univariable linear regression for hepcidin/ferritin ratio (pmol/µg)

	Males				
		95% CI			
Variable	**Beta[a]**	**Lower limit**	**Upper limit**	**R^2, %**	*p*
Hb, g/dL	-0.143	-0.203	-0.083	12.0	0.000
Reticulocytes, x10^9/L	-0.004	-0.009	0.002	0.3	0.219
MCV, fL	-0.041	-0.060	-0.023	10.3	0.000
Iron, µmol/L	-0.007	-0.021	0.007	0.7	0.340
TIBC, µmol/L	-0.004	-0.014	0.006	0.2	0.398
TSAT, %	-0.002	-0.011	0.007	4.8	0.620
sTfR, µg/mL	0.159	-0.162	0.480	0.0	0.328
ALT, IU/L[b]	0.273	-0.289	0.834	0.6	0.339
CRP, mg/L[b]	0.135	0.008	0.262	2.1	0.038
Age, years	-0.049	-0.065	-0.033	19.5	0.000
Age group, years					
0-< 2	ref	ref	ref		
2-<6	-0.034	-0.340	0.282	22.2	0.834
6-<12	-0.166	-0.454	0.121		0.254
12-<18	-0.643	-0.941	-0.345		0.000
Age, years					
<12 12-< 18	-0.527	-0.683	-0.371	21.9	0.000
BMI[c]					
Normal weight	ref	ref	ref		
Underweight	0.725	0.145	1.305	2.4	0.015
Overweight	0.120	-0.130	0.369		0.346
Adipose	0.060	-0.393	0.513		0.794
Time blood sampling					
8-12 PM	ref	ref	ref		
12-3 PM	0.109	-0.065	0.284	1.7	0.217
3- 6 PM	0.251	0.013	0.488		0.039

[a] Beta expresses the change in the dependent variable-log transformed hepcidin/ferritin ratio- that is associated with a 1-unit change in the independent variable. Independent variables marked with [b] were log-transformed as well; the interpretation of the regression coefficients for these variables is as follows: a 1% change in the independent variable corresponds to a beta % change in the ferritin/hepcidin ratio.

[c] BMI was assessed according to international standards established by Cole *et al*[11]

Abbreviations: ref denotes reference category

Females				
	95% CI			
Beta[a]	Lower limit	Upper limit	R^2, %	*p*
-0.067	-0.155	0.021	1.2	0.136
-0.004	-0.010	0.002	0.6	0.198
-0.034	-0.057	-0.010	6.0	0.006
-0.002	-0.019	0.015	0.1	0.807
-0.008	-0.019	0.003	0.9	0.163
0.002	-0.010	0.013	0.1	0.767
0.538	0.153	0.924	5.1	0.007
-0.021	-0.787	0.745	0.0	0.957
0.078	-0.072	0.228	0.6	0.303
-0.041	-0.062	-0.020	11.6	0.000
ref	ref	ref		
0.088	-0.383	0.5559	10.4	0.712
-0.277	-0.718	0.164		0.215
-0.425	-0.857	0.007		0.054
-0.287	-0.485	-0.090	6.9	0.005
ref	ref	ref		
0.139	-0.311	0.590	0.4	0.541
0.012	-0.344	0.368		0.947
-0.034	-0.798	0.730		0.730
ref	ref	ref		
0.134	-0.084	0.352	1.4	0.226
0.272	-0.036	0.581		0.083

Supplemental Table 5. Univariable linear regression for TSAT/hepcidin ratio (%/nmol/L)

	Males				
		95% CI			*p*
Variable	Beta[a]	Lower limit	Upper limit	R^2, %	
Hb, g/dL	0.142	0.071	0.214	8.6	0.000
Reticulocytes, x10^9/L	-0.001	-0.008	0.006	0.0	0.815
MCV, fL	0.047	0.025	0.069	9.8	0.000
Ferritin, µg/L[b]	-1.024	-1.475	-0.573	11.0	0.000
Iron, µmol/L	0.034	0.018	0.050	9.7	0.000
TIBC, µmol/L	0.005	-0.006	0.016	0.5	0.396
sTfR, µg/mL	-0.324	-0.695	0.046	1.3	0.086
ALT, IU/L[b]	-0.828	-1.468	-0.189	3.5	0.011
CRP, mg/L[b]	-0.329	-0.470	-0.188	11.6	0.000
Age, years	0.050	0.031	0.069	14.9	0.000
Age group, years					
0-< 2	ref	ref	ref		
2-<6	0.377	0.002	0.752	18.0	0.049
6-<12	0.430	0.088	0.771		0.014
12-<18	0.896	0.542	1.250		0.000
Age, years					
<12 12-< 18	0.523	0.334	0.712	15.8	0.000
BMI[c]					
Normal weight	ref	ref	ref		
Underweight	-0.787	-1.454	-0.120	3.6	0.021
Overweight	-0.222	-0.510	0.065		0.129
Adipose	-0.350	-0.871	0.171		0.186
Time blood sampling					
8-12 PM	ref	ref	ref		
12-3 PM	-0.095	-0.298	0.107	1.6	0.355
3- 6 PM	-0.292	-0.568	-0.017		0.038

[a] Beta expresses the change in the dependent variable- log transformed TSAT/hepcidin ratio- that is associated with a 1-unit change in the independent variable. Independent variables marked with [b] were log-transformed; the interpretation of the regression coefficients for these variables is as follows: a 1% change in the independent variable corresponds to a beta/100 unit change in the TSAT/hepcidin ratio.
[c] BMI was assessed according to international standards established by Cole *et al*[11]
Abbreviations: ref denotes reference category

Females				
	95% CI			*p*
Beta[a]	Lower limit	Upper limit	R^2, %	
0.061	-0.038	0.160	0.5	0.226
0.004	-0.003	0.012	0.5	0.220
0.042	0.016	0.067	8.4	0.002
-0.541	-1.056	-0.039	3.3	0.035
0.020	0.002	0.038	3.6	0.029
0.010	-0.002	0.022	1.7	0.095
-0.660	-1.079	-0.241	7.8	0.002
0.047	-0.806	0.901	0.0	0.913
-0.173	-0.335	-0.010	3.2	0.037
0.036	0.012	0.059	7.2	0.003
ref	ref	ref		
0.134	-0.394	0.663	5.2	0.615
0.363	-0.131	0.857		0.148
0.513	0.031	0.995		0.037
0.258	0.038	0.478	4.1	0.022
ref	ref	ref		
0.021	-0.512	0.555	0.1	0.937
-0.061	-0.448	0.326		0.756
-0.012	-0.842	0.817		0.976
ref	ref	ref		
-0.051	-0.259	0.192	0.8	0.676
-0.160	-0.508	0.189		0.366

Supplemental Table 6. Results of multivariable linear regression analyses for hepcidin/ferritin concentrations (pmol/µg) stratified by sex

	Males			
		95% CI		
Variable	**Beta**	**Lower limit**	**Upper limit**	*p*
Age, years				
0-<2	ref	ref	ref	
2-<6	-0.090	-0.392	0.211	0.555
6-<12	-0.254	-0.529	0.022	0.071
12-<18	-0.768	-1.054	-0.482	0.000
Time Blood sampling				
7.30 AM-12 PM	ref	ref	ref	
12 -3 PM	0.169	0.022	0.315	0.024
3- 6 PM	0.421	0.211	0.631	0.000
CRP, mg/L[b,c]	0.103	-0.008	0.215	0.070
BMI[d]				
Normal weight	ref	ref	ref	
Underweight	0.508	0.008	1.007	0.046
Overweight	0.139	-0.073	0.350	0.197
Adipose	0.056	-0.331	0.443	0.776

[a] Beta expresses the change in the dependent variable -log-transformed hepcidin/ferritin ratio- that is associated with a 1-unit change in the independent variable. Independent variables marked with [b] were log-transformed as well; the interpretation of the regression coefficients for these variables is as follows: a 1% change in the independent variable corresponds to a beta % change in the hepcidin/ferritin ratio

[c] CRP levels of children were between > 0.1 and < 5 mg/l

[d] BMI was assessed according to international standards established by Cole *et al*[11]

Abbreviations: na denotes not applicable; ref, reference category

Females			
	95% CI		
Beta	Lower limit	Upper limit	*p*
ref	ref	ref	
-0.018	-0.486	0.450	0.939
-0.421	-0.865	0.023	0.063
-0.533	-0.964	-0.102	0.016
ref	ref	ref	
0.178	-0.028	0.384	0.090
0.320	0.024	0.616	0.034
0.124	-0.019	0.266	0.088
ref	ref	ref	
na	na	na	
na	na	na	
na	na	na	

Supplemental Table 7. Results of multivariable linear regression analyses for TSAT/hepcidin (%/nmoL) ratio stratified by sex

	Males			
		95% CI		
Variable	**Beta[a]**	**Lower limit**	**Upper limit**	*p*
Age, years				
0-<2	ref	ref	ref	
2-<6	0.221	-0.108	0.550	0.186
6-<12	0.411	0.106	0.716	0.009
12-<18	0.912	0.589	1.236	0.000
Time Blood sampling				
7.30 AM-12 PM	ref	ref	ref	
12 -3 PM	-0.137	-0.297	0.022	0.090
3- 6 PM	-0.408	-0.626	-0.189	0.000
Ferritin, µg/L[b]	-0.972	-1.388	-0.556	0.000
Iron, µmol/L	0.012	-0.003	0.027	0.104
sTfR, mg/L	na	na	na	na
ALT, IU/L[b]	0.075	-0.484	0.635	0.790
CRP, mg/L[b,c]	-0.170	-0.303	-0.038	0.012
BMI[d]				
Normal weight	ref	ref	ref	
Underweight	-0.636	-1.159	-0.112	0.018
Overweight	-0.197	-0.423	0.029	0.087
Adipose	-0.096	-0.522	0.331	0.659

[a] Beta expresses the change in the dependent variable – log transformed TSAT/hepcidin ratio- that is associated with a 1-unit change in the independent variable. Independent variables marked with [b] were log-transformed; the interpretation of the regression coefficients for these variables is as follows: a 1% change in the independent variable corresponds to a beta % change in the TSAT/hepcidin ratio

[c] CRP levels of children were between > 0.1 and < 5 mg/L

[d] BMI was assessed according to international standards established by Cole *et al*[11]

Abbreviations: na denotes not applicable; ref, reference category

	Females		
	95% CI		
Beta[a]	Lower limit	Upper limit	*p*
ref	ref	ref	
-0.011	-0.514	0.490	0.965
0.427	-0.050	0.903	0.079
0.398	-0.090	0.886	0.109
ref	ref	ref	
-0.151	-0.371	0.068	0.174
-0.263	-0.580	0.054	0.103
-0.892	-1.388	-0.396	0.001
0.021	0.002	0.039	0.027
-0.431	-0.874	0.011	0.056
na	na	na	na
-0.134	-0.290	0.021	0.089
ref	ref	ref	
na	na	na	na
na	na	na	na
na	na	na	na

REFERENCES

1. Girelli D, Nemeth E, Swinkels DW. Hepcidin in the diagnosis of iron disorders. *Blood.* 2016;127(23):2809-2813.
2. Laarakkers CM, Wiegerinck ET, Klaver S, et al. Improved mass spectrometry assay for plasma hepcidin: detection and characterization of a novel hepcidin isoform. *PloS one.* 2013;8(10):e75518.
3. Diepeveen LE, Laarakkers CMM, Martos G, et al. Provisional standardization of hepcidin assays: creating a traceability chain with a primary reference material, candidate reference method and a commutable secondary reference material. *Clin Chem Lab Med.* 2018.
4. Schaap CC, Hendriks JC, Kortman GA, et al. Diurnal rhythm rather than dietary iron mediates daily hepcidin variations. *Clinical chemistry.* 2013;59(3):527-535.
5. Sdogou T, Tsentidis C, Gourgiotis D, et al. Immunoassay-based serum hepcidin reference range measurements in healthy children: differences among age groups. *J Clin Lab Anal.* 2015;29(1):10-14.
6. Ridefelt P, Larsson A, Rehman JU, Axelsson J. Influences of sleep and the circadian rhythm on iron-status indices. *Clinical biochemistry.* 2010;43(16-17):1323-1328.
7. Troutt JS, Rudling M, Persson L, et al. Circulating human hepcidin-25 concentrations display a diurnal rhythm, increase with prolonged fasting, and are reduced by growth hormone administration. *Clinical chemistry.* 2012;58(8):1225-1232.
8. Galesloot TE, Vermeulen SH, Geurts-Moespot AJ, et al. Serum hepcidin: reference ranges and biochemical correlates in the general population. *Blood.* 2011;117(25):e218-225.
9. Sanad M, Osman M, Gharib A. Obesity modulate serum hepcidin and treatment outcome of iron deficiency anemia in children: a case control study. *Ital J Pediatr.* 2011;37:34.
10. Mirciov CSG, Wilkins SJ, Anderson GJ, Frazer DM. Food deprivation increases hepatic hepcidin expression and can overcome the effect of Hfe deletion in male mice. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology.* 2018:fj201701497RR.
11. Cole TJ, Lobstein T. Extended international (IOTF) body mass index cut-offs for thinness, overweight and obesity. *Pediatr Obes.* 2012;7(4):284-294.

Chapter 8

Transferrin Saturation/Hepcidin Ratio discriminates *TMPRSS6*-related Iron Refractory Iron Deficiency Anemia from Patients with Multi-causal Iron Deficiency Anemia with High Sensitivity and Specificity

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ABSTRACT

Pathogenic *TMPRSS6* variants impairing matriptase 2 function result in inappropriately high serum hepcidin levels relative to body iron status, leading to the clinical phenotype of Iron Refractory Iron Deficiency Anemia (IRIDA). Due to its genotypical and phenotypical heterogeneity, diagnosing IRIDA can be challenging. Therefore, we aimed to assess the transferrin saturation (TSAT)/hepcidin ratio as tool to distinguish *TMPRSS6*-related IRIDA from *TMPRSS6*-unrelated iron deficiency anemia (IDA).

TSAT/hepcidin ratios were determined in 21 IRIDA patients and 39 IDA controls, both with CRP ≤10 mg/L. IRIDA cases had mono- or biallelic pathogenic *TMPRSS6* variants combined with microcytic anemia, TSAT <10% and a poor response to oral iron treatment. IDA controls had microcytic anemia, TSAT ≤15%, absence of iron therapy <3 months, severe renal impairment or chronic liver disease. In all patients and controls, serum hepcidin-25 levels were measured by the same isotope dilution mass spectrometry assay.

IRIDA cases showed significantly lower TSAT/hepcidin ratios compared to IDA controls, median 0.7 %/nM (range 0.1-9.1 %/nM) and 16.7 %/nM (8.0-44.0 %/nM, p=0.000), respectively. The area under the ROC-curve for the TSAT/hepcidin ratio was 0.99 with a specificity of 100% (95%-CI, 93-100%) and a sensitivity of 95% (95% CI, 79-100%) at an optimal cut-off point of 5.9%/nM to differentiate between IRIDA and IDA.

We conclude that the TSAT/hepcidin ratio is an effective tool in distinghuishing *TMPRSS6*-related IRIDA from multi-causal IDA with high specificity, in which a ratio ≤5.9%/nM strongly indicates the presence of IRIDA, provided inflammatory parameters are low and no iron therapy has been given recently.

INTRODUCTION

Matriptase 2, a transmembrane serine protease encoded by *TMPRSS6,* plays a key role in down-regulating hepcidin expression through modulation of the BMP-SMAD pathway when iron stores are low.[1-4] Pathogenic *TMPRSS6* variants result in impaired matriptase 2 function, leading to inappropriately high serum hepcidin levels in relation to body iron status.[5,6] Since hepcidin impairs intestinal iron absorption and recycling by inhibiting ferroportin-mediated iron export from enterocytes and macrophages, patients with *TMPRSS6*-related IRIDA develop a microcytic anemia with remarkably low transferrin saturation (TSAT<10%), low-to-normal ferritin levels and a poor response to oral iron treatment.[5,7-12]

Usually bi-allelic *TMPRSS6* variants are found in IRIDA patients, therefore the disease is considered as autosomal recessive. However, anecdotal data are available of phenotypically affected IRIDA patients in whom only a heterozygous *TMPRSS6* variant was found, (reviewed in[12]), corroborating our data.[11] Bi-allelic affected patients typically present in childhood, while mono-allelic affected patients generally present later in life with a milder phenotype regarding hemoglobin (Hb) and mean corpuscular volume (MCV).[11]

Diagnosing *TMPRSS6*-related IRIDA may be challenging, as the disorder is phenotypically and genotypically heterogeneous.[11] In our case series, time from the identification of anemia to the diagnosis of IRIDA ranged from 1-17 years in the bi-allelic patients and from 1-37 years in the mono-allelic patients.[11] The majority of patients went through extensive diagnostic work-up with invasive diagnostic tests (e.g. gastrointestinal endoscopy and video capsule endoscopy) before IRIDA was diagnosed and effective treatment (primarily parenteral iron) could be initiated.[11] Therefore, we sought to develop a tool to assist clinicians in recognizing and differentiating *TMPRSS6*-related IRIDA from other common causes of iron deficiency anemia (IDA), in order to ensure timely diagnosis and prevention of unnecessary invasive diagnostic work-up. Since the cardinal feature of IRIDA is discrepantly high serum hepcidin levels in relation to a low body iron status in general and a low circulating iron pool in particular, the TSAT/hepcidin ratio has been suggested a promising tool in diagnosing IRIDA.[11-14] Our previous case study, comprising 18 IRIDA patients and 20 phenotypically non-affected relatives with or without the relevant *TMPRSS6* defect, supported this idea, e.g. the ratio was able to discriminate between

bi-allelic and mono-allelic IRIDA patients, and between mono-allelic IRIDA patients and their phenotypically unaffected relatives with the same heterozygous *TMPRSS6* defect, even after parenteral iron therapy had been given.[11]

In this study, we assessed the ability of the TSAT/hepcidin ratio to discriminate between genotypically and phenotypically affected IRIDA patients and patients with IDA due to other reasons than IRIDA (e.g. menstrual blood loss, gastro-intestinal hemorrhage or malabsorption). Applying standardized hepcidin values, we aimed to establish a cut-off point for the TSAT/hepcidin ratio to distinguish bi-allelic and mono-allelic affected IRIDA patients from a control group with IDA, provided moderate or severe inflammation (defined as CRP >10 mg/L) was absent and no recent iron therapy had been provided, as these factors have been described to increase hepcidin production. [8,15-19] We observed that IRIDA cases had significantly lower TSAT/hepcidin ratios compared to IDA controls, making this ratio a specific tool for discriminating *TMPRSS6*-related IRIDA in a broad iron-deficient population

METHODS

IRIDA patients

We screened the Radboudumc Iron Registry Biobank, Nijmegen, the Netherlands for patients who were diagnosed with *TMPRSS6*-related IRIDA (called 'IRIDA' hereafter) between 2010 and May 2019.[20] This registry is a facility to optimize the use and distribution of biomaterial and/or demographic and clinical patient data for scientific research of iron disorders, for which ethical permission has been obtained. IRIDA probands were defined as patients having both an 'IRIDA phenotype' and an 'IRIDA genotype', i.e. microcytic anemia, TSAT<10%, Hb not or partially responsive to oral iron (<2g/dL Hb increment after three weeks of oral iron therapy),[21] and mono- or bi-allelic pathogenic *TMPRSS6* variants. Only patients living in the Netherlands for which the TSAT/hepcidin ratio was assessed in absence of moderate or severe inflammation (defined as CRP≤10 mg/L) were included.

Genotyping of IRIDA patients was performed by PCR, DNA Sanger sequencing (until March 2014) and by Ion Torrent sequencing (after March 2014) of the exons of *TMPRSS6*, thereby analyzing the coding part of the gene. Multiplex ligation-dependent probe amplification (MLPA) was conducted in patients with an IRIDA phenotype in whom only a mono-allelic pathogenic *TMPRSS6* variant was found, in order to exclude large deletions and/or duplications in the 'healthy' allele.[22] The pathogenicity of genetic variants was assessed by review of the literature on previous reported cases and functional studies, association of the variant with the phenotype within a family and by bioinformatic tools (SIFT, Align GVGD, Polyphen, SpliceSiteFinder-like, MaxEntScan, NNSplice, GeneSplicer, and Human Splicing Find, all as part of Alamut software, Alamut), as described in detail before. [11,23,24]

IDA controls

Participants of the control group were consecutively enrolled between February 2018 and May 2019 while attending different departments of the Máxima MC (MMC) Veldhoven, the Netherlands, for work-up of IDA. For ethical reasons, we only included IDA controls aged ≥18 years.

A total of 161 patients underwent laboratory testing for IDA and were screened for potential study inclusion by checking laboratory results, medical history and medication use. IDA

controls were included if they had microcytic anemia, according to WHO guidelines,[25] with TSAT ≤15% and ferritin levels ≤40 µg/L (which is higher than the WHO-defined cut-off value of <15 µg/L).

Use of either oral or parenteral iron supplementation during the last three months, CRP >10 mg/L, severe renal impairment (defined as an estimated Glomerular Filtration Rate (eGFR) < 30 ml/min/1.73 m2)[26] and chronic liver disease (defined as ALT > 40 IU/L) were exclusion criteria, as these factors have been described to potentially influence hepcidin production.[8,27-29]

Of the 161 eligible patients, we excluded 122 subjects because of (a combination of) the above-mentioned exclusion criteria. This resulted in the inclusion of 39 IDA controls. The study was conducted according to the principles of the Declaration of Helsinki and approved by the local ethics committee of the MMC. For all participants, oral and written informed consent was obtained. The study was registered at the Netherlands Trial Register as 'SATURNUS Study', an acronym for the 'Transferrin **Satur**ation/Hepcidi**n** ratio: a st**u**dy on the diagno**s**tic utility in the differentiation of Iron Refractory Iron Deficiency Anemia from Iron Deficiency Anemia'.[30]

Laboratory measurements

For IRIDA and IDA patients Hb, red blood cell indices, serum iron parameters, CRP and if applicable serum creatinine and ALT were measured in accredited Dutch hospital laboratories. Glomerular Filtration Rate was estimated (eGFR) with the CKD-EPI formula in patients in whom a serum creatinine was measured.[26]

In both IRIDA patients and IDA controls, hepcidin-25 was measured by weak cation exchange time-of-flight mass spectrometry (WCX-TOF MS) in freshly-thawed samples, as described before.[31]This assay was recently standardized, using a secondary reference material (RM) that was value assigned by a provisional primary RM.[32] Reference values for this standardized assay are available.[33] Measurements of serum hepcidin in the IRIDA group were performed before standardization of our assay, while measurements of serum hepcidin in the IDA control group were performed with the same assay, but after standardization. Standardization slightly altered serum hepcidin values, that is, standardized results were found to be 5.4% higher compared to historic results obtained without standardization.[33] Stability of the assay in time was ensured by the use of serum matrix based quality controls.

Statistical analysis

Descriptive statistics were reported as medians, 25th to 75th percentiles (Interquartile Range, IQR), and/or ranges for continuous variables and as absolute frequencies and percentages for categorical variables, using original untransformed values. Normality of distributions of the continuous variables was visually checked using histograms. Baseline characteristics between the IRIDA group and the non-IRIDA group were compared and tested by using the Chi-squared test for categorical variables and the Mann-Whitney U test for continuous variables to determine whether there were significant differences between the groups. Measurements of hepcidin levels and the TSAT/hepcidin ratio were stratified by sex with for female subjects a distinction between premenopausal and postmenopausal age for female subjects. In addition, in the IRIDA group a distinction was made between children and adults, since sex- and age-specific differences in hepcidin levels have been previously described between children and adults.[34] [33]

Hepcidin values below the lower limit of detection (<0.5 nM) were imputated with the average value of 0.25 nM. We assessed the performance of the TSAT/hepcidin ratio in distinguishing *TMPRSS6*-related IRIDA from *TMPRSS6*-unrelated IDA by Receiver Operating Characteristic (ROC) analysis.

In order to avoid negative values, we used the TSAT/hepcidin ratio, instead of the TSAT/loghepcidin ratio as others did.[14] The area under the curve (AUC) was calculated, including the sensitivity and specificity for each possible cut-off point of the TSAT/hepcidin ratio. An AUC value of 1 represents the perfect discriminating test. The Youden index ([sensitivity + specificity -1]) was used to select the optimal cut-off value.[35] *P* values less than 0.05 were considered statistically significant. SPSS version 22 was used for data analysis.

RESULTS

Patient characteristics IRIDA patients

We included 21 patients in the IRIDA group. Seventeen out of 21 patients were diagnosed between 2010 and 2015 and were described earlier.[11] Four out of 21 IRIDA patients were diagnosed between 2015 and 2019 and have not been described elsewhere. Clinical, biochemical and genetic characteristics of the IRIDA group are presented in Table 1A. The bi-allelic affected group consisted of 7 females and 4 men, the mono-allelic affected group of 9 females and 1 male. MLPA showed no large deletions and/or duplications in the as 'healthy' considered *TMPRSS6* allele. Age at time of genotypic confirmation of IRIDA ranged from 1 to 38 years in the bi-allelic affected group, with a median age of diagnosis at 9 years old (IQR 6-21 years). In the mono-allelic group evaluation of IRIDA took place at a median age of 40.5 years (IQR 31-48 years).

Patient characteristics IDA controls

The IDA group consisted of 39 patients, of whom 29 (74 %) were female. Clinical, biochemical and treatment characteristics of the IDA patients are presented in Table 1B. All controls had a microcytic anemia according to the WHO definition (MCV ≤80 fL and Hb <13.5 g/dL for men; <12 g/dL for women)[36] with a TSAT <15%, of which 95 % had TSAT levels ≤10%. Median ferritin level was 9.0 µg/L (range 2-38 µg/l), median CRP was 1.8 mg/L (range 0.2-8.1 mg/L). Remarkably, 9 out of 39 IDA were overweight (23%) and 17 out of 39 IDA controls were obese (44%) according to international cut-off points for BMI.[37] Apart from IDA, 18 out of 39 (46%) patients had comorbidity, whereby diabetes mellitus was the most common condition (12 out of 18=67%). Sixteen out of 39 IDA cases (41%) had a normal kidney function (defined as eGFR >90 ml/min/1.73 m^2) and 17 out of 39 patients (44%) had a mild to moderately decreased kidney function (eGFR >30-< 90 ml/min/1.73 m^2). In the other 6 out of 39 (15%) renal function was not determined.

Causes of IDA of the control subjects are summarized in Table 1B. In 21 out of 39 patients (54%) and underlying cause for IDA was found with gastrointestinal bleeding being the most prevalent cause (31%) In 18 out of 39 patients (46%), IDA was unexplained after extensive workup, including gastroscopy, colonoscopy and video capsule endoscopy. In these cases, IDA was attributed to an insufficient diet and/or the use of certain medication that enhances the risk of gastrointestinal bleeding (e.g. anticoagulants, antithrombotic agents and NSAIDs) or diminishes iron absorption (e.g. proton pomp inhibitors). In the

unexplained IDA controls responsiveness to oral or parenteral iron was assessed. Thirteen out of 18 patients responded to iron supplementation, suggesting IDA not related to inappropriately increased serum hepcidin levels relative to body iron related to a *TMPRSS6* defect. In 3 out of 18 patients no iron therapy was given because of spontaneous Hb normalization without treatment, gastrointestinal side-effects after iron supplementation in the past and refusal of iron therapy. In all IDA controls, iron therapy was initiated after blood withdrawal for assessment of the TSAT/hepcidin ratio.

Comparison of baseline characteristics.

At the time of TSAT/hepcidin assessment, IRIDA patients had a median age 29 years (range 1-58 years), which was significantly younger than the IDA controls, who had a median age of 62 years (range 18-90 years, *p*=0.000, 2-sided Mann-Whitney U test). Women were equally represented in both groups (*p*=0.797, 2-sided Chi-Square test. In IRIDA patients, data on BMI and eGFR were not available, but we anticipated that the proportion of IRIDA patients with overweight or obesity and impaired eGFR is lower because of the younger age compared to the IDA controls. In the IRIDA group MCV was significantly lower and ferritin significantly higher in comparison to the IDA group (p=0.010, 2-sided Mann-Whitney U test).

Table 1A. Clinical, biochemical and genetic characteristics of patients with *TMPRSS6*-related IRIDA

Patient characteristics	N	Median or count	Range or percentage
Age at presentation with anemia, years	21	10	0-57
Age at time of assessment for IRIDA and TSAT/ hepcidin determination, years	20	29	1-58
Women	21	16	76
Systemic disease (i.e. diabetes mellitus)	21	1	5
Hb, g/dL[a]	20	11.5	6.4-13.8
MCV, fL[a]	20	60.5	52.0-87.0
Ferritin, µg/L[a]	20	93	23.0-934
TSAT, %[a]	21	6.1	1.9-20.0
CRP, mg/L	15	<0.5	0.5-8.0
TMPRSS defect	21		
• Bi-allelec	11	11	52
• Mono-allelic	10	10	48

[a] at time of assessment of diagnosis of IRIDA and determination of TSAT/hepcidin ratio, after iron treatment had been given

Table 1B. Clinical, biochemical and treatment characteristics of control patients with iron deficient anemia

Patient characteristics	N	Median or count	Range or percentage
Age at time of TSAT/ hepcidin assessment, years	39	62	18-90
Women	39	29	74
Systemic disease:		18	46
• Diabetes mellitus		12[b]	24
• Hypothyroidism		4[b]	5
• IBD (complete remission)		1	2
Rheumatic disease (complete remission)		1	2
Hb, g/dL[a]	39	9.3	3.8-13.1
MCV, fL[a]	39	73.0	58-80
Ferritin, µg/L[a]	39	9.0	2.0-38.0
TSAT, %[a]	39	5.0	2.0-11.0
CRP, mg/L[a]	39	1.8	0.2-8.1
ALT, U/L[a]	23	18.0	12.0-42.0
eGFR (CKD-EPI), mL/min/1.73m^2 [a,b]	33	67	42->90
BMI, kg/m^2 [a]	39	28	19-42
• Normal weight (18.5-25)		13	33
• Overweight (25-30)		9	23
• Obese (>30)		17	44
Assessment of underlying disorder of IDA	39		
• Unexplained		18	46
• Gastrointestinal bleeding		11	33
• Gynaecological bleeding		5	13
• Malabsorption		4	10
Assessment of medication use	39		
• Anticoagulants		6	15
• Antithrombotic agents		12	31
• NSAIDs		1	3
• Proton pomp inhibitors		14	36
• Corticosteroids		1[c]	3
Assessment of response to iron therapy in unexplained IDA[c]	18		
• Hb increase <2g/dL after oral iron		1	6
• Hb increase <2g/dL after IV iron		1	6
• Hb increase >2g/dL after oral iron		4	22.
• Hb increase >2g/dl after IV iron		9	50
• No iron supplementation		3	17

[a] Before initiation of iron supplementation, at the time of TSAT/hepcidin assessment

[b] eGFR was >90 mL/min/1.73m^2 in 49% of IDA controls, 60-89 mL/min/1.73m^2 in 30% of IDA controls, 45-59 mL/min/1.73m^2 in 18% of IDA controls and 30-44 mL/min/1.73m^2 in 3% of IDA controls.

[c] Response to iron therapy 3 weeks after initiation of iron supplementation

Abbreviations: Hb denotes hemoglobin; MCV, mean corpuscular volume; TSAT, transferrin saturation; CRP, C-reactive protein; ALT, alanine aminotransferase; eGFR, estimated glomerular filtration rate

TSAT/hepcidin ratio siginificantly differs between IRIDA and IDA

The TSAT/hepcidin ratio was significantly lower in IRIDA patients (median 0.7 %/nM, range 0.1-9.1 %/nM) than in IDA controls (median 16.7 %/nM, range 8.0-44.0 %/nM, p=0.000) (Table 2, Figure 1).

Furthermore, we divided the IRIDA group in mono-allelic and bi-allelic affected cases, since patients with a pathogenic mono-allelic *TMPRSS6* defect tend to have lower hepcidin levels compared to bi-allelic affected patients according to our previous observations.[11] Indeed, the TSAT/hepcidin ratio was statistically lower in the mono-allelic affected group, median 0.5 %/nM (range 0.1-1.0 %/nM) versus median 1.4 %/nM (range 0.3-9.1 %/nM, p=0.003) in the bi-allelic affected group. For both bi-allelic and mono-allelic IRIDA patients the TSAT/hepcidin ratio was significantly lower than in IDA controls (p=0.000 and p=0.000 respectively).

For comparison of our results with previously published results by Heeney *et al*[13], who uses non-standardized hepcidin values, we also converted our data to the TSAT/$\log_{10}$(hepcidin). However, using this ratio for much lower standardized hepcidin values, after log transformation the ability to discriminate between IRIDA and IDA disappeared.

Table 2A. Hepcidin values (nM) in IRIDA patients and IDA controls

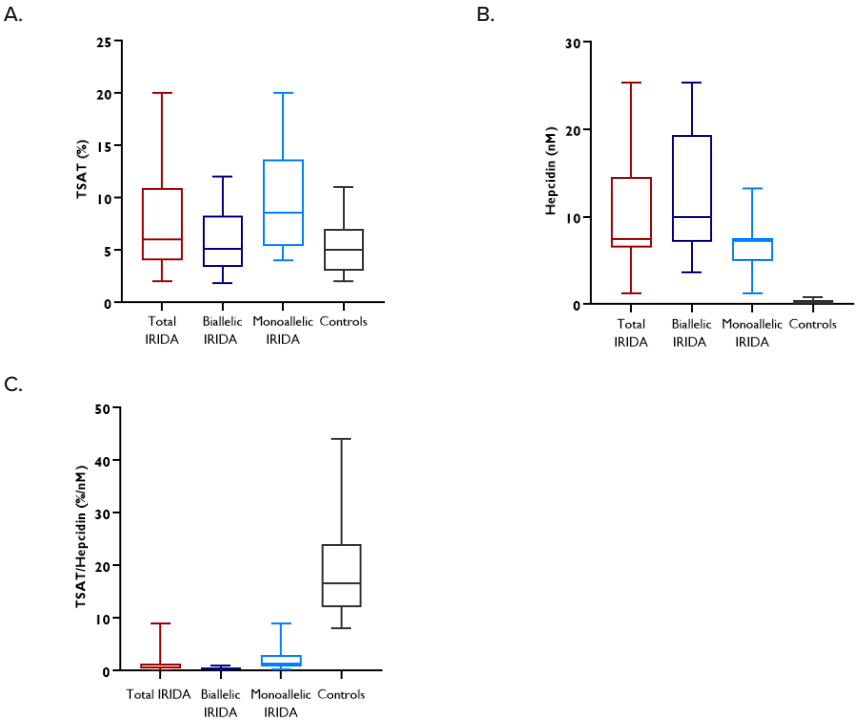
	IRIDA patients			IDA controls			
	n	Median	Min-Max	n	Median	Min-Max	*p*
Total group	21	7.5	1.3-25.4	39	0.3	0.3-0.8	0.000
Men	5	7.6	6.6-22.5	10	0.3	0.3-0.3	0.000
Premenopausal women	15	7.5	1.3-25.4	10	0.3	0.3-0.3	0.000
Postmenopausal women	1	2.4	na	19	0.3	0.3-0.8	na
Adults	13	7.5	1.3-19.4	39	0.3	0.3-0.8	0.000
Children < 12 years	7	7.6	6.6-25.4	na	na	na	na
Bi-allelic *TMPRSS6* defect	11	10.0	3.6-25.4	na	na	na	na
Mono-allelic *TMPRSS6* defect	10	7.3	1.3-13.3	na	na	na	na

Table 2B. TSAT/hepcidin ratios (%/nM) in IRIDA patients and IDA controls

	IRIDA patients			IDA controls			
	n	Median	Min-Max	n	Median	Min-Max	*p*
Total group	21	0.7	0.1-19.1	39	16.7	8.0-44.0	0.000
Men	5	0.5	0.1-1.7	10	20.0	8.0-44.0	0.002
Premenopausal women	15	0.8	0.3-9.1	10	16.0	8.0-28.0	0.000
Postmenopausal women	1	3.8	na	19	20.0	8.0-44.0	na
Adults	13	1.0	0.3-9.1	39	16.7	8.0-44.0	0.000
Children < 12 years	7	0.5	0.3-1.7	na	na	na	na
Bi-allelic *TMPRSS6* defect	11	0.5	0.1-1.0	na	na	na	na
Mono-allelic *TMPRSS6* defect	10	1.4	0.3-9.1	na	na	na	na

For hepcidin levels below the detection limit of 0.5 nM were imputated a value of 0.25 nM.
For reference values of hepcidin and TSAT/hepcidin ratio, see www.hepcidinanalys.com and Donker *et al*.[33,34] Since our data indicate that serum hepcidin relative to body iron significantly drops in both boys and girls after the age of 12 years, we intended to stratify for age <12 years and age 12-17 years. However, since our IRIDA case series contained only one case ≤12 years, comparison of the hepcidin and TSAT/hepcidin ratio between IRIDA cases <12 versus >12 years was not possible.
Abbreviations: Min-Max denotes minimum-maximum

Figure 1. TSAT levels (A), hepcidin levels (B) and TSAT/hepcidin levels (C) in IRIDA patients and control patients



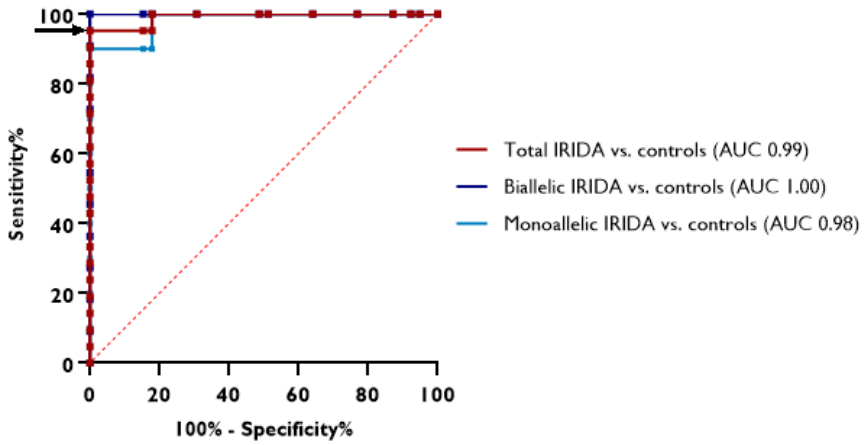
A. TSAT, transferrin saturation in the total IRIDA group (n=21, red box plot), biallelic IRIDA group (n=11, dark blue box plot), monoallelic group (n=10, light blue blox plot) and IDA controls (n=39, black box plot); B. Serum hepcidin levels in the toal IRIDA group (n=21), biallelic IRIDA group (n=11), monoallelic group (n=10) and IDA controls (n=39); C. TSAT/hepcidin ratio in IRIDA group (n=21). biallelic IRIDA group (n=11) monoallelic group (n=10) and IDA controls (n=39). Box and whisker plots present the quartiles (box), the medians (bold line) and the minimum and maximum (whiskers).

TSAT/hepcidin ratio distinguishes IRIDA from IDA with high specificiy

ROC-analysis was performed to assess the ability of the TSAT/hepcidin ratio to differentiate between IRIDA and IDA (**Figure 2**). The area under the curve (AUC) for the TSAT/hepcidin ratio in the total IRIDA group versus the IDA group was 0.991 (p=0.000). By using the Youden index,[35] an optimal cut-off value of 5.9%/nM was established. At this cut-off, a TSAT/hepcidin ratio of 5.9%/nM or lower distinguished IRIDA patients from IDA controls with a sensitivity of 95% (95% CI, 79-100%) and a specificity of 100% (95% CI, 93-100%). The AUC for TSAT/hepcidin in the bi-allelic IRIDA group versus the IDA group was 1.000 (p=0.000) with a sensitivity of 100%

(95% CI, 87-100%) and the same specificity as in the total study population. The AUC in the mono-allelic affected group versus the IDA group was 0.982 (p=0.000) with a sensitivity of 90% (95% CI, 72-98%) and a comparable specificity of 100% (95% CI, 93-100%). There was one IRIDA patient with a TSAT/hepcidin ratio of >5.9%, concerning a monoallelic affected patients with a ratio of 9.1%nM, based on a TSAT of 11.8% and a hepcidin level of 1.3 nM after parenteral iron treatment (i.e. intramuscular iron in combination with vitamin C) had been given. Since our choice to imputate hepcidin levels below the lower limit of detection (LLOD) with a value of 0.25 nM might over-estimate the differences between the TSAT/hepcidin ratios of the IRIDA group and IDA control group, we also performed ROC-analysis of the ratio with imputated hepcidin levels of 0.49 nM. Using the latter level for imputation for levels < LLOD, the TSAT/hepcidin ratio also discriminates between IRIDA and IDA with high sensitivity and specificity (Supplemental Table 1).

Figure 2. ROC curve of TSAT/hepcidin ratio in IRIDA patients and IDA controls.



Receiver operator characteristic curve analysis comparing the TSAT/hepcidin ratio in the non-IRIDA group versus the total IRIDA group (red), the bi-allelic IRIDA group (dark blue) and the mono-allelic IRIDA group (light blue). The arrow indicates the cut-off point of 5.9%/nM, which renders a specificity of 100% and a sensitivity of 95%.

DISCUSSION

In this study we aimed to (i) validate the TSAT/hepcidin ratio as a diagnostic tool to distinguish *TMPRSS6*-related IRIDA from other disorders presenting with IDA unrelated to *TMPRSS6* and (ii) to establish a cutt-off value for the ratio to differentiate between both groups with high specificity.

Using standardized hepcidin values, we confirmed that the TSAT/hepcidin ratio is able to distinguish IRIDA patients from IDA controls in a broad iron deficient population, provided moderate or severe inflammation is absent and no recent iron therapy has been given. Based on our findings, we reasoned that using our standardized hepcidin assay a ratio of 5.9%/nM or lower strongly indicates the presence of IRIDA, as a low TSAT/hepcidin ratio was highly specific for *TMPRSS6*-related IRIDA.

To our knowledge, there is only one research group that elaborated a comparable biochemical method to distinguish IRIDA from other forms of IDA. Heeney *et al*[13] evaluated the TSAT/$\log_{10}$(hepcidin) ratio to predict which patients were most likely to have bi-allelic *TMPRSS6* mutations in a group of patients who had a high pre-test probability of having IRIDA, as they had chronic iron deficiency with TSAT≤15% and a poor response to at least one course of oral iron supplementation. After genetic evaluation of *TMPRSS6*, they included for further analysis 44 patients with bi-allelic *TMPRSS6* defects and 59 IDA controls without identifiable *TMPRSS6* mutations by Sanger sequencing. In agreement with their observations,[13] we also found that hepcidin levels were significantly higher in *TMPRSS6*-related IRIDA patients compared to IDA controls. Moreover, while Heeney *et al* apply the TSAT/$\log_{10}$(hepcidin), we observed better ROC characteristics with higher specificity using the TSAT/hepcidin ratio, to distinguish IRIDA patients from IDA controls. A higher pre-test probability of having *TMPRSS6*-related IRIDA in the study population of Heeney *et al* might explain the difference in ROC characteristics, since they included IDA patients that failed to respond to oral iron supplementation, while we did not test responsiveness to oral iron in our IDA controls.[38] In addition, we argue that our IDA controls might have been more iron deficient compared to Heeney's *et al* controls, resulting in lower hepcidin levels and that recent iron therapy could have increased hepcidin levels in their study population.

Another factor that makes comparison of results between the two studies challenging, is that we reported the TSAT/hepcidin ratio rather than the log-transformed TSAT/$\log_{10}$(hepcidin) ratio, and that both studies used differently calibrated assays. Previous round robin studies show that thepcidin levels of the group of Heeney *et al* are measured up to approximately a factor 7 higher due to the use of a calibrator in their assay that is not traceable to primary reference material. [39] Indeed, after multiplying our hepcidin values 7 times, we observed a comparable ability of the TSAT/$\log_{10}$(hepcidin) ratio in distinguishing IRIDA from IDA as in our untransformed TSAT/hepcidin data.

The strengths of our study are: i) we validated the ability of the TSAT/hepcidin ratio to detect both mono-allelic affected IRIDA patients as bi-allelic affected IRIDA patients from IDA controls in a broad iron-deficient population, ii) we established a cut-off value for use of the TSAT/hepcidin ratio as diagnostic test in the work up of iron deficient microcytic anemic patients suspected for the presence of IRIDA, iii) we used a recently standardized assay for hepcidin measurements of our IDA controls enabling future comparison of results of hepcidin assays used elsewhere, provided these are standardized by using the same reference material. This will ultimately allow global uniform decision-making based on TSAT/hepcidin ratios. iv) We described baseline characteristics and comorbid conditions that could have influenced hepcidin production in IDA controls in detail.

Our study has several limitations. As we used a case-control design in which IDA controls had an alternative diagnosis explaining their microcytic anemia and the prevalence of *TMPRSS6*-related IRIDA is unknown, we could not calculate the positive and negative predictive value. However, since a low TSAT/hepcidin ratio was highly specific for *TMPRSS6*-related IRIDA, we argue that a ratio of 5.9%/nM or lower strongly indicates the presence of IRIDA. Nevertheless, there was one IRIDA patient who was diagnosed with IRIDA based on microcytic anemia unresponsive to oral iron treatment in combination with a mono-allelic pathogenic *TMPRSS6* variant, despite the serum hepcidin level being not discrepantly high (i.e. an hepcidin value of 1.3 nM). This resulted in a TSAT/hepcidin ratio of 9.1%/nM, which would be considered as not suspect for having *TMPRSS6*-related IRIDA according to our established cut-off value of 5.9%/nM. One could argue this patient has been diagnosed with IRIDA incorrectly, as inappropriately increased serum hepcidin levels in relation to body iron stores are the cornerstone of diagnosing IRIDA, even after iron supplementation has been

given.[11,12] We hypothesize that in this case another gene or pathway irrespective of hepcidin expression might be involved, explaining the 'normal' hepcidin level despite the IRIDA phenotype.[40]

Secondly, comorbid conditions could have interfered with the performance of the test. Nevertheless, although factors that have been described to stimulate hepcidin production (i.e. advanced age, high BMI, diabetes mellitus, renal impairment) were more present in the control group, serum hepcidin levels remained very low in our IDA controls.[8] This suggests that iron deficiency in IDA patients seems to be a stronger determinant on net circulating hepcidin levels than these comorbid conditions, as has been demonstrated by others as well.[18,41-43] However, we did not assess the performance of the test in controls who had moderate-to-severe signs of inflammation (CRP >10 mg/L), underlying inflammatory disorders (e.g. malignancy, active IBD), severe kidney impairment (eGFR <90 ml/min/1.73m2) and/or recent iron therapy (<3 months). We expect specificity to be lower in these subgroups, since those conditions are described to increase hepcidin synthesis, which could result in TSAT/hepcidin ratios more resembling those found in IRIDA patients.

In addition, due to restricted sample size we could not assess the performance of the TSAT/hepcidin ratio between IRIDA patients of postmenopausal age compared to their IDA counterparts. Moreover, since we did not include children in the IDA control group for ethical reasons, we could also not assess the ratio between children diagnosed with IRIDA and IDA peers.

At last, we did not perform genetic testing of *TMPRSS6* in our control group, which renders us unable to state with certainty that the controls with 'unexplained IDA' had no pathogenic *TMPRSS6* variants.

Taken together, our findings demonstrated that the TSAT/hepcidin ratio could be a useful tool for differentiating *TMPRSS6*-related IRIDA from other causes of microcytic anemia early in the diagnostic work-up of IDA. Our observations show that a TSAT/hepcidin ratio ≤5.9%/nM strongly indicates the presence of IRIDA, which could assist clinicians in distinguishing IRIDA from other disorders presenting with IDA, thereby ensuring timely referral for *TMPRSS6* testing and early initiation of parenteral iron therapy when *TMPRSS6*-related IRIDA has been confirmed after genetic analysis. Moreover, in patients with IDA unresponsive to oral iron treatment in

which endoscopic evaluation of the gastrointestinal tract is not necessary to exclude malignancy as possible cause of IDA, as is mainly the case when it concerns children, *TMPRSS6*-related IRIDA should be considered when a ratio ≤5.9%/nM has been found before performing invasive gastrocolonoscopy as this procedure carries a higher risk in children than in adults (1.1 % versus 0.3%), predominantly related to the use of general anesthesia and sedation.[44]

Further studies are required to i) assess the performance of the TSAT/hepcidin ratio in IDA controls with comorbid inflammatory conditions and patients who received recent iron therapy (<3 months) and in IDA patients < 18 years and ii) evaluate the value of the TSAT/hepcidin ratio as screening tool for detecting *TMPRSS6*-related IRIDA from multi-causal IDA in a prospective cohort study.

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Author Contributions

AED, DLB, and DWS designed the research. HS, AED, and DLB coordinated the data collection. JS, LM and MB coordinated the inclusion of the subjects at the Department of Gastro-Enterology, the Emergency Room Department and the Department of Gynecology of the Máxima Medical Center respectively. SM and CML performed the hepcidin analyses. AED, HS, JPD, DLB and DWS analyzed the data and interpreted the results. HS and AED wrote the manuscript.

Conflicts of Interest

SMK, CML and DWS are employees of Radboudumc, which offers hepcidin assays and hepcidin reference material to the research, clinical, and pharmaceutical community at a fee for service via the Hepcidinanalysis initiative (www.hepcidinanalysis.com).[33] All other authors have no conflict of interest to declare.

Data Sharing Statement

For original data, please contact Dorine W. Swinkels (dorine. swinkels@radboudumc.nl).

REFERENCES

1. Hentze MW, Muckenthaler MU, Galy B, Camaschella C. Two to tango: regulation of Mammalian iron metabolism. *Cell*. 2010;142(1):24-38.
2. Muckenthaler MU, Rivella S, Hentze MW, Galy B. A Red Carpet for Iron Metabolism. *Cell*. 2017;168(3):344-361.
3. Silvestri L, Pagani A, Nai A, De Domenico I, Kaplan J, Camaschella C. The serine protease matriptase-2 (TMPRSS6) inhibits hepcidin activation by cleaving membrane hemojuvelin. *Cell metabolism*. 2008;8(6):502-511.
4. Silvestri L, Nai A, Dulja A, Pagani A. Hepcidin and the BMP-SMAD pathway: An unexpected liaison. *Vitamins and hormones*. 2019;110:71-99.
5. Finberg KE, Heeney MM, Campagna DR, et al. Mutations in TMPRSS6 cause iron-refractory iron deficiency anemia (IRIDA). *Nature genetics*. 2008;40(5):569-571.
6. Silvestri L, Guillem F, Pagani A, et al. Molecular mechanisms of the defective hepcidin inhibition in TMPRSS6 mutations associated with iron-refractory iron deficiency anemia. *Blood*. 2009;113(22):5605-5608.
7. De Falco L, Sanchez M, Silvestri L, et al. Iron refractory iron deficiency anemia. *Haematologica*. 2013;98(6):845-853.
8. Girelli D, Nemeth E, Swinkels DW. Hepcidin in the diagnosis of iron disorders. *Blood*. 2016;127(23):2809-2813.
9. Heeney MM, Finberg KE. Iron-refractory iron deficiency anemia (IRIDA). *Hematology/oncology clinics of North America*. 2014;28(4):637-652, v.
10. Melis MA, Cau M, Congiu R, et al. A mutation in the TMPRSS6 gene, encoding a transmembrane serine protease that suppresses hepcidin production, in familial iron deficiency anemia refractory to oral iron. *Haematologica*. 2008;93(10):1473-1479.
11. Donker AE, Schaap CC, Novotny VM, et al. Iron Refractory Iron Deficiency Anemia (IRIDA): A heterogeneous disease that is not always iron refractory. *American journal of hematology*. 2016.
12. Donker AE, Raymakers RA, Vlasveld LT, et al. Practice guidelines for the diagnosis and management of microcytic anemias due to genetic disorders of iron metabolism or heme synthesis. *Blood*. 2014;123(25):3873-3886; quiz 4005.
13. Heeney MM, Guo D, De Falco L, et al. Normalizing hepcidin predicts TMPRSS6 mutation status in patients with chronic iron deficiency. *Blood*. 2018;132(4):448-452.
14. Heeney MM, Campagna DR, Westerman M, Fleming MD. The clinical and genetic spectrum of TMPRSS6 mutations leading to inappropriate hepcidin expression and iron refractory iron deficiency anemia (IRIDA). Paper presented at: Blood2009.
15. Zimmermann MB, Troesch B, Biebinger R, Egli I, Zeder C, Hurrell RF. Plasma hepcidin is a modest predictor of dietary iron bioavailability in humans, whereas oral iron loading, measured by stable-isotope appearance curves, increases plasma hepcidin. *The American journal of clinical nutrition*. 2009;90(5):1280-1287.
16. Bregman DB, Morris D, Koch TA, He A, Goodnough LT. Hepcidin levels predict nonresponsiveness to oral iron therapy in patients with iron deficiency anemia. *American journal of hematology*. 2013;88(2):97-101.
17. Moretti D, Goede JS, Zeder C, et al. Oral iron supplements increase hepcidin and decrease iron absorption from daily or twice-daily doses in iron-depleted young women. *Blood*. 2015;126(17):1981-1989.
18. Stoffel NU, Lazrak M, Bellitir S, et al. The opposing effects of acute inflammation and iron deficiency anemia on serum hepcidin and iron absorption in young women.

Haematologica. 2019;104(6):1143-1149.
19. Camaschella C. Iron deficiency: new insights into diagnosis and treatment. *Hematology American Society of Hematology Education Program.* 2015;2015:8-13.
20. Manders P, Lutomski, J.E., Smit, C., Swinkels, D.W. and Zielhuis, G.A. The Radboud Biobank: A Central Facility for Disease-Based Biobanks to Optimise Use and Distribution of Biomaterial for Scientific Research in the Radboud 2018.
21. Schrier SL. So you know how to treat iron deficiency anemia. *Blood.* 2015;126(17):1971.
22. White SJ, Breuning MH, den Dunnen JT. Detecting copy number changes in genomic DNA: MAPH and MLPA. *Methods in cell biology.* 2004;75:751-768.
23. Richards S, Aziz N, Bale S, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genetics in medicine : official journal of the American College of Medical Genetics.* 2015;17(5):405-424.
24. https://www.interactive-biosoftware.com/alamut-visual/. Accessed 19 June 2019.
25. http://www.who.int/vmnis/anaemia/prevalence/summary/anaemia_data_status_t2/en/. Accessed 3 July 2019.
26. Levey AS, Coresh J, Balk E, et al. National Kidney Foundation practice guidelines for chronic kidney disease: evaluation, classification, and stratification. *Annals of internal medicine.* 2003;139(2):137-147.
27. Tan TC, Crawford DH, Franklin ME, et al. The serum hepcidin:ferritin ratio is a potential biomarker for cirrhosis. *Liver international : official journal of the International Association for the Study of the Liver.* 2012;32(9):1391-1399.
28. Nemeth E, Rivera S, Gabayan V, et al. IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin. *The Journal of clinical investigation.* 2004;113(9):1271-1276.
29. Kemna E, Pickkers P, Nemeth E, van der Hoeven H, Swinkels D. Time-course analysis of hepcidin, serum iron, and plasma cytokine levels in humans injected with LPS. *Blood.* 2005;106(5):1864-1866.
30. The Transferrin Saturation/Hepcidin ratio: a study on the diagnostic utility in the differentiation of *TMPRSS6*-related Iron Refractory Iron Deficiency Anemia from *TMPRSS6*-unrelated Iron Deficiency Anemia (SATURNUS study) https://www.trialregister.nl/trial/6845
31. Laarakkers CM, Wiegerinck ET, Klaver S, et al. Improved mass spectrometry assay for plasma hepcidin: detection and characterization of a novel hepcidin isoform. *PloS one.* 2013;8(10):e75518.
32. Diepeveen LE, Laarakkers CMM, Martos G, et al. Provisional standardization of hepcidin assays: creating a traceability chain with a primary reference material, candidate reference method and a commutable secondary reference material. *Clinical chemistry and laboratory medicine.* 2019;57(6):864-872.
33. http://www.hepcidinanalysis.com. Accessed 2 March 2019.
34. Donker AE, Galesloot TE, Laarakkers CM, Klaver SM, Bakkeren DL, Swinkels DW. Standardized serum hepcidin values in Dutch children: Set point relative to body iron changes during childhood. *Pediatric blood & cancer.* 2020;67(3):e28038.
35. Yin J, Tian L. Joint confidence region estimation for area under ROC curve and Youden index. *Stat Med.* 2014;33(6):985-1000.
36. http://www.who.int/nutrition/publications/en/ida_assessment_prevention_control.pdf.Accessed 3 July 2019.
37. https://www.who.int/gho/ncd/risk_factors/bmi_text/en/. Accessed at 23 May 2019.
38. Rutjes AW, Reitsma JB, Vandenbroucke JP, Glas AS, Bossuyt PM. Case-control and two-gate designs in diagnostic accuracy studies. *Clin Chem.* 2005;51(8):1335-1341.

39. Diepeveen LE, Laarakkers CMM, Martos G, et al. Provisional standardization of hepcidin assays: creating a traceability chain with a primary reference material, candidate reference method and a commutable secondary reference material. *Clin Chem Lab Med*. 2018.
40. Pagani A, Colucci S, Bocciardi R, et al. A new form of IRIDA due to combined heterozygous mutations of TMPRSS6 and ACVR1A encoding the BMP receptor ALK2. *Blood*. 2017;129(25):3392-3395.
41. Theurl I, Aigner E, Theurl M, et al. Regulation of iron homeostasis in anemia of chronic disease and iron deficiency anemia: diagnostic and therapeutic implications. *Blood*. 2009;113(21):5277-5286.
42. Kearney SL, Nemeth E, Neufeld EJ, et al. Urinary hepcidin in congenital chronic anemias. *Pediatric blood & cancer*. 2007;48(1):57-63.
43. Gardenghi S, Marongiu MF, Ramos P, et al. Ineffective erythropoiesis in beta-thalassemia is characterized by increased iron absorption mediated by down-regulation of hepcidin and up-regulation of ferroportin. *Blood*. 2007;109(11):5027-5035.
44. Thakkar K, El-Serag HB, Mattek N, Gilger M. Complications of pediatric colonoscopy: a five-year multicenter experience. *Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association*. 2008;6(5):515-520.

SUPPLEMENTAL FILE

Supplemental Table 1. Performance characteristics of the TSAT/hepcidin ratio to distinguish subjects with IRIDA (n=21) from patients with IDA other than IRIDA (n=39), provided that inflammation is absent, and no iron therapy has been given < 3 months.

		IRIDA group vs. non-IRIDA group (n=21 and n=39)		
TSAT/hepcidin ratio:		AUC	SE of AUC	95% CI of AUC
A.	*Hepcidin <0.5 nM = 0.25 nM*			
	Total study population	0.991	0.009	0.973-1.000
	Bi-allelic IRIDA group vs. non-IRIDA group	1.000	0.000	1.000-1.000
	Mono-allelic IRIDA group vs. non-IRIDA group	0.982	0.019	0.945-1.000
B.	*Hepcidin <0.5 nM = 0.49 nM ('worst-case')*			
	Total study population	0.977	0.000	0.931-1.000
	Bi-allelic IRIDA group vs. non-IRIDA group	1.000	0.000	1.000-1.000
	Mono-allelic IRIDA group vs. non-IRIDA group	0.951	0.048	0.858-1.000

.SE and 95% CI of AUC calculated with SPSS version 22.0 and 95% CI of sensitivity and specificity calculated with Ken Rothman's Episheet, 2011.[1]

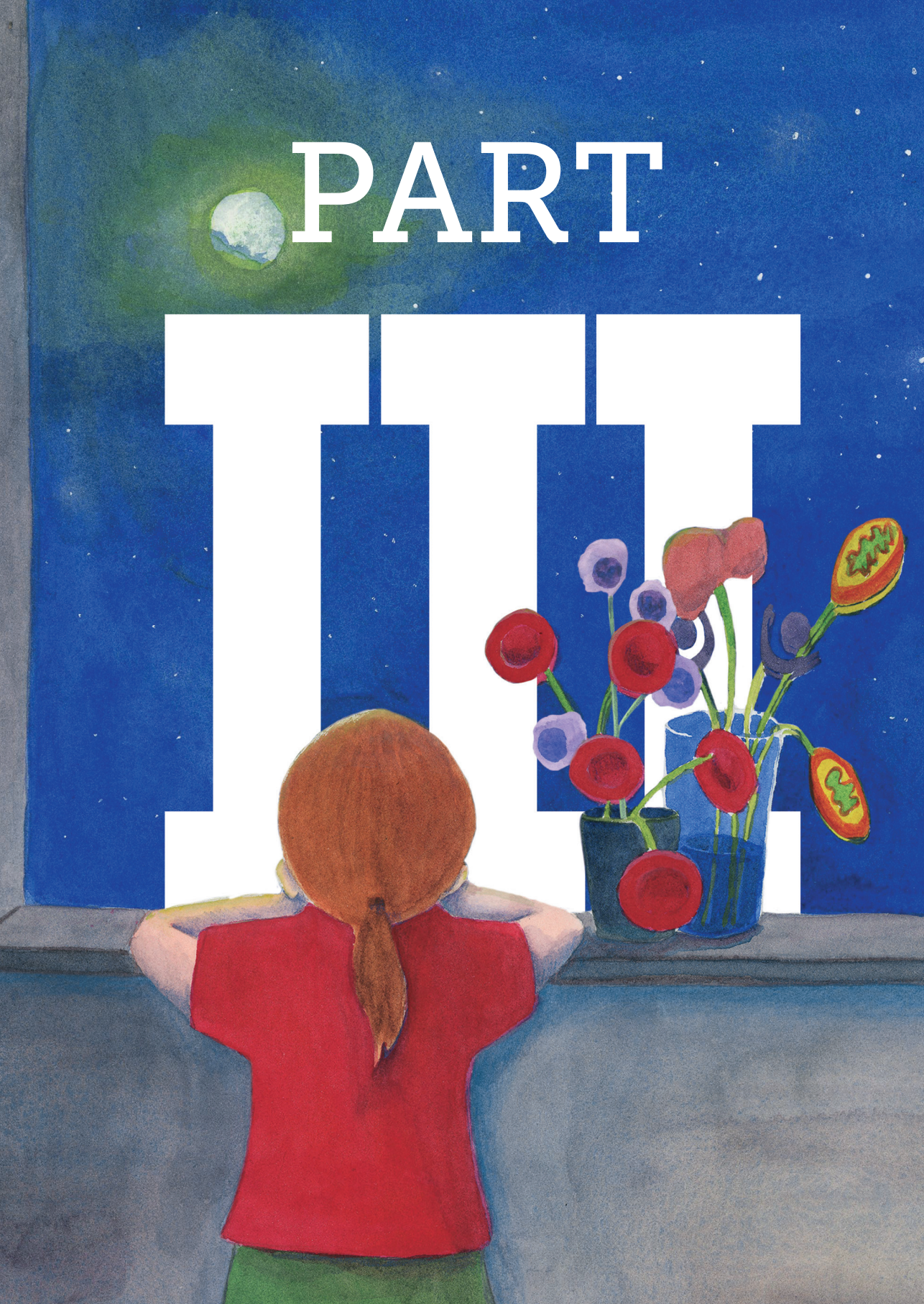
Abbreviations: AUC denotes area under the curve; SE, standard error; CI, confidence intervals

REFERENCE

1. Kenneth J. Rothman JDB. Spreadsheets for the analysis of epidemiologic data. Version 4 October 2012. Available at: http://krothman.hostbyet2.com/Episheet.xls. Accessed 3 June 2019.

	Cut-off value	Sensitivity	95% CI of Sensitivity	Specificity	95% CI of Specificity
	<5.9 %/nM	95%	79-100%	100%	93-100%
	<4.5 %/nM	100%	87-100%	100%	93-100%
	<5.8 %/nM	90%	72-98%	100%	93-100%
	<3.9 %/nM	95%	79-100%	100%	93-100%
	<2.6 %/nM	100%	87-100%	100%	93-100%
	<3.9%/nM	90%	72-98%	100%	93-100%

DISCUSSION, SUMMARY AND ADDENDA


PART
III

Chapter 9

General Discussion

GENERAL DISCUSSION

In this thesis, we explored several clinical and diagnostic aspects of microcytic anemias due to a genetic disorder of iron metabolism or heme synthesis. We intended to create awareness among clinicians for these rare disorders, and to provide them with information and tools that facilitate distinction from the far more common causes of microcytic anemias as iron deficiency and hemoglobinopathies. This is of great importance because timely diagnosis of these anemias may prevent an unnecessarily long diagnostic process and a not effective or even harmful treatment, thereby reducing the disease burden and avoiding lifelong sequelae of either iron deficiency or iron overload for the individual patient.

In this concluding chapter, we review our main findings and reflect upon the implications for clinical practice. We discuss the strengths and limitations of the studies and consider future research challenges with a focus on Iron Refractory Iron Deficiency Anemia (IRIDA).

STUDIES AT A GLANCE

PART I: LITERATURE AND CLINICAL STUDIES

We started with a narrative review with the intention to identify the critical roles of iron for the different iron-demanding organ systems during the journey from fetus to adult and to assess the developmental aspects of iron handling, with a focus on the systemic level. We aimed to increase the understanding of iron biology in childhood, therewith enabling a timely and accurate diagnosis of both genetic and acquired iron disorders in children (Chapter 2). After a comprehensive survey of the literature we concluded that iron homeostasis in the growing and developing child is still poorly understood and that appropiate biomarkers for the various iron-consuming organs, especially the central nervous system, are largely absent. This limited comprehension of iron physiology of childhood complicates the diagnosis and management of both acquired iron disorders as iron deficiency (anemia), anemia of inflammation or iron loading due to ineffective erythropoiesis and also of anemias due to genetic disorders of iron metabolism that are addressed in this thesis. For example, lack of pediatric serum hepcidin reference values hamper the diagnosis of *TMPRSS6*-related IRIDA. Limited understanding of the optimal iron status for the different iron-consuming tissues in the growing and developing child impede adequate treatment concerning both iron deficiency (ID) and iron overload (IO). Considerations for future research in order to unravel iron handling in childhood are reviewed in the discussion section of Chapter 2.

We continued with a systematic review of the literature on microcytic anemias due to a genetic disorder of iron metabolism or heme synthesis, with the aim of developing evidence-based and multidisciplinary guidelines, aiding the clinician in the diagnosis, treatment and family screening of these rare disorders (Chapter 3[1]). We presented such guidelines on 12 disorders of microcytic anemia due to defects in 13 different genes involved in iron metabolism and heme synthesis. We concluded that many aspects of these orphan diseases regarding the pathophysiology, clinical presentation and optimal diagnostic workup and treatment are still unknown, making the level of evidence relatively low. Therefore, we suggested that national and international collaboration between both clinicians and researchers is crucial, in order to improve the clinical care for the involved patients. Suggestions how to shape this cooperation in the field of microcytic anemias due to a genetic disorder of iron metabolism or heme synthesis, are addressed in this chapter. In the next chapters (Chapter 4[2] and Chapter 6[3]) we retrospectively described Dutch case series of IRIDA due to *TMPRSS6* defects and of

X-linked Sideroblastic Anemia (XLSA) due to *ALAS2* defects respectively, assessing age of presentation, clinical and biochemical features, genotype and treatment characteristics of the patients. The case study on IRIDA illustrates the clinical and genetic characteristics of inappropriately increased hepcidin levels relative to body iron. Conversely, the case series of XLSA demonstrates the clinical and genetic characteristics of inappropriately decreased hepcidin levels relative to body iron. Both studies exemplify the phenotypic and genotypic heterogeneity of genetic disorders of iron metabolism and heme synthesis, suggesting a complex interplay between (epi)genetic and environmental factors in the pathogenesis of these disorders. These factors still need elucidation, with the ultimate goal of determining the prognosis, optimal diagnostic approach and treatment regimen, tailored to the individual patient. Suggestions to achieve this goal, in particular for IRIDA, are discussed in this chapter.

PART II: DIAGNOSTIC STUDIES: FOCUS ON IRON REFRACTORY IRON DEFICIENCY ANEMIA

In the diagnostic part of this thesis (PART II) we described a study on serum hepcidin levels, hepcidin/ferritin ratios and transferrin saturation (TSAT)/hepcidin ratios in 266 healthy children aged 0.3-17 years, measured with a standardized assay, with the intention to facilitate the diagnosis of iron disorders in childhood (Chapter 7[4]). We demonstrated that serum hepcidin and also hepcidin relative to ferritin and TSAT is age dependent in children, suggesting that the set point of serum hepcidin relative to stored and circulating iron changes during childhood. This suggestion needs confirmation in a broader population as we elaborate on in this chapter.

In Chapter 8 we performed a study assessing the value of the TSAT/hepcidin ratio in discriminating IRIDA from iron deficiency anemia (IDA) due to acquired causes e.g. gastro-intestinal or vaginal blood loss or malabsorption in 21 IRIDA patients and 39 IDA controls. We observed that the TSAT/hepcidin ratio was significantly lower in IRIDA patients compared to IDA controls. We therefore concluded that the TSAT/hepcidin ratio is able to differentiate between IRIDA and IDA-controls due to other reasons with high specificity and sensitivity in a broad iron-deficient population, provided moderate to severe inflammation is absent and no recent iron therapy has been given. Recommendations to validate the TSAT/hepcidin ratio in a population without the above-mentioned restrictions are important in order to increase its usability in clinical practice. This is discussed in the discussion section of Chapter 8.

LOOKING BACKWARDS: STRENGTHS AND LIMITATIONS

Strengths of this thesis

Extensive laboratory research during the last 20 years has answered many questions regarding both the physiology and pathophysiology of systemic and cellular iron homeostasis.[5,6] Several genes have been discovered that play a pivotal role in microcytic anemias due to genetic disorders of iron metabolism and heme synthesis.[7-10] However, clinical studies addressing the accurate diagnosis and adequate treatment of these anemias are relatively scarce. The power of the descriptive and diagnostic studies in this thesis lies in our patient-centered point of view, thereby bridging the gap between basis science and clinical practice. The systematic approach of our evidence-based guidelines in combination (Chapter 3[1]) with the extensive description of the phenotypes and genotypes in our case series (Chapter 4[2] and Chapter 6[3]) will help the clinician in distinguishing these rare disorders from the far more common causes of microcytic anemia due to an acquired iron deficit or due to a thalassemia syndrome.

Apart from adults, we paid attention to children in our studies, especially in Chapter 2 and Chapter 7.[4] This is crucial; the very first lesson we learned as residents in pediatrics was: children are not just small adults. Children have specific anatomical and physiological characteristics that change and mature during the journey from fetal life to adulthood. That is also true for the regulation of systemic and cellular iron handling (Chapter 2); the dynamic process of growth and development provides an extra challenge for clinicians to accurately diagnose and treat iron disorders in childhood. Therefore, the establishment of standardized pediatric serum hepcidin ranges and hepcidin ratios relative to indicators of body iron is an important step in elucidating the physiology of iron metabolism during the successive maturation stages of human life and in facilitating the pediatrician dealing with children with (suspected) genetic and acquired iron disorders.

Importantly, for the determination of serum hepcidin levels in our diagnostic studies (Chapter 7[4] and Chapter 8) we used a hepcidin assay that was standardized with our commutable 2nd reference material (RM), value-assigned by a candidate primary RM.[11] Both clinical care and research in the field of genetic and acquired iron disorders would benefit from comparability of laboratory results independent of time, place,

and measurement procedure. As a first step, harmonization ensures traceability to a reference system agreed on by convention. The next step is standardization, which leads to the "true" value, by ensuring traceability to the International System of Units. [12,13] Standardization, but at least harmonization, is paramount regarding serum hepcidin measurements procedures. Until now, serum hepcidin measurements procedures had not been standardized nor harmonized.[14,15] Therefore, our studies performed with a standardized hepcidin assay are unique and allow the definition of international reference values and clinical decision limits, and as such have the potential of bringing clinical care and research regarding iron disorders to a higher level.

Limitations of this thesis

Our studies have several limitations that should be taken into account. These include the retrospective description of patients in our case series. Furthermore, in the diagnostic studies, the majority of patients were of Dutch ancestry and thus of Western European origin. Moreover, in the study on serum reference values of hepcidin in children (**Chapter 7**[4]) the age distribution of the participants was skewed; young children were underrepresented.

Another important point is that our patients with IRIDA and XLSA were diagnosed by traditional pathways, following the conventional sequence of history, examination, standard hematological and biochemical assays, possibly a bone marrow aspirate and at last genetic analysis, revealing the relevant gene defect. This approach requires a high index of suspicion from the clinician and might result in selection bias regarding instigating genetic testing for IRIDA, XLSA and other microcytic anemias due to disorders of iron metabolism and heme synthesis. Nowadays, the quick advance of next generation sequence (NGS) techniques (particular whole exome sequencing (WES)) [16-18] is changing the work-up of patients with rare diseases, also in the field of benign hematology.[18,19] Employing WES in the diagnostic process is altering the known phenotypic spectrum of diseases, as illustrated by the case series of a family with multiple females with a macrocytic dyserythropoietic anemia that turned out to be caused by an X-linked *ALAS2* defect. This presentation is different from the known classical phenotype of *ALAS2* defects as a combination of microcytic anemia and iron loading in male patients.[19] Another example is the description by Khuong-Quang of two siblings suffering from unexplained severe microcytic anemia, hypoferremia and hyperferritinemia, responding to some extent

to oral iron supplementation.[20] The clinical and biochemical characteristics of the children did not fit any previously described microcytic anemia known at that time. WES revealed compound heterozygous *TMPRSS6* defects in both patients that previously were described as pathogenic, thereby elucidating the genetic basis of this unusual clinical presentation of *TMPRSS6* defects. [20] It also further uncovers the pathophysiology of IRIDA, which is characterized by a distribution of the absorbed orally supplemented iron to the RES, resulting in relatively high ferritin levels for the amount of body iron.

Furthermore, the unbiased analysis of coding exons by WES might reveal genotypic variability of disorders, meaning that defects in multiple genes lead to the same clinical phenotype and that defects in the same gene might result in a variation in clinical phenotype, or that a combination of defects in genes involved in iron metabolism modulates the phenotype. Anecdotal data suggesting that co-inheritance of *HFE* and *ALAS2* variants might worsen the iron loading in patients with XLSA are a good example of the latter,[21] as further corroborated by the clinical course of patient 2B (**Chapter 6**[3]) who was diagnosed with both a p.Arg452His *ALAS2* defect and a homozygous p.Cys282Tyr *HFE* defect. He developed iron loading already in his teens, while in the majority of XLSA patients IO does not become evident until the third or fourth decade.

Since the patients we studied were diagnosed by a clinical presentation, followed by classical Sanger sequencing of the candidate gene, we might have assessed selected subpopulations of patients with a certain phenotype of a microcytic genetic disorder of iron metabolism or heme synthesis, and we might have missed defects in other disease-causing or modulating genes that explain the phenotype of the patients.

LOOKING FORWARDS: FUTURE PERSPECTIVES

Biobanking is a promising tool in unrevealing the pathogenesis of rare genetic anemias and improving the clinical care of the involved patients

In 2009, Time Journal published an article entitled '10 ideas changing the world right now; number 8: Biobanking'. [22] Ten years later, biobanking has indeed brought a lot of discoveries and new knowledge in the whole process of medical research in the twenty-first century. [23,24] The collection of bio-specimens along with clinical data in biobanks facilitates the stratification of patients with the same disorder, accelerating the new area of personalized medicine from curing to prevention.[23]

The upcoming of the science of biobanking is of great importance against the background of the limitations of this thesis as mentioned above; the retrospective design of our case series and the small study populations of predominantly Western-European descent. Therefore, a first suggestion to allow future research, from bench to bedside, would be the establishments of a European and ultimately international biobank of patients with a diagnosed or suspected microcytic anemia due to a genetic defect of iron homeostasis or heme synthesis. Such a biobank, comprising both biomaterial, demographic and clinical data, will enable the assessment of the natural course of these anemias for a broad population, the identification of disease-modifying genetic and environmental factors and hopefully the development of effective treatment regimens. International biobanking might also help in identifying epidemiologic differences in the prevalence and incidence of diseases between various populations across the world and the underlying pathophysiological mechanisms for such differences. To date, 115 IRIDA patients in 85 families have been described worldwide, harboring 78 different TMPRSS6 variants.[2,25-27] Forty-one out of these 115 patients are Dutch inhabitants (**Chapter 4[2], Chapter 8**), which raises the question whether IRIDA is associated with ethnicity or whether IRIDA is an under-diagnosed disorder. Furthermore, 5 out of the 21 patients who are described in detail in **Chapter 4[2]** were immigrants with a Mediterranean origin, e.g. Morocco and Turkey. In addition, according to the literature, many IRIDA cases with a pathologic *TMPRSS6* defect are from thalassemia or malaria endemic regions.[1,25,28] This might suggest a survival benefit for the combination of thalassemia (carrier ship) and *TMPRSS6* defects, which might result in a higher incidence of IRIDA in thalassemia endemic regions. Possible explanations for this hypothetical relation are the findings that low

body iron status protects against malaria, [29-31] and that both α –thalassemia (HbH disease) and β-thalassemia major and intermedia, but also carriers of α –thalassemia have relatively low serum hepcidin levels due to an ineffective erythropoiesis.[32-34] In β-thalassemia mice, *TMRPSS6* defects counteract the low serum hepcidin levels, thereby attenuating iron overload and anemia.[35-37] Biobanking on an international level would be helpful in exploring these hypotheses.

The Iron Biobank, as part of the Radboud Biobank that involves a central storage facility of patient biomaterials, designed in accordance with the standards set by a Dutch national biobank (Parelsnoer Institute[38]), is a first and important step in the direction of biobanking in the field of iron disorders, although a lot of work needs to be done.[39,40] This Iron Biobank prospectively collects clinical data and bio-specimens of Dutch individuals, referred to the Radboud Center of Iron Disorders (RCID[41]), with (suspected) inherited disorders of iron metabolism (hereditary hemochromatosis or anemia due to disorders of iron metabolism and heme synthesis) and hemoglobinopathies, and also of their family members in a standardized way.[39-41] However, the recent enormous advances in molecular medicine and genomic research have raised a range of ethical, legal and social implications related to biobank-based research, facing both researchers and clinicians with major challenges that still need to be overcome.[42] Main areas of concern involve the transparency to biobank participants and stakeholders regarding the use and sharing of health data for research, the active involvement of participants in biobank activities, including the selection of research topics and the establishment of public and private partnerships, which constitutes a risk of getting into conflicts of interest around profit-based versus public health activities.[42]

Moreover, the recent introduction of two major legal instruments in Europe has great impact on the practice of biobank medicine. First, the European Union General Data Protection Regulation 2016/679 (GDPR) dictates new and strict rules in terms of transparency, data subjects' rights and information about data uses, and introduces a legal framework regarding direct responsibility of the processor of (health) data to the individual subject.[43] Second, Article 27 of The Additional Protocol to the Convention on Human Rights and Biomedicine, concerning Biomedical Research, establishes legal and ethical obligations in the countries that have ratified this Council of Europe instrument.[44,45]

The translation of the above-mentioned legislation to daily practice is costly and time-consuming. Practical solutions, especially with regards to informed consent (IC) procedures that reasonably take account of the rights of the involved patients without interfering with the conduction of relevant research are urgently needed. This is of particular interest for minors, for which the procedure regarding participation in clinical research is even more complicated from a legal point of view.[46]

Apart from biobanking, (virtual) networks with health care providers involved in the care for patients with complex and/or rare disorders that require concentrated knowledge and resources and highly specialized treatments, is crucial. The European Reference Network (ERN) EuroBloodNet, of which the RCID is a part, is a promising example of such collaboration.[41,47,48] Further developing this network will certainly benefit (European) patients with rare disorders, such as the genetic anemias addressed in this thesis.

Reference values of serum hepcidin are required for children of all age groups from various origins

As discussed earlier, our study on serum values of hepcidin in children (Chapter 7[4]) predominantly contained older children of Western-European descent; young children and children of non-Western European origin were underrepresented. Therefore, broadening the study population regarding children of different age groups and ethnic origins is essential in order to establish usable and widely applicable reference values of serum hepcidin and its ratio to the iron parameters ferritin and TSAT, which form the starting point of the diagnosis and treatment of iron disorders in childhood. Noticeably, our data on serum hepcidin values relative to indicators of body iron show evidence for a changing set point of iron handling during childhood. This point needs further exploration, preferably in prospective cohort studies that assess the course of serum hepcidin levels from fetal life until young adulthood in a healthy population. However, this will present a challenge since children who visit the clinic periodically for check-ups including blood tests are often diagnosed with chronic conditions that are known to affect serum hepcidin values by either organ dysfunction as liver- or renal impairment or by inflammation. Moreover, clinical research in children is challenging because of the stringent Dutch legislation applying to medical research in minors; at this point practical and workable guidelines are urgently needed for this purpose.

Dutch legislation regarding minimal-risk pediatric research needs adjustments

First of all, we are certainly aware of the important historical roots of the legislation around human research, roots that should never be forgotten. On December 9, 1946, an important conference took place at the Nuremberg Palace of Justice that would define the course and nature of permissible human research. The conference was part of the Nuremberg trials, conducted by a military tribunal, held after World War II by the Allied Forces under international law. This was the start of the so-called Doctors' trial for war crimes of German doctors, accused of having been involved in human experiments including exposure to simulated high altitudes, freezing, malaria and mustard gas amongst other cruelties. Fifteen of the 23 defendants were found guilty and sentenced to death by hanging.[49] This historical indictment resulted in a list of conditions (Nuremberg Code[50] and later in the Declaration of Helsinki[51]) required for the conduction of 'permissible human experiments', which became the cornerstone of the ethics of human research and a template for all informed consent forms regarding participation in clinical trials. Autonomy, beneficence and justice are leading principles in these documents. Importantly, in pediatric research self-determination is exercised by proxy (parents or guardians), requiring even more careful balancing of the benefits versus the disadvantages of participation in clinical trials.[52]

Taking into account the dramatic violations of human rights in human experiments during the past, strict rules regarding research involving human subjects are warranted. In the Netherlands, this legislation is defined in the Medical Research Involving Human Subjects Acts (Wet Medisch Onderzoek (WMO)).[53] Apart from this law, also European legislation regarding conducting human research exists, as the guideline for Good Clinical Practice (GCP).[54] In line with the United Nation Convention of the Rights of the Child (UNCRN)[55], including minors in clinical trials is subject to stringent rules in order to protect vulnerable children who are not able yet to stand-up for themselves.

Against the background of the historical abuses around human research we definitely agree with the strict legislation as defined in the WMO and GCP guideline. We also agree with the even more strict rules regarding research that involves minors since the very important principle of autonomy might be compromised in children. However, in our opinion, research ethics committees should apply various degrees of stringency regarding the relevant ethical and regulatory structures, dependent

of the impact of the study. Such an approach would be in line with the legislation in the United States (US).[56] In the US, different categories of research are identified regarding the risks and the anticipated direct benefits for the subject. In case of a minimal risk study, anticipated direct benefit is not required and a simplified informed consent procedure will suffice. Importantly, the signature of only one parent is required. Procedures under the minimal-risk threshold include collection of blood (3 mL/kg with a maximum of 50 mL) and psychological examinations and tests amongst others.[56] On the basis of these criteria, the study we performed on serum hepcidin values in Dutch children could be classified as such a minimal risk study (**Chapter 7**[4]). However, the Central Committee on Research Involving Human Subjects (Centrale Commissie Mensgebonden Onderzoek (CCMO)),[57] which is charged with accrediting and supervising the medical ethics review committees in the Netherlands, does not acknowledge such a division of studies with respect to low- or high risk. Therefore the consent of both parents/caregivers is required, regardless of the impact of the study for the child. This is remarkable since the WMO dictates that the stringent GCP standards only apply to pharmaceutical research.[53,54]

Our experience (and also the experience of other Dutch researchers[58]) is that this lack of proportionality hampers the conduction of important research in children. Moreover, we are concerned about the implications of the current developments in the legislation regarding multi-parenting on the possibility to include children in clinical trials.[59] If the recommendations of Wolfsen *et al*[59] regarding multi-parenting indeed will be followed, a child might have four legal representatives in the future. As yet, the Dutch Government has not accepted this recommendation.[60] However, if they do so, this will render the conduction of pediatric clinical research practically impossible.

We think a balance between reasonable legislation and practical feasibility is crucial, by tailoring the rules regarding pediatric research to the impact of inclusion in a clinical study, without compromising the protection of the subject. The very comprehensive Dutch legislation around the inclusion of minors in clinical studies entails the risk that children as subjects are protected in such extent that pediatric research will become impossible, with the inevitable consequence that children have to be exposed to diagnostic and therapeutic procedures without evidence for its effectiveness in this particular age group.

Therefore, in our opinion, Dutch legislation regarding minimal-risk pediatric research needs relaxation, especially of the informed consent procedure. In the interest of children, we highly agree with the following recent statement in the Lancet: 'The time has come to protect children and young people *through* research, not *from* research'.[61]

High-throughput DNA sequencing technology in the field of iron disorders holds significant promise for the future

Since the use of traditional diagnostic approaches was also an important limitation of our studies, a next suggestion to strengthen future research would be the wider application of NGS, especially WES, in patients with (suggested) microcytic anemias due to genetic disorders of iron metabolism or heme synthesis. As mentioned above, WES might shed new light on the phenotypic and genotypic spectrum of particular diseases. Importantly, various studies have shown a high concordance with Sanger sequencing, making WES non-inferior and potentially even more accurate than the classical sequencing techniques.[62,63]

During the last decades, enormous progress has been made in the mapping of genes directly causing diseases or conferring susceptibility to complex disorders in general,[16] also in the field or iron disorders.[18] Apart from WES in patients, genome wide association studies (GWAS) in a broad population have discovered common genetic variants associated with iron metabolism that might confer a susceptibility to iron disorders.[64,65] Although such associations simply represent a statistical relation between specific *alleles* and phenotypes, which might have various reasons (totally different from the phenomenon of linkage that represents a relation between specific *loci* e.g. physical sites on the chromosome and phenotypes), GWAS has provided useful insights regarding iron handling, which are helpful in the formulation of hypotheses regarding iron disorders. Good examples are the studies of Traglia[66] and Galesloot,[67] who disproved the hypothesis that the association of common *HFE* and *TMPRSS6* with body iron parameters can be explained by the intermediate effect on hepcidin concentration. By contrast, their data suggest that serum hepcidin independent mechanisms play a role in the association of certain *HFE* and *TMPRRS* variants with serum iron parameters. [66,67]

However, data obtained from GWAS explain only a small fraction of the genetic risk for particular diseases.[68] Therefore, substantial challenges remain for researchers and clinicians that aim to unravel the architecture of susceptibility of complex diseases, such as the disorders addressed in this thesis, and to translate the information gathered into improvements in clinical guidelines. One of these challenges involves the identification of disease-modifying genetic variants that lie in the middle of the spectrum of at one side very rare DNA sequences directly causing disease with a Mendelian inheritance pattern (high penetrance), and on the opposite side common DNA variants that have very weak effects (low penetrance), which can be identified by GWAS. These variants with both an intermediate allele frequency and penetrance can neither be detected by linkage analysis because of the relatively low penetrance nor by association studies because of the relatively low allele frequency.[68,69] Such variants could be identified by sequencing of candidate genes by WES in patients that have been diagnosed with a specific genetic defect by classical Sanger sequencing. We would suggest such an approach in our patients with microcytic anemias due to inherited disorders of iron metabolism or heme synthesis, for example in the patients with a phenotype of IRIDA in whom only a heterozygous *TMPRSS6* defect was found. We would prefer a targeted WES, filtering only the relevant candidate genes since a major concern of genome-wide DNA sequencing techniques is still the identification of secondary, actionable findings. Those unintended findings might reveal genetic information not pertaining to the patient's presenting conditioning, possibly raising negative consequences such as stigmatization, financial burden, discrimination in insurance and employment.[70] This is particularly relevant and challenging in the case of children, since the parents have to serve as substitute decision makers, weighing the potential benefits against the potential undesirable above-mentioned consequences of the disclosure of secondary findings.[71]

KNOWLEDGE GAPS CONCERNING IRON REFRACTORY IRON DEFICIENCY ANEMIA

The genetic and molecular background of IRIDA needs further elucidation

The genetic aspects and molecular pathways underlying the pathogenesis of IRIDA are poorly understood. In our IRIDA case series (Chapter 4[2]) 14 out of 21 of our IRIDA patients had a homozygous or compound heterozygous *TMPRSS6* defect, consistent with an autosomal recessive disease, in line with the majority of IRIDA cases that have been described in the literature.[1] However, in the other seven patients both Sanger sequencing and Multiplex ligation-dependent probe amplification (MLPA) identified only a heterozygous *TMPRSS6* defect, challenging the idea of Mendelian inheritance due to rare *TMPRSS6* variants with a high penetrance. These findings corroborate previous reports of mono-allelic patients presenting with an IRIDA phenotype (reviewed in[1]). Also, the question remains whether others and we did not miss small deletions or insertions in the coding sequences, defects in the introns and promoter region or balanced translocations of chromosome 22, affecting expression or function of the wild type considered allele. Several IRIDA probands in our series in whom only a heterozygous *TMPRSS6* defect was found, had first-degree relatives without an IRIDA phenotype but with identical *TMPRSS6* genotypes, supporting this hypothesis. Moreover, the results of parental haplotype analysis that we performed in two IRIDA families suggested that differences in phenotype between probands and unaffected siblings with the same heterozygous *TMPRSS6* defect could be attributed to differences in the as wild type considered inherited parental allele, also supporting this idea.

Apart from still undetected pathogenic variants in this as wild type considered *TMPRSS6* allele, *cis*-acting mechanisms may also explain the different phenotypes in IRIDA patients and individuals with identical *TMPRSS6* genotypes. Epigenetic processes as imprinting, involving DNA and histone methylation, are known to influence mRNA transcription and may result in a difference concerning the relative expression of the paternal-inherited versus the maternal-inherited allele. [72] Furthermore, the presence of polymorphisms in regulatory elements of the *TMPRSS6* gene may play a role in these *cis*-acting mechanisms.[72] Interestingly, mice studies indicate that expression of *HAMP* is regulated by histone acetylation and de-acetylation,[73] suggesting that indeed epigenetic phenomena may play a role in the etiology of IRIDA. In addition to the above-mentioned genetic factors, environmental

conditions may determine the phenotype of IRIDA, as suggested by mice studies that show and increases susceptibility to the development of ID after exposure to an iron-deficient diet in *TMPRSS6* haplo-insufficient mice during periods of rapid growth and increased erythropoiesis.

Concerning the possible intronic variants in the as wild type considered allele in IRIDA patients in whom only a mono-allelic *TMPRSS6* defect has been identified, sequencing of the whole *TMPRSS6* gene including the introns may be an appropriate approach. However, the interpretation of deep intronic alterations still forms a big challenge, compared to modifications in coding regions that cause clearly interpretable changes in amino acid sequence or splicing sites.[18] Addition of RNA studies could be helpful in determining (non)-pathogenicity of variants, especially with regards to identifying aberrant splicing. However, since RNA functions as a temporary intermediate, enabling the transfer of data from the genome to the organism, it is subject to targeted degradation by various ribonucleases. This intrinsic unstable character of RNA renders its laboratory use for diagnostic purposes challenging, especially with regards to bio-specimens stored in biobanks, since commonly used banking modalities are not able to adequately preserve RNA molecules.[74] We therefore think that at the current state of knowledge sequencing of the whole *TMPRSS6* gene is actually not a feasible approach.

Apart from the possibility we missed defects in the as wild type considered *TMPRSS6* allele, we might have overlooked pathogenic variants in other *trans*-acting genes that affect *TMPRSS6* expression or genes that modulate hepcidin levels independently from matriptase 2. This latter suggestion is supported by the case description of Pagani *et al* who described a digenic form of IRIDA due to a combination of defects in both *TMPRSS6* defect and in *ACVR1A*, encoding the BMP receptor ALK2.[75] Therefore, we propose WES sequencing of these candidate genes that are described to be involved in hepcidin regulation in mono-allelic IRIDA cases. Of note, also this approach will inevitably generate findings that are difficult to interpret. Deletions, frameshifts, nonsense mutations and changes to splice sites are very likely to be pathogenic, but deciding whether a nucleotide substitution that has not been described earlier in SNP databases (as the NCBI dbSNP, describing information on allele frequency, functional consequence and clinical significance[76]) is pathogenic and hence represents one of the sought after defects, can be very challenging.[76,77] Co-segregation of a certain allele variant with the relevant IRIDA phenotype within

a family will support but not prove pathogenicity since the true cause of the disease might be located elsewhere in the gene, in the *cis* position. If SNP databases and co-segregation data within a family do not answer the question whether a new variant is pathogenic or not, *in silico* predictions can be helpful.[78] However, performing functional studies is still the gold standard for determining the (non)pathogenicity of variants of unknown significance (VUS). For this reason, we suggest to perform functional studies of VUS that will be found by WES sequencing of candidate genes in mono-allelic IRIDA patients by the above-mentioned approach.

Functional studies might also be helpful in determining the pathogenicity of VUS in *TMPRSS6* variants that are often found in suspected IRIDA cases. However, the development of functional *in vitro* assays that represent the *in vivo* situation is challenging. Moreover, the exact function of matriptase 2 is still not completely certain. As reviewed earlier in this thesis, *in vitro* experiments have shown that matriptase 2 (MT2) acts by cleaving hemojuvelin, the BMP co-receptor that upregulates *HAMP* transcription. [79] IRIDA-causing *TMPRSS6* variants result in aberrant MT2 with impaired HJV-cleaving capacity, resulting in inappropriately increased hepcidin levels. [79] These data and other observations [80] suggest that HJV is the substrate of MT2. However, recent *in vivo* studies in *Hjv* knock out mice overexpressing *TMPRSS6* suggest that also BMP receptors of the BMP/SMAD pathway are cleaved by MT2.[81] Whether BMP receptors are substrates of MT2, next to HJV, needs further exploration in functional studies. Performing these studies might identify novel working mechanism for matriptase 2, form the basis for novel functional assays for *TMPRSS6* variants, and might help to further elucidate the pathophysiology of IRIDA, with the ultimate goal of improving the diagnosis and treatment for the individual patient.

The lifetime course of IRIDA is intriguing and raises questions concerning the effects of *TMPRSS6* defects during the journey from fetus to adult

The lifetime course of IRIDA in human life is intriguing. According to our observations and those of others,[82] suspicion of IRIDA because of unexplained and therapy-resistant IDA predominantly occurs during childhood, but not at birth or during the first postnatal months. This raises the following questions: i) Is hepcidin suppressed by other, MT2 independent pathways in the fetus and neonate? Noteworthy, in *TMPRSS6 -/-* fetal mice mRNA expression of liver *HAMP* is significantly higher than in control mice, arguing against this suggestion.[83,84] ii) Are the fetus and neonate protected for overexpression of hepcidin, and if so, by which mechanism(s)? One

might argue that infants are born with sufficient iron stores at birth to overcome the first months of infancy, also in case of an IRIDA due to a pathogenic *TMPRSS6* defect. However, in IRIDA patients, iron stores are often sufficient irrespective of age. The main problem is the maldistribution of iron since the high hepcidin levels favor entrapment of iron inside the macrophages. This mechanism plays an even more important role in early life because of the relatively short life span and consequently high turnover of fetal Hb. The occurrence of sufficient iron stores at birth does therefore not sufficiently explain the apparent absence of complaints during the first postnatal months of an IRIDA patient. Interestingly, studies in suckling mice suggest that enterocyte ferroportin is hypo-responsive to hepcidin during infancy.[85] Other mice studies indicate that in the fetus and infant DMT1 and ferroportin are expressed in all areas of the small intestines and colon, in contrast to the situation in adults in whom iron absorption predominantly occurs in the duodenum.[86] These studies indicate that both the sensitivity of ferroportin to degradation by hepcidin and the surface area available to absorb iron are age-dependent, possibly explaining the onset of IRIDA symptoms only a couple of months after birth in the majority of patients.

According to our observations and those of others (reviewed in [1]), in most patients, especially in whom a bi-allelic *TMPRSS6* defect is found, the signs and symptoms of IRIDA, including iron deficiency anemia that is not responsive to oral iron and only partially responsive to parenteral iron, typically persist into early adulthood. Afterwards, the phenotype becomes milder in many IRIDA patients, with declining requirements for (parenteral) iron treatment. One might hypothesize that iron needs of the erythroblasts and/or other iron-demanding tissues decrease with decline of growth in adults. Another explanation might be the increasing TSAT/hepcidin ratio during childhood, as we observed in our reference values study on serum hepcidin values (**Chapter 7**[4]), indicating a changing set point of systemic iron homeostasis, with an increased tendency to absorb and release iron into the circulation with advancing age.

As a first step, our own observations regarding this typical course need confirmation, underpinning the importance of longitudinally following IRIDA patients due to *TMPRSS6* defects and collecting clinical, biochemical and genetic data in national and international biobanks, as proposed in this chapter.

Decreased energy metabolism on the cellular level affecting multiple organ systems may play a role in the clinical phenotype of IRIDA

Since the majority of iron in the human body is attributed to hemoglobin synthesis, a persistent deficit of iron will inevitably lead to IDA, with a decreased oxygen-transporting capacity to the tissues. Clinically, this will result in fatigue and decreased exercise intolerance, which are the most commonly heard complaints in IRIDA patients. However, in general we observe a more pronounced tiredness in our IRIDA patients compared to other patients suffering from a chronic anemia, e.g. sickle cell disease.

The reason for the relatively mild complaints of fatigue in patients with chronic anemia lies in the shifting of the oxygen dissociation curve to the right, with a decreased affinity of Hb for oxygen. This is a physiologic adaptation mechanism in order to ensure sufficient oxygen supply to the tissues in conditions of impaired oxygen transporting capacity.

However, ID does not only affect the hematopoietic system; virtually all organ systems in the human body are dependent of iron (see also **Table 1** in **Chapter 2**). Importantly, myoglobin, which is crucial for the function of the muscular system, contains heme of which iron is a critical component. Iron is also involved in thyroid metabolism, whereas IDA is associated with thyroid dysfunction, especially (subclinical) hypothyroidism.[87] The mechanism by which IDA influences thyroid metabolism is still incompletely understood but seems to include an impaired function of the heme-containing enzyme thyroid peroxidase (TPO), a reduced tissue conversion of T4 to T3 and a decreased pituitary TSH secretion.[87] Importantly, on the cellular level, iron is indispensable for mitochondrial respiration, the process that is essential for the generation of adenosine tri phosphate (ATP) as the universal energy donor in the cell.[88] Furthermore, iron plays a crucial role in the citric acid cycle and oxidative phosphorylation.[89]

We therefore hypothesize that the tiredness in IRIDA patients can be attributed to iron deficiency on multiple organ systems, apart from the iron deficit of the erythroblasts, leading to IDA. Results of *in vitro* and animal studies indeed indicate that ID negatively affects cellular oxidative metabolism, especially in the skeletal system that contains a very high concentration of mitochondria. [90,91] Noteworthy, in adult patients with heart failure, the administration of intravenous iron is associated with an improvement

of exercise capacity, clinical status and quality of life, irrespective of concomitant anemia,[92] supporting the idea that ID on the muscular and cellular level plays a role in the symptomatology of IRIDA, independent of IDA.

An important question is whether ID and IDA affect central nervous development and neurocognitive functioning of IRIDA patients. As reviewed earlier in this thesis (Chapter 2), iron is essential for the development of the central nervous system. Iron and iron-containing enzymes are indispensable for neuronal and glial energy metabolism, myelin and neurotransmitter synthesis and degradation.[88,93] According to both animal and human studies, ID and IDA in fetal life and infancy are associated with longlasting and possibly irreversible neurocognitive impairment. [94] However, anecdotal data do not report neuropsychological consequences of IRIDA, even in the presence of a severe phenotype.[95] This corroborates the observations in our IRIDA patients, with the remark that we did not perform neuropsychological assessment and thus might have overlooked minor neurocognitive deficits.

Taken together, the effect of *TMPRSS6* defects and subsequent ID on multiple organs and physiologic systems, including the muscular system, the thyroid gland and the central nervous needs further elucidation in longitudinal clinical studies of IRIDA patients. Insights in the degree and the effect of ID regarding the different iron-demanding tissues might help in tailoring treatment to the individual IRIDA patient, weighing the benefits of (parenteral) iron treatment on the health and wellbeing against the risks of iron loading, especially of the reticulo-endothelial (RES) system.

Balancing between adequate treatment of iron deficiency (anemia) and risk of iron loading of the reticulo-endothelial system is a challenge in IRIDA

As described in this thesis (Chapter 4[2]), and also observed by others (Chapter 3[1]), most IRIDA patients due to *TMPRSS6* defects require parenteral iron in order to correct anemia to a tolerable level. However, response to parenteral iron is still sluggish and incomplete since the inappropriately high serum hepcidin levels keep the iron trapped inside the RES in especially the spleen and liver. Therefore, intravenous iron supplementation poses the IRIDA patient at risk for iron loading of the macrophages. This pattern of iron loading is also characteristic for patients with loss of function variants in *SLC40A1* encoding ferroportin, but differs from observations in patients with hereditary hemochromatosis due to variants in *HFE, TfR2, HJV* and *HAMP* and gain of function variants in *SLC40A1*, which are conversely associated with inappropriately low serum hepcidin levels,

resulting in iron release by the RES and iron accumulation in the parenchymal cells, as the hepatocytes. While parenchymal iron overload may result in liver cirrhosis and hepatocellular carcinoma, this form of iron loading is considered more harmful than RES iron loading.[96-98] However, increased levels of RES iron may impair the cytotoxic response of the macrophage against pathogens and promote the survival of intracellular microbes as Salmonella, Mycobacteria and Legionalla.[99-101] Moreover, recent data suggest that iron loading of the RES might exacerbate the progression of atherosclerosis by inducing inflammation and enhancing the glycolysis inside the macrophages, although the evidence is not unambigious.[102,103] For these reasons, caution should be exercised when using chronic intravenous iron in IRIDA patients, in our opinion. Since macrophages in the human body are predominantly located in the spleen and to a lesser extent in the liver, we suggest that the application of MRI of both the liver and spleen might be helpful in monitoring iron loading of the RES in IRIDA patients in the future. [104-106] We would expect more IO in the spleen than in the liver in these patients, while we anticipate the opposite in patients with mainly parenchymal iron loading due to for example hereditary hemochromatosis. The feasibility of this approach still needs exploration. A potential technical constraint might be the sensitivity of the current MRI scans to discern (minor) differences of iron loading between the liver and the spleen.

Noteworthy, others and we observed that some IRIDA patients to a certain extent respond to prolonged oral iron supplementation, with or without the addition of vitamin C, despite a severe *TMPRSS6* genotype. [2,3,107] Of note, this chronic oral iron supplements might result in unabsorbed iron entering the colon, causing unwanted side effects on the intestinal host-microbiota interface,[108,109] although in the future this problem might be overcome by the administration of liposomal iron or nano-compound iron, both new generations of oral iron with a high gastrointestinal absorption and bioavailability.[110-115] The benefits of these types or oral iron need further exploration in IRIDA patients.

Taking into account the possible side effects of both parenteral en enteral iron supplementation, clinical studies are warranted in IRIDA patients, assessing the benefits of iron administration on Hb but also on exercise tolerance and thyroid function, against the possible side effects regarding infections with intracellular pathogens, atherosclerosis, or dysbiosis of the gut microbiota, dependent on the route of administration and dosing scheme.

A novel and promising approach regarding the treatment of disorders associated with overexpression of hepcidin such as anemia of inflammation (AI)[116] and IRIDA would be the application of hepcidin antagonists.[117] Clinically relevant hepcidin inhibitors currently undergo evaluation in randomized trials in adult patients with AI.[117] To the best of our knowledge, no studies addressing hepcidin inhibitors in children are underway yet.

Regarding the implementation of hepcidin modulating therapies – either hepcidin antagonists for the above-mentioned indications or hepcidin agonists for diseases characterized by hepcidin underexpression due to ineffective erythropoiesis such as the thalassemia syndromes- we have certain reservations. As mentioned earlier, we found indications of a changing set point of hepcidin relative to body iron indicators during childhood. We suggest that the relatively high serum hepcidin levels we observed in young children might support the innate immune system of the infant and toddler, thereby providing a survival advantage in a critical and vulnerable period. We therefore recommend that this topic will be further explored before the eventual implementation of hepcidin suppressors in children diagnosed with IRIDA, in order to define safe target levels of serum hepcidin relative to ferritin and transferrin saturation for this age group.

CONCLUDING REMARKS AND SUMMARY OF FUTURE PERSPECTIVES

Unexplained microcytic anemias might be caused by defects in genes involved in iron metabolism or heme synthesis. An important message of this thesis is that these genetic anemias need attention from both clinicians and researchers. Awareness among clinicians for these rare, heterogeneous disorders will result in a timely diagnosis, thereby avoiding unnecessary long diagnostic processes and allowing the initiation of adequate treatment modalities. Hopefully this will ultimately result in the prevention of both (severe) iron deficiency but also iron loading and its possibly life-long sequelae. As explained in this thesis, this iron loading concerns two different patterns, which might overlap. First, low hepcidin expression followed by increased intestinal iron absorption and iron release by the macrophages results in parenchymal iron excess. Second, hepcidin overexpression with subsequent iron entrapment in the macrophages, particularly after the administration of parenteral iron, leads to RES iron excess. The first mentioned pathophysiologic mechanism is thought to play a role in XLSA, underpinning the importance of a prompt diagnosis in order to prevent severe complications as liver cirrhosis or hepatocellular carcinoma. The second mentioned pathophysiologic mechanism is an important factor in the development of iron loading of the macrophages in IRIDA patients, especially after the administration of intravenous iron supplementation. Noteworthy, there are, although not equivocal, concerns about the association between RES iron loading and atherosclerosis.

The results of the retrospective and observational studies in this thesis provide clinicians with essential information and practical clinical tools, which facilitate the diagnostic and therapeutic process regarding both children and adults with microcytic anemias due to a genetic disorder of iron metabolism or heme synthesis. However, lots of work needs to be done, both on the preclinical and clinical level, as we discussed in this chapter and as we will summarize in the next paragraphs.

First, collaboration on the national and international level by expanding networks and biobanks is an important precondition because of the rarity of the disorders addressed in this thesis. Accompanying ethical, legal and social issues that nowadays hamper the process of biobanking need to be addressed urgently in our opinion. A second prerequisite is the establishment of standardized serum hepcidin

references ranges for children of all age groups and of different origins that can be used worldwide, since the starting point of diagnosing iron disorders, such as IRIDA, is the determination of a serum hepcidin level. Third, we argue for a broader application of NGS with the aim of expanding our knowledge on the genotypic and phenotypic spectrum of the rare anemias addressed in this work.

Next steps regarding the research on IRIDA involve the further elucidation of the genetic and molecular background of this disorder by performing WES sequencing of candidate genes in patients with a mono-allelic *TMPRSS6* defect, and by studies that further confirm or contradict possible other cleavage targets in the BMP-SMAD hepcidin regulatory pathway of MT2. Furthermore, the clinical consequences of ID on the function of the various iron-demanding organ systems such as the muscular system (especially the heart), the thyroid gland and the brain need longitudinal assessment in IRIDA patients. In addition, there is an unmet need for biomarkers to assess functional consequences of iron deficiency beyond anemia. This will help to further elucidate the pathophysiology of IRIDA and to guide decisions on the application of different treatment modalities and dosing schemes tailored to a specific patient.

The ultimate aim to achieve is the establishment of an evidence-based guideline, based on the molecular, genetic and clinical information gathered in internationally collaborating biobanks, allowing a tailored diagnosis and treatment of the individual IRIDA patient of all ages. This will limit the injury induced by both iron deficit and iron excess to a variety of organ systems, preventing the patients from lifelong sequelae that might have a considerable impact on quality of life and longevity.

“Sola dosis facit venenum” (“Only the dose makes the poison”)

Paracelsus (1493-1541)

REFERENCES

1. Donker AE, Raymakers RA, Vlasveld LT, et al. Practice guidelines for the diagnosis and management of microcytic anemias due to genetic disorders of iron metabolism or heme synthesis. *Blood.* 2014;123(25):3873-3886; quiz 4005.
2. Donker AE, Schaap CC, Novotny VM, et al. Iron Refractory Iron Deficiency Anemia (IRIDA): A heterogeneous disease that is not always iron refractory. *American journal of hematology.* 2016.
3. Donker AE, Raymakers RA, Nieuwenhuis HK, et al. X-linked sideroblastic anaemia due to ALAS(2) mutations in the Netherlands: a disease in disguise. *The Netherlands journal of medicine.* 2014;72(4):210-217.
4. Donker AE, Galesloot TE, Laarakkers CM, Klaver SM, Bakkeren DL, Swinkels DW. Standardized serum hepcidin values in Dutch children: Set point relative to body iron changes during childhood. *Pediatric blood & cancer.* 2019:e28038.
5. Hentze MW, Muckenthaler MU, Galy B, Camaschella C. Two to tango: regulation of Mammalian iron metabolism. *Cell.* 2010;142(1):24-38.
6. Muckenthaler MU, Rivella S, Hentze MW, Galy B. A Red Carpet for Iron Metabolism. *Cell.* 2017;168(3):344-361.
7. Iolascon A, De Falco L, Beaumont C. Molecular basis of inherited microcytic anemia due to defects in iron acquisition or heme synthesis. *Haematologica.* 2009;94(3):395-408.
8 .van Rooijen KL, Raymakers RA, Cuijpers ML, Brons PP, Janssen MC, Swinkels DW. [New causes of microcytic anaemia: hereditary disorders of iron homeostasis]. *Nederlands tijdschrift voor geneeskunde.* 2010;154:A1039.
9. Camaschella C. Recent advances in the understanding of inherited sideroblastic anaemia. *British journal of haematology.* 2008;143(1):27-38.
10. Fleming MD. Congenital sideroblastic anemias: iron and heme lost in mitochondrial translation. *Hematology / the Education Program of the American Society of Hematology American Society of Hematology Education Program.* 2011;2011:525-531.
11. Diepeveen LE, Laarakkers CMM, Martos G, et al. Provisional standardization of hepcidin assays: creating a traceability chain with a primary reference material, candidate reference method and a commutable secondary reference material. *Clin Chem Lab Med.* 2018.
12. Greg Miller W, Myers GL, Lou Gantzer M, et al. Roadmap for Harmonization of Clinical Laboratory Measurement Procedures. *Clinical chemistry.* 2011;57(8):1108-1117.
13. Vesper HW, Myers GL, Miller WG. Current practices and challenges in the standardization and harmonization of clinical laboratory tests. *The American journal of clinical nutrition.* 2016;104 Suppl 3:907s-912s.
14. Kroot JJ, van Herwaarden AE, Tjalsma H, Jansen RT, Hendriks JC, Swinkels DW. Second round robin for plasma hepcidin methods: first steps toward harmonization. *American journal of hematology.* 2012;87(10):977-983.
15. van der Vorm LN, Hendriks JC, Laarakkers CM, et al. Toward Worldwide Hepcidin Assay Harmonization: Identification of a Commutable Secondary Reference Material. *Clinical chemistry.* 2016;62(7):993-1001.
16. Zhang J, Chiodini R, Badr A, Zhang G. The impact of next-generation sequencing on genomics. *Journal of genetics and genomics = Yi chuan xue bao.* 2011;38(3):95-109.
17. Ducamp S, Fleming MD. The molecular genetics of sideroblastic anemia. *Blood.* 2019;133(1):59-69.
18. Sankaran VG, Gallagher PG. Applications of high-throughput DNA sequencing to benign hematology. *Blood.* 2013;122(22):3575-3582.
19. Sankaran VG, Ulirsch JC, Tchaikovskii V, et

al. X-linked macrocytic dyserythropoietic anemia in females with an ALAS2 mutation. *The Journal of clinical investigation*. 2015;125(4):1665-1669.
20. Khuong-Quang DA, Schwartzentruber J, Westerman M, et al. Iron refractory iron deficiency anemia: presentation with hyperferritinemia and response to oral iron therapy. *Pediatrics*. 2013;131(2):e620-625.
21. Barron R, Grace ND, Sherwood G, Powell LW. Iron overload complicating sideroblastic anemia--is the gene for hemochromatosis responsible? *Gastroenterology*. 1989;96(4):1204-1206.
22. Time Journal. 12 March 2009. http://content.time.com/time/specials/packages/article/0,28804,1884779_1884782_1884766,00.html. Accessed 23 October 2019.
23. Kinkorova J. Biobanks in the era of personalized medicine: objectives, challenges, and innovation: Overview. *The EPMA journal*. 2015;7:4.
24 Kinkorova J, Topolcan O. Biobanks in Horizon 2020: sustainability and attractive perspectives. *The EPMA journal*. 2018;9(4):345-353.
25. De Falco L, Sanchez M, Silvestri L, et al. Iron refractory iron deficiency anemia. *Haematologica*. 2013;98(6):845-853.
26. De Falco L, Silvestri L, Kannengiesser C, et al. Functional and clinical impact of novel TMPRSS6 variants in iron-refractory iron-deficiency anemia patients and genotype-phenotype studies. *Human mutation*. 2014;35(11):1321-1329.
27. Wang CY, Meynard D, Lin HY. The role of TMPRSS6/matriptase-2 in iron regulation and anemia. *Frontiers in pharmacology*. 2014;5:114.
28. Finberg KE, Heeney MM, Campagna DR, et al. Mutations in TMPRSS6 cause iron-refractory iron deficiency anemia (IRIDA). *Nature genetics*. 2008;40(5):569-571.
29. Clark MA, Goheen MM, Fulford A, et al. Host iron status and iron supplementation mediate susceptibility to erythrocytic stage Plasmodium falciparum. *Nature communications*. 2014;5:4446.
30. Goheen MM, Wegmuller R, Bah A, et al. Anemia Offers Stronger Protection Than Sickle Cell Trait Against the Erythrocytic Stage of Falciparum Malaria and This Protection Is Reversed by Iron Supplementation. *EBioMedicine*. 2016;14:123-130.
31. Goheen MM, Bah A, Wegmuller R, et al. Host iron status and erythropoietic response to iron supplementation determines susceptibility to the RBC stage of falciparum malaria during pregnancy. *Scientific reports*. 2017;7(1):17674.
32. Nemeth E. Hepcidin and beta-thalassemia major. *Blood*. 2013;122(1):3-4.
33. Origa R, Cazzola M, Mereu E, et al. Differences in the erythropoiesis-hepcidin-iron store axis between hemoglobin H disease and beta-thalassemia intermedia. *Haematologica*. 2015;100(5):e169-171.
34. Guimaraes JS, Cominal JG, Silva-Pinto AC, et al. Altered erythropoiesis and iron metabolism in carriers of thalassemia. *European journal of haematology*. 2015;94(6):511-518.
35. Nai A, Pagani A, Silvestri L, Camaschella C. Increased susceptibility to iron deficiency of Tmprss6-haploinsufficient mice. *Blood*. 2010;116(5):851-852.
36. Gardenghi S, Ramos P, Marongiu MF, et al. Hepcidin as a therapeutic tool to limit iron overload and improve anemia in beta-thalassemic mice. *The Journal of clinical investigation*. 2010;120(12):4466-4477.
37. Ginzburg Y, Rivella S. beta-thalassemia: a model for elucidating the dynamic regulation of ineffective erythropoiesis and iron metabolism. *Blood*. 2011;118(16):4321-4330.
38. Parelsnoer Institute. https://parelsnoer.org/page/nl/. Accessed 23 October 2019.
39. Manders P, Siezen AE, Gazzoli S, Smit C, Swinkels DW, Zielhuis GA. Radboud Biobank: a central facility for prospective clinical biobanking in the Radboud university medical center, Nijmegen. OA Epidemiology 2014 Feb 10;2(1):4.
40. Manders P, Peters TMA, Siezen AE, et

al. A Stepwise Procedure to Define a Data Collection Framework for a Clinical Biobank. *Biopreservation and biobanking*. 2018;16(2):138-147.
41. Radboud Iron Center. http://www.radboud-ironcenter.com. Accessed 23 October 2019.
42. Goisauf M, Martin G, Bentzen HB, et al. Data in question: A survey of European biobank professionals on ethical, legal and societal challenges of biobank research. *PloS one*. 2019;14(9):e0221496.
43. European Union General Data Protection Regulation. https://gdpr-info.eu/. Accessed 23 October 2019.
44. Additional Protocol to the Convention on Human Rights and Biomedicine. Article 27 https://rm.coe.int/1680081562. Accessed 23 October 2019.
45. Gefenas E, Cekanauskaite A, Tuzaite E, Dranseika V, Characiejus D. Does the "new philosophy" in predictive, preventive and personalised medicine require new ethics? *The EPMA journal.* 2011;2(2):141-147.
46. Paquette ED, Derrington SF, Shukla A, et al. Biobanking in the Pediatric Critical Care Setting: Adolescent/Young Adult Perspectives. *Journal of empirical research on human research ethics : JERHRE*. 2018;13(4):391-401.
47. Heon-Klin V. European Reference networks for rare diseases: what is the conceptual framework? *Orphanet journal of rare diseases.* 2017;12(1):137.
48. EuroBloodnet. http://eurobloodnet.eu/. Accessed 23 October 2019
49. Rose CD. Ethical Conduct of Research in Children: Pediatricians and Their IRB (Part 1 of 2). *Pediatrics.* 2017;139(5).
50. Nuremberg Code, 1946. https://history.nih.gov/research/downloads/nuremberg.pdf. Accessed 23 October 2019.
51. World Medical Association. Declaration of Helsinki. 64th WMA General Assembly, Fortaleza, Brazil, October 2013. https://www.wma.net/policies-post/wma-declaration-of-helsinki-ethical-principles-for-medical-research involving-human-subjects/ Accessed 23 October 2019.
52. Roberts HM, Liabo K. Research with children and young people: not on them. What can we learn from the non-clinical research? *Arch Dis Child.* 2018.
53. Wet Medisch Onderzoek. https://wetten.overheid.nl/BWBR0009408/2019-04-02. Accessed 23 October 2019.
54. Good Clinical Practice. https://www.ema.europa.eu/en/ich-e6-r2-good-clinical-practice. Accessed 23 October 2019.
55. United Nation Convention of the Rights of the Child. https://www.ohchr.org/documents/professionalinterest/crc.pdf. Accessed 23 October 2019.
56. Rose CD. Ethical Conduct of Research in Children: Pediatricians and Their IRB (Part 2 of 2). *Pediatrics.* 2017;139(6).
57. Centrale Commissie MensgebondenOnderzoek. https://www.ccmo.nl/. Accessed 23 October 2019.
58. Schot MJ, Broekhuizen BD, Cals JW. [Rules and regulations threaten non-pharmacological studies in children]. *Nederlands tijdschrift voor geneeskunde.* 2015;160:A9354.
59. Wolfsen et al, 2016. Rapport van de Staatscommissie Herijking ouderschap "Kind en ouders in de 21ste eeuw". https://www.rijksoverheid.nl/documenten/rapporten/2016/12/07/rapport-van-de-staatsommissie-herijking-ouderschap-kind-en-ouders-in-de-21ste-eeuw. Accessed 23 October 2019
60. Kamerbrief Herijking Ouderschap, 2019. https://www.rijksoverheid.nl/onderwerpen/ouderlijk-gezag/documenten/kamerstukken/2019/07/12/kabinetsreactie-op-de-aanbevelingen-op-het-terrein-van-draagmoederschap-meerouderschap-en-meerpersoonsgezag-van-de-staatscommissie-herijking-ouderschap. Accessed 23 October 2019.
61. Strengthening clinical research in children and young people. *Lancet.* 2015;385(9982):2015.
62. Hamilton A, Tetreault M, Dyment DA, et

al. Concordance between whole-exome sequencing and clinical Sanger sequencing: implications for patient care. *Molecular genetics & genomic medicine.* 2016;4(5):504-512.
63. Zeng X, Lian T, Lin J, et al. Whole-exome sequencing improves genetic testing accuracy in pulmonary artery hypertension. *Pulmonary circulation.* 2018;8(2):2045894018763682.
64. Benyamin B, Esko T, Ried JS, et al. Novel loci affecting iron homeostasis and their effects in individuals at risk for hemochromatosis. *Nature communications.* 2014;5:4926.
65. Benyamin B, Ferreira MA, Willemsen G, et al. Common variants in TMPRSS6 are associated with iron status and erythrocyte volume. *Nature genetics.* 2009;41(11):1173-1175.
66. Traglia M, Girelli D, Biino G, et al. Association of HFE and TMPRSS6 genetic variants with iron and erythrocyte parameters is only in part dependent on serum hepcidin concentrations. *J Med Genet.* 2011;48(9):629-634.
67. Galesloot TE, Geurts-Moespot AJ, den Heijer M, et al. Associations of common variants in HFE and TMPRSS6 with iron parameters are independent of serum hepcidin in a general population: a replication study. *J Med Genet.* 2013;50(9):593-598.
68. de Los Campos G, Vazquez AI, Hsu S, Lello L. Complex-Trait Prediction in the Era of Big Data. *Trends in genetics : TIG.* 2018;34(10):746-754.
69. McCarthy MI, Abecasis GR, Cardon LR, et al. Genome-wide association studies for complex traits: consensus, uncertainty and challenges. *Nature reviews Genetics.* 2008;9(5):356-369.
70. Mackley MP, Fletcher B, Parker M, Watkins H, Ormondroyd E. Stakeholder views on secondary findings in whole-genome and whole-exome sequencing: a systematic review of quantitative and qualitative studies. *Genetics in medicine : official journal of the American College of Medical Genetics.* 2017;19(3):283-293.
71. Kleiderman E, Knoppers BM, Fernandez CV, et al. Returning incidental findings from genetic research to children: views of parents of children affected by rare diseases. *Journal of medical ethics.* 2014;40(10):691-696.
72. Serre D, Gurd S, Ge B, et al. Differential Allelic Expression in the Human Genome: A Robust Approach To Identify Genetic and Epigenetic <italic>Cis</italic>-Acting Mechanisms Regulating Gene Expression. *PLoS Genet.* 2008;4(2):e1000006.
73. Pasricha SR, Lim PJ, Duarte TL, et al. Hepcidin is regulated by promoter-associated histone acetylation and HDAC3. *Nature communications.* 2017;8(1):403.
74. Strom SP. Fundamentals of RNA Analysis on Biobanked Specimens. *Methods in molecular biology (Clifton, NJ).* 2019;1897:345-357.
75. Pagani A, Colucci S, Bocciardi R, et al. A new form of IRIDA due to combined heterozygous mutations of TMPRSS6 and ACVR1A encoding the BMP receptor ALK2. *Blood.* 2017;129(25):3392-3395.
76. SNP database. NCBI. https://www.ncbi.nlm.nih.gov/snp. Accessed 23 October2019.
77. Richards S, Aziz N, Bale S, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genetics in medicine : official journal of the American College of Medical Genetics.* 2015;17(5):405-424.
78. Alamut http://www.interactive-biosoftware.com/doc/alamut-visual/2.7/. Accessed 23 October 2019.
79. Silvestri L, Pagani A, Nai A, De Domenico I, Kaplan J, Camaschella C. The serine protease matriptase-2 (TMPRSS6) inhibits hepcidin activation by cleaving membrane hemojuvelin. *Cell metabolism.* 2008;8(6):502-511.
80. Finberg KE, Whittlesey RL, Fleming MD, Andrews NC. Down-regulation of Bmp/

Smad signaling by Tmprss6 is required for maintenance of systemic iron homeostasis. *Blood.* 2010;115(18):3817-3826.

81. Wahedi M, Wortham AM, Kleven MD, et al. Matriptase-2 suppresses hepcidin expression by cleaving multiple components of the hepcidin induction pathway. *The Journal of biological chemistry.* 2017;292(44):18354-18371.
82. De Falco L, Bruno M, Yilmaz-Keskin E, et al. The role of Matriptase-2 during the early postnatal development in humans. *Haematologica.* 2016;101(4):e126-128.
83. Willemetz A, Lenoir A, Deschemin JC, et al. Matriptase-2 is essential for hepcidin repression during fetal life and postnatal development in mice to maintain iron homeostasis. *Blood.* 2014;124(3):441-444.
84. Nicolas G, Bennoun M, Porteu A, et al. Severe iron deficiency anemia in transgenic mice expressing liver hepcidin. *Proceedings of the National Academy of Sciences of the United States of America.* 2002;99(7):4596-4601.
85. Frazer DM, Wilkins SJ, Darshan D, Mirciov CSG, Dunn LA, Anderson GJ. Ferroportin Is Essential for Iron Absorption During Suckling, But Is Hyporesponsive to the Regulatory Hormone Hepcidin. *Cellular and molecular gastroenterology and hepatology.* 2017;3(3):410-421.
86. Frazer DM, Wilkins SJ, Anderson GJ. Elevated iron absorption in the neonatal rat reflects high expression of iron transport genes in the distal alimentary tract. *American journal of physiology Gastrointestinal and liver physiology.* 2007;293(3):G525-531.
87. Soliman AT, De Sanctis V, Yassin M, Wagdy M, Soliman N. Chronic anemia and thyroid function. *Acta bio-medica : Atenei Parmensis.* 2017;88(1):119-127.
88. Beard JL. Iron biology in immune function, muscle metabolism and neuronal functioning. *The Journal of nutrition.* 2001;131(2s-2):568S-579S; discussion 580S.
89. Oexle H, Gnaiger E, Weiss G. Iron-dependent changes in cellular energy metabolism: influence on citric acid cycle and oxidative phosphorylation. *Biochimica et biophysica acta.* 1999;1413(3):99-107.
90. Dziegala M, Josiak K, Kasztura M, et al. Iron deficiency as energetic insult to skeletal muscle in chronic diseases. *Journal of cachexia, sarcopenia and muscle.* 2018;9(5):802-815.
91. Stugiewicz M, Tkaczyszyn M, Kasztura M, Banasiak W, Ponikowski P, Jankowska EA. The influence of iron deficiency on the functioning of skeletal muscles: experimental evidence and clinical implications. *European journal of heart failure.* 2016;18(7):762-773.
92. Lam CSP, Doehner W, Comin-Colet J. Iron deficiency in chronic heart failure: case-based practical guidance. *ESC heart failure.* 2018;5(5):764-771.
93. Georgieff MK. Long-term brain and behavioral consequences of early iron deficiency. *Nutrition reviews.* 2011;69 Suppl 1:S43-48.
94. Lozoff B, Beard J, Connor J, Barbara F, Georgieff M, Schallert T. Long-lasting neural and behavioral effects of iron deficiency in infancy. *Nutrition reviews.* 2006;64(5 Pt 2):S34-43; discussion S72-91.
95. Arsenault V, Mailloux C, Bonnefoy A, Lemyre E, Pastore Y. Iron-Refractory Iron Deficiency Anemia May Not Lead to Neurocognitive Dysfunction: A Case Report. *Pediatrics.* 2016;138(1).
96. Gualdi R, Casalgrandi G, Montosi G, Ventura E, Pietrangelo A. Excess iron into hepatocytes is required for activation of collagen type I gene during experimental siderosis. *Gastroenterology.* 1994;107(4):1118-1124.
97. Pietrangelo A, Montosi G, Totaro A, et al. Hereditary hemochromatosis in adults without pathogenic mutations in the hemochromatosis gene. *The New England journal of medicine.* 1999;341(10):725-732.
98. Pietrangelo A, Caleffi A, Corradini E. Non-HFE hepatic iron overload. *Seminars in liver disease.* 2011;31(3):302-318.
99. Ganz T. Iron and infection. *International journal of hematology.* 2017.

100. Soares MP, Hamza I. Macrophages and Iron Metabolism. *Immunity.* 2016;44(3):492-504.
101. Armitage AE, Drakesmith H. Genetics. The battle for iron. *Science.* 2014;346(6215):1299-1300.
102. Hu X, Cai X, Ma R, Fu W, Zhang C, Du X. Iron-load exacerbates the severity of atherosclerosis via inducing inflammation and enhancing the glycolysis in macrophages. *Journal of cellular physiology.* 2019.
103. van Swelm RPL, Wetzels JFM, Swinkels DW. The multifaceted role of iron in renal health and disease. *Nature reviews Nephrology.* 2019.
104. Franca M, Marti-Bonmati L, Porto G, et al. Tissue iron quantification in chronic liver diseases using MRI shows a relationship between iron accumulation in liver, spleen, and bone marrow. *Clinical radiology.* 2018;73(2):215.e211-215.e219.
105. Brewer CJ, Coates TD, Wood JC. Spleen R2 and R2* in iron-overloaded patients with sickle cell disease and thalassemia major. *Journal of magnetic resonance imaging : JMRI.* 2009;29(2):357-364.
106. Wood JC. Use of magnetic resonance imaging to monitor iron overload. *Hematology/oncology clinics of North America.* 2014;28(4):747-764, vii.
107. Cau M, Galanello R, Giagu N, Melis MA. Responsiveness to oral iron and ascorbic acid in a patient with IRIDA. *Blood cells, molecules & diseases.* 2012;48(2):121-123.
108. Kortman GA, Raffatellu M, Swinkels DW, Tjalsma H. Nutritional iron turned inside out: intestinal stress from a gut microbial perspective. *FEMS microbiology reviews.* 2014;38(6):1202-1234.
109. Kortman GA, Duilh BE, Maathuis AJH etal. Microbial metabolism shifts towards an adverse profile with supplementary iron in the TIM-2 in vitro model of the human colon. *Frontiers in microbiology.* 2016;6: 1481.
110. Pisani A, Riccio E, Sabbatini M, Andreucci M, Del Rio A, Visciano B. Effect of oral liposomal iron versus intravenous iron for treatment of iron deficiency anaemia in CKD patients: a randomized trial. *Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association.* 2015;30(4):645-652.
111. Capra AP, Ferro E, Cannavo L, La Rosa MA, Zirilli G. A child with severe iron-deficiency anemia and a complex TMPRSS6 genotype. *Hematology.* 2017;22(9):559-564.
112. Hilty FM, Arnold M, Hilbe M, et al. Iron from nanocompounds containing iron and zinc is highly bioavailable in rats without tissue accumulation. *Nature nanotechnology.* 2010;5(5):374-380.
113. Pereira DI, Mergler BI, Faria N, et al. Caco-2 cell acquisition of dietary iron(III) invokes a nanoparticulate endocytic pathway. *PloS one.* 2013;8(11):e81250.
114. Powell JJ, Bruggraber SF, Faria N, et al. A nano-disperse ferritin-core mimetic that efficiently corrects anemia without luminal iron redox activity. *Nanomedicine : nanotechnology, biology, and medicine.* 2014;10(7):1529-1538.
115. Kortman GAM, Reijnders D, Swinkels DW. Oral iron supplementation: Potential implications for the gut microbiome and metabolome in patients with CKD. *Hemodialysis international International Symposium on Home Hemodialysis.* 2017;21 Suppl 1:S28-s36.
116. Weiss G, Ganz T, Goodnough LT. Anemia of inflammation. *Blood.* 2019;133(1):40-50.
117. Katsarou A, Pantopoulos K. Hepcidin Therapeutics. *Pharmaceuticals (Basel).* 2018;11(4).

Chapter 10

Summary

SUMMARY

Every general practitioner, internist, pediatrician or any other physician will meet patients suffering from anemia during his of her medical career. The worldwide high prevalence of approximately 1.62 billion people, corresponding to 24.8% of the population, makes anemia one of the most common reasons for seeking medical attention in daily clinical practice.

Although this condition has numerous causes, diagnostic evaluation is usually straightforward, starting with the determination of the mean corpuscular volume (MCV) of the erytrocytes in order to classify the anemia as microcytic, normocytic of macrocytic.

The majority of anemias are microcytic, primarily caused by iron deficiency due to nutritional deficits, gastro-intestinal or gynecological blood loss or iron malabsorption. Furthermore, thalassemia syndromes need to be considered as the cause of microcytic anemias within specific populations.

However, in some patients the microcytic anemias cannot be explained by iron deficiency or a hemoglobinopathy. In those cases a genetic disorder of iron metabolism or heme synthesis due to defects in genes involved in systemic and cellular iron metabolism and heme synthesis should be suspected. It is a diagnostic challenge for the clinician to distinguish such rare disorders from the far more common forms of acquired iron deficiency anemia as a result of for example celiac disease or from α- or ß thalassemias.

If the treating physician overlooks a microcytic anemia due to a genetic disorder of iron metabolism or heme synthesis, the individual patient may encounter an unnecessarily long diagnostic process and not effective or even harmful treatments. Moreover, in some genetic anemias, such as the sideroblastic anemias, iron overload is of greater clinical consequence than the anemia itself since unrecognized tissue iron loading might lead to severe morbidity and even mortality.

The objective of this thesis was to provide the clinician with information on several clinical aspects of microcytic anemias due to a genetic disorder of iron metabolism or heme synthesis with a particular focus on Iron Refractory Iron Deficiency Anemia

(IRIDA) and x-linked sideroblastic anemia (XLSA). We aimed to increase awareness for these rare disorders, to facilitate the diagnostic process, enabling the timely start of an adequate treatment, with the ultimate goal of reducing the disease burden and preventing lifelong sequelae of either iron deficiency or iron overload for the individual patient.

Chapter 1 is the general introduction to this thesis, describing the (patho)physiology of systemic and cellular iron metabolism and heme synthesis.

Iron can participate in electron transfer by the interconversion between Fe^{2+} and Fe^{3} and this fundamental biochemical characteric makes virtually all tissues in the human body dependent of iron. However, the majority of iron is dedicated to the synthesis of the iron-containing hemoglobin inside the erythrocytes, crucial for oxygen transport.

Therefore, deficit of iron results in the clinical condition of iron deficiency anemia, affecting over 1.2 billion people globally. On the other hand, iron is also potentially toxic to cells because unbound iron can catalyze the formation of oxidative radicals that damage proteins, lipids and nucleic acids. Furthermore, many pathogens are dependent of iron for their survival. Because both iron deficiency and iron overload may have detrimental effects, a highly sophisticated regulatory system is required to maintain iron homeostasis on both the systemic and cellular level.

In this introductory chapter, the two regulatory systems responsible for body iron homeostasis are described, one that functions systemically and is dependent on the liver-derived hormone hepcidin, and the other that predominantly controls cellular iron metabolism through iron-regulatory proteins (IRP) that bind iron-responsive elements (IRE) in regulated messenger RNA's of cellular iron importers, exporters and storage genes. Although the machineries of systemic and cellular iron homeostasis are separated, crosstalk exists between the two distinct control systems, which is explained.

Since this thesis also addresses genetic disorders of heme synthesis, especially XLSA due to *ALAS2* defects, we explain the eight steps of heme biosynthesis, for which ALAS2 is crucial for the first and rate-limiting step.

After depicting the physiology of systemic and cellular iron homeostasis and heme synthesis, we discuss the clinical sequelae of a defective systemic iron homeostasis or heme synthesis in terms of iron deficiency and iron overload, with a focus on IRIDA and XLSA.

PART I: LITERATURE AND CLINICAL STUDIES

In Chapter 2 we narratively reviewed the critical roles of iron during the different phases of childhood and the developmental aspects of iron homeostasis throughout the journey from fetus to adult. The aim of this review was to increase the understanding of iron biology from fetal life to adulthood since this is essential for the timely and accurate diagnosis of both genetic and acquired iron disorders in childhood. We assessed the importance of iron for the growth of the body and the accompanying increase of circulating blood volume and bone mass, for the maturation of the central nervous system, the immune system and the gut microbiota. We reviewed the current knowledge on the systemic iron homeostasis from fetus to infant to middle-aged child to adolescent. After extensive exploration of the literature, we concluded that many questions remain on iron handling in childhood and on the ideal iron status for the different iron-demanding tissues in the growing and developing child. Understanding the way the human body prioritizes and distributes iron between the erythroblasts in the bone marrow, the brain and other iron-dependent organs systems during the different phases of development from fetus to adult is limited. Moreover, the lack of appropiate biomarkers for the various iron-consuming organs, and especially the central nervous system, hampers adequate assessment of iron status of the different iron-containing compartments. This limited understanding of iron physiology of childhood complicates the diagnosis and management of both acquired iron disorders as iron deficiency (anemia), anemia of inflammation or iron loading due to ineffective erythropoiesis and also anemias due to genetic disorders of iron metabolism. We proposed research ideas addressing these knowledge gaps regarding iron and iron handling in childhood, the assessment of body iron status and the treatment of iron deficiency, some of which are adressed in this thesis (establishment of hepcidin reference values in children, Chapter 7).

Chapter 3 is a systematic review of the literature on microcytic anemias due to a genetic disorder of iron metabolism or heme synthesis, with the intention to develop evidence-based and multidisciplinary guidelines that assist the clinician in the diagnosis and treatment of these rare disorders and provide recommendations on

family screening of the index patient. We presented evidence-based, multidisciplinary guidelines on the diagnosis, the management and the recommended family screening of 12 disorders of microcytic anemia due to defects in 13 different genes involved in iron metabolism and heme synthesis. In order to help the clinician in discriminating this diseases from the fare more common hemoglobinopathies and acquired iron deficiency anemias, we added a flow chart with a step-by-step plan supporting the diagnostic process and a table summarizing the key features characterizing these disorders. We recommended the collaboration between centers of excellence with special expertise of these particular disorders join in order to enlarge pathophysiological insights and to ultimately improve the diagnostic workup and treatment.

In **Chapter 4** we retrospectively described a case series of 21 Dutch patients diagnosed with Iron Refractory Iron Deficiency Anemia (IRIDA) due to 14 different *TMPRSS6* defects, resulting in decreased matriptase 2 activity with inappropriately increased hepcidin levels relative to body iron parameters. In fourteen patients we found a homozygous or compound heterozygous *TMPRSS6* defect. Interestingly, in the other 7 patients we only found a heterozygous *TMPRSS6* defect after both DNA sequencing and multiplex ligation dependent probe amplification (MLPA), challenging the idea of IRIDA being a disease with an autosomal recessive inheritance pattern. Nine out of 14 *TMPRSS6* defects in our population had not been described earlier. We assessed pathogenicity of the *TMPRSS6* variants in our patients by review on the literature on previous reports and functional studies in case of known defects, and by considering the association of the *TMPRSS6* variants with the phenotype within a family and by bio-informatic tools in case of novel defects.

We explored genotype-phenotype correlation, age of presentation, disease severity and response to oral and parenteral iron treatment. We observed that most patients were dependent of parenteral iron. However, a severe genotype did not preclude responsiveness to oral iron. We found that the transferrin saturation (TSAT)/hepcidin ratios were lower in IRIDA patients than in healthy relatives, suggesting that the TSAT/hepcidin ratio is a promising diagnostic tool for discriminating IRIDA from the far more common acquired iron deficiency anemias (we expanded upon this issue in **Chapter 8**). Since we found a noticeable discrepancy between the phenotypes of probands and of relatives with the same genotype, we suggest that a complex interplay between genetic and environmental factors plays a role in the pathogenesis or IRIDA.

In **Chapter 5** we commented on a case report of a suggested IRIDA case of a 10-years old Chinese girl, at the age of 10 months diagnosed with a splenomegaly and a severe microcytic anemia (Hb 5.8 g/dL, MCV 61 fL), with a decreased TSAT of 7%. The child was neither responding to oral iron nor to a combination of intravenous iron and erythropoietin. IRIDA was diagnosed in this child because of increased hepcidin levels (12.0 nmol/L) compared to the normal ranges for females for this specific hepcidin assay (4.0 ± 1.5 nmol/L) and because of the non-synonymous *TMPRSS6* changes c.757A>G (p.Lys253Glu, K253E, rs2235324) and c.2207 T>C (p.Val736Ala, V736A, rs855791). Because of the refractoriness to oral iron, parenteral iron and erythropoietin, a steroid trial was performed. Strikingly, hepcidin levels dropped to 2.7 nmol/L and Hb increased to 8.9 g/dL on this treatment. We argued that the clinical course was more consistent with anemia of inflammation than with IRIDA because of the sharp decline of serum hepcidin levels after the initiation of the anti-inflammatory drug methylprednisolone and because of the occurrence of splenomegaly that is not typical of IRIDA. We debated the pathogenicity of the above-mentioned *TMPRSS6* variants that are common non-synonymous polymorphisms, also for the Han Chinese population, and therefore unlikely to be the cause of IRIDA with such a severe microcytic anemia.

Moreover, we discussed the functional studies indicating that the *TMPRSS6* 736A variant inhibits hepcidin more efficiently than 736V variant, implicating that the presence of the 736A variant in the patient protects against instead of causes elevated hepcidin levels and iron deficiency.

In **Chapter 6** we presented clinical and genetic data of 15 Dutch patients from 11 unrelated families diagnosed with XLSA patients, which is the most common inherited form of sideroblastic anemia, characterized by mostly mild hypochromic microcytic anemia with bone marrow ring sideroblasts, in combination with mild to severe parenchymal iron loading due to ineffective erythropoiesis. The disease is associated with several mutations in the erythroid specific 5-aminolevulinate synthase gene (*ALAS2*). We reviewed age of presentation, clinical and biochemical features, ALAS-2 defects and treatment characteristics (e.g. pyridoxine, blood transfusion, chelation, phlebotomy) of the patients. Although 10 out of 11 families shared the same previously described c.1355G>A (p.Arg452His) *ALAS2* defect, phenotype regarding severity of anemia and iron loading was very different, even for those six patients with the p.Arg452His defect in which haplotype analysis strongly suggested ancestral

relationship. In two brothers we found a novel c.1412G>A (p.Cys471Tyr) variant. Despite the results of the different bio-informatic tools that were not consistent in its prediction of pathogenicity, we considered this *ALAS2* variant as pathogenic since both brothers shared the same phenotype of microcytic anemia and iron overload. Of note, the two affected brothers had no unaffected brothers without the relevant *ALAS2* variant. This would have supported our hypothesis concerning its pathogenicity.

Although XLSA is an X-linked disease, we illustrate that female carriers of an *ALAS2* defect may develop a phenotype of sideroblastic anemia later in life, probably due to a predominant inactivation of the normal X-chromosome and physiological age-related skewed X-inactivation in hematopoietic cells. Regarding effect of treatment, we observed an increase in Hb in one of our patients after commencement of phlebotomies, illustrating that blood drawing not only benefits the reduction of systemic iron overload but may also improve erythropoiesis in XLSA patients. We ended our article with practical recommendations for the clinician regarding a timely diagnosis and adequate treatment of probands with XLSA in combination with accurate screening of relatives, in order to reduce the disease burden for the individual patient and to avoid lifelong sequelae of iron deficiency or iron overload for both the proband and the possibly affected relatives.

PART II: DIAGNOSTIC STUDIES: FOCUS ON IRON REFRACTORY IRON DEFICIENCY ANEMIA

For the second part of this thesis we performed studies aimed at improving the diagnosis of microcytic disorders due to disorders of iron metabolism, with the focus on IRIDA.

In Chapter 7 we described a study on ranges of serum hepcidin in healthy children with the objective of facilitating the diagnosis of iron disorders in childhood. We measured serum hepcidin-25 levels in 266 healthy Dutch children, aged 0.3-17 years, attending the day care unit of the Máxima MC, Veldhoven, the Netherlands for minor surgical or diagnostic procedures. We used an isotope dilution mass spectrometry hepcidin assay, standardized with our commutable 2nd reference material (RM), assigned by a candidate primary RM. Since we aimed to cover the different key periods of human growth and development for both sexes, we divided our study population in subgroups for age and sex; infancy and toddler stage (0-<2 years), early childhood (2-<6 years), middle childhood (6 -<12 years) and adolescence (12-17 years). We constructed age- and sex-specific values for serum hepcidin and its ratio

with ferritin and transferrin saturation (TSAT). Serum hepcidin levels, hepcidin/ferritin and TSAT/hepcidin ratios were similar for both sexes. Serum hepcidin and hepcidin/ferritin ratio substantially declined after the age of 12 years, TSAT/hepcidin ratio gradually increased with increasing age. Corroborating with data in adults, we found that serum ferritin was the most significant correlate of serum hepcidin in our study population, explaining 15.1 % and 7.9 % of variance in boys and girls, respectively. Multivariable linear regression analysis including age, blood sampling time, iron parameters, ALT, CRP and BMI as independent variables showed a statistically significant negative association between age as dichotomous variable (≤ 12 years *versus* >12 years) and log-transformed serum hepcidin levels in both sexes. With this study we demonstrated that serum hepcidin relative to indicators of body iron is age dependent in children, suggesting that the set point of serum hepcidin relative to stored and circulating iron changes during childhood.

In Chapter 8 we described a study assessing the value of the TSAT/hepcidin ratio in discriminating IRIDA from iron deficiency anemia due to acquired causes, e.g. gastro-intestinal or vaginal blood loss of malabsorption. We included 21 IRIDA patients from various hospitals in the Netherlands and 39 IDA controls from the Gastroenterology, Gynecology and Emergency Department of the Máxima Medical Center, Veldhoven, the Netherlands. We measured serum hepcidin-25 levels in all patients by an isotope dilution mass spectrometry assay and calculated the TSAT/hepcidin ratio. We observed that the TSAT/hepcidin ratio was significantly lower in IRIDA patients compared to IDA controls. Area under the receiver operating characteristic (ROC) curve for the TSAT/hepcidin ratio was 0.991 with a specificity of 100% (95% Confidence Interval, 93-100%), a sensitivity of 95% (95% CI, 79-100%) at an optimal cut-off point of 5.9%/nM to distinguish IRIDA from IDA because of other reasons. We therefore concluded that the TSAT/hepcidin ratio is able to differentiate between IRIDA and IDA-controls due to other reasons with high specificity in a broad iron-deficient population, provided inflammation is absent and no recent iron therapy has been given.

PART III: DISCUSSION, SUMMARY AND ADDENDA

In Chapter 9 we discuss our main findings and reflect upon the implications for clinical practice. We comment on the strengths and limitations of the studies and consider future research challenges. In the current Chapter 10 we summarize this thesis. Chapter 11 contains the addenda of this paper.

Chapter 11

Addenda

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LIST OF ABBREVIATIONS

ABCB6	ATP Binding Cassette Subfamily B Member 6
ABCB10	ATP Binding Cassette Subfamily B Member 10
ACVR2A	Activin receptor type 2A
ACP	Aceruloplasminemia
ALA	Aminolevulinic acid
ALAD	Aminolevulinate dehydratase
ALAS2	5-Aminolevulinic acid synthase 2
ALK2	Activin receptor-like kinase 2
ALK3	Activin receptor-like kinase 3
BMP	Bone morphogenetic protein
BMPR2	BMP receptor 2
BMP-RE	BMP-responsive element
CDC14A	Cell division cycle 14A
CEP	Congenital Erythropoietic Porphyria
CP	Ceruloplasmin
CPOX	Coproporphyrinogen oxidase
DMT1	Divalent metal transporter 1
DCYTB	Duodenal cytochrome B
EPO	Erythropoietin
EPP	Erythropoietic protoporphyria
ERFE	Erythroferrone
Fe/S cluster	Iron sulfur cluster
FLVCR1b	Feline leukemia virus subgroup C receptor 1b
FECH	Ferrochelatase
FPN	Ferroportin
FPP	Free protoporphyrin
GDF15	Growth differentiation factor 15
GH	Growth hormone
GLRX	Glutaredoxin
GOF	Gain of function
GWAS	Genome wide association studies
Hb	Hemoglobin
HCP1	Heme carrier protein 1
HH	Hereditary hemochromatosis

HIF2α	Hypoxia inducible factor 2 alpha
HJV	Hemojuvelin
HMBS	Hydroxymethylbilane synthase
HPG axis	Hypothalamic pituitary gonadal axis
HSCT	Hematopoietic stem cell transplantation
ID	Iron deficiency
IDA	Iron deficiency anemia
IGF1	Insulin-like growth factor 1
IL6	Interleukin 6
IO	Iron overload
IRIDA	Iron Refractory Iron Deficiency Anemia
IRE	Iron-responsive element
IRP1	Iron-regulatory protein 1
IRP2	Iron-regulatory protein 2
IRT	Iron-regulated transporter
JAK	Janus kinase
LIP	Labile iron pool
LOF	Loss of function
LSEC	Liver sinusoidal endothelial cell
MCH	Mean corpuscular hemoglobin
MCV	Mean corpuscular volume
MDS	Myelodysplastic syndrome
MDS-RA	Myeodysplastic syndrome-refractory anemia
MDS-RARS	Myeodysplastic syndrome-refractory anemia with ring sideroblasts
MDS- RCMD	Myelodysplastic syndrome- refractory cytopenia with multilineage dysplasia
MFRN	Mitoferrin
MLPA	Multiplex ligation-dependent probe amplification
MT2	Matriptase 2
NADPH	Nicotinamide adenine dinucleotide phosphate
NGS	Next generation sequencing
NOS	Nitric oxide synthase
NTBI	Non-transferrin-bound iron
OIAT	Oral iron absorption test
PHC	Peak height velocity

PPIX	Protoporphyrin IX
RBC	Red blood cell
RDW	Red cell distribution width
RES	Reticulo-endothelial system
RM	Reference material
SA	Sideroblastic anemia
sHJV	soluble Hemojuvelin
SMAD	Sons of mothers against decapentaplegic
SLC25A38	Solute carrier 25A38
STAT3	Signal transducer and activator of transcription 3
STAT-RE	STAT-responsive element
STEAP3	Six-transmembrane epithelial antigen of prostate 3
STR	Short tandem repeat
TBI	Transferrin-bound iron
Tf	Transferrin
TfR1	Transferrin receptor 1
TfR2	Transferrin receptor 2
TIBC	Total iron biding capacity
TPO	Thyroid peroxidase
TSAT	Transferrin saturation
TWSG1	Twisted gastrulation 1
UROD	Uroporphyrinogen decarboxylase
UROS	Uroporphyrinogen synthase
UTR	Un-translated region
VUS	Variance of unknown significance
WCX-MS-TOF	Weak cation exchange chromatography followed by time of flight mass spectrometry
WES	Whole exome sequencing
XLDPP	X-Linked Dominant Protoporphyria
XLSA	X-Linked Sideroblastic Anemia
ZIP14	ZRT/IRT protein 14
ZnPP	Zinc protoporphyrin
ZRT	Zinc-regulated transporter

ABOUT THE AUTHOR

Albertine Donker was born on May 3rd 1977 in Leiden, the Netherlands. After completing secondary school at the Driestar College in Gouda, she started her medical training at the Leiden University. She obtained her medical degree *cum laude* in 2002, after which she started as an intern in pediatric medicine. In 2004, Albertine started the residency programme in pediatrics, successively at the Máxima MC Veldhoven (supervision prof. dr. Sidarto Bambang Oetomo) and at the Radboud University Medical Center (supervision dr. Jos M.Th. Draaisma). After her graduation as a pediatrician in 2009, she continued her training as a fellow in pediatric hemato-oncology at the Radboud University Medical Center (supervision prof. dr. Peter M. Hoogerbrugge) and the Amsterdam UMC, AMC (supervision dr. Marjolein Peters), until her registration as a pediatric hemato-oncologist in 2011. During this fellowship, Albertine came into contact with the Radboud Center for Iron Disorders (RCID), a national and European expertise center, involved in both research activities and patient care in the field of genetic disorders of iron metabolism. In 2012, she started with her PhD trajectory at the RCID and at the Department of Pediatrics of the Máxima MC, Veldhoven, resulting in this thesis (supervision prof. dr. Dorine W. Swinkels, dr. Dirk L. Bakkeren).

Since 2011 Albertine has been working as a pediatric hematologist at the Department of Pediatrics of the Máxima MC, Veldhoven, with a special interest in benign hematologic disorders, including anemias, clotting disorders and hemoglobinopathies.

LIST OF PUBLICATIONS

Publications related to this thesis

Donker AE, Raymakers RA, Nieuwenhuis HK, Coenen MJ, Janssen MC, MacKenzie MA, Brons PP, Swinkels DW. X-linked sideroblastic anaemia due to ALAS2 mutations in the Netherlands: a disease in disguise. *The Netherlands Journal of Medicine*. 2014;72(4):210-217.

Donker AE, Raymakers RA, Vlasveld LT, van Barneveld T, Terink R, Dors N, Brons PP, Knoers NV, Swinkels DW. Practice guidelines for the diagnosis and management of microcytic anemias due to genetic disorders of iron metabolism or heme synthesis. *Blood.* 2014;123(25):3873-3886; quiz 4005.

Donker AE, Brons PP, Swinkels DW. Microcytic anaemia with low transferrin saturation, increased serum hepcidin and non-synonymous TMPRSS6 variants: not always iron-refractory iron deficiency anaemia. *British Journal of Haematology.* 2015;169(1):150-151.

Donker AE, Schaap CC, Novotny VM, Smeets R, Peters TM, van den Heuvel BL, Raphael MF, Rijneveld AW, Appel IM, Vlot AJ, Versluijs AB, van Gelder M, Granzen B, Janssen MC, Rennings AJ, van de Veerdonk FL, Brons PP, Bakkeren DL, Nijziel MR, Vlasveld LT, Swinkels DW. Iron Refractory Iron Deficiency Anemia (IRIDA): A heterogeneous disease that is not always iron refractory. *American Journal of Hematology.* 2016;91(12):E482-E490.

Donker AE, Galesloot TE, Laarakkers CM, Klaver SM, Bakkeren DL, Swinkels DW. Standardized serum hepcidin values in Dutch children: set point relative to body iron changes during childhood. *Pediatric Blood and Cancer 2020;67(3):e28038.*

Other publications

Donker AE, van Merkesteyn JP, Bredius RG, van Weel-Sipman MH. Value of panoramic radiographs in paediatric pre-bone marrow transplantation oral evaluation. *International Journal of Oral and Maxillofacial Surgery.* 2002;31(2):170-172.

Heemskerk MB, van Walraven SM, Cornelissen JJ, Barge RM, Bredius RG, Egeler RM, Tj Lie JL, Révész T, Sintnicolaas K, Wulffraat NM, **Donker AE**, Hoogerbrugge PM, van Rood JJ, Claas FH, Oudshoorn M. How to improve the search for an unrelated haematopoietic stem cell donor. Faster is better than more! *Bone Marrow Transplantation.* 2005;35(7):645-652.

Donker AE, Bocca G. Chance finding of foetal and neonatal ovarian cysts on echography. *Nederlands Tijdschrift voor Geneeskunde.* 2006;150(47):2577-2581.

Donker AE, Hoogerbrugge PM, Mavinkurve-Groothuis AM, et al. Metastatic rhabdomyosarcoma cured after chemotherapy and allogeneic SCT. *Bone Marrow Transplantation.* 2009;43(2):179-180.

Vossen JM, **Donker AE**, Heemskerk MB, et al. Unrelated donor marrow transplantation in children: transplant policy and outcome in Leiden Paediatrics SCT-Centre. *Bone Marrow Transplantation.* 2010;45(1):87-95.

Donker AE, Mavinkurve-Groothuis AM, van Die LE, Verweij PE, Hoogerbrugge PM, Warris A. Favorable outcome of chronic disseminated candidiasis in four pediatric patients with hematological malignancies. *Medical Mycology.* 2012;50(3):315-319.

Jansen E, Emmen J, Mohns T, **Donker AE**. Extreme hyperleucocytosis of the premature. *BMJ Case Reports.* 2013 doi10.1136/bcr-2012-008385.

Stocker M, van Herk W, El Helou S, Dutta S, Fontana MS, Schuerman FABA, van den Tooren-de Groot RK, Wieringa JW, Janota J, van der Meer-Kappelle LH, Moonen R, Sie SD, de Vries E, **Donker AE**, Zimmerman U, Schlapbach LJ, de Mol AC, Hoffman-Haringsma A, Roy M, Tomaske M, Kornelisse RF, van Gijsel J, Visser EG, Willemsen SP, van Rossum AMC; NeoPInS Study Group. Procalcitonin-guided decision making for duration of antibiotic therapy in neonates with suspected early-onset sepsis: a multicentre, randomised controlled trial (NeoPIns). *Lancet.* 2017;390(10097):871-881.

Sinnema M, Song D, Guan W, Janssen JWH, van Wijk R, Navalsky BE, Peng K, **Donker AE**, Stegmann APA, Lee FS. Loss-of-function zinc finger mutation in the *EGLN1* gene associated with erythrocytosis. *Blood.* 2018;132(13):1455-1458.

Sol JJ, van de Loo M, Boerma M, Bergman KA, **Donker AE**, van der Hoeven MAHBM, Hulzebos CV, Knol R, Djien Liem K, van Lingen RA, Lopriore E, Suijker MH, Vijlbrief DC, Visser R, Veening MA, van Weissenbruch MM, van Ommen CH. NEOnatal Central-venous Line Observational study on Thrombosis (NEOCLOT): evaluation of a national guideline on management of neonatal catheter-related thrombosis. *BMC Pediatrics.* 2018;18(1):84.

van Dooijeweert B, van Ommen CH, Smiers FJ, Tamminga RYJ, Te Loo MW, **Donker AE**, Peters M, Granzen B, Gille HJJP, Bierings MB, MacInnes AW, Bartels M. Pediatric Diamond-Blackfan anemia in the Netherlands: An overview of clinical characteristics and underlying molecular defects. *European Journal of Haematology.* 2018;100(2):163-170.

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2015 Pediatric Hematology Course European Hematology Association (EHA), Sorrento, Italy

2016 Recertification Good Clinical Practice MMC (first course in 2012)

National Conferences: Oral Presentations

2015 Dutch Hematology Congress, Papendal
Practice guidelines for the diagnosis and management of microcytic anemias due to genetic disorders of iron metabolism or heme synthesis (Invited oral presentation, nomination TOP publication)

2018 Herfstpalet, Amsterdam UMC, Amsterdam
IJzerstofwisseling bij kinderen, presentatie voor algemeen kinderartsen

2019 Congres Nederlandse Vereniging voor Kindergeneeskunde, Papendal
Symposium Kind en IJzer, organisatie van symposium en presentatie voor algemeen kinderartsen en jeugdartsen

International Conferences: Oral Presentations

2017 EHA Scientific Working Group on Anemias, Barcelona, Spain
Iron Refractory Iron Deficiency Anemia: a heterogeneous disease that is not always iron refractory

2019 BioIron, Heidelberg, Germany
Standardized serum hepcidin values in Dutch children: set point relative to body iron changes during childhood

International Conferences: Poster Presentations

2012 EHA, Amsterdam
Homozygous absence of the *TMPRSS6* gene in an 11-year-old child resulting in severe Iron Refractory Iron Deficiency Anemia

2012 EHA, Amsterdam
Phenotype of heterozygous *TMPRSS6* defects in 2 Dutch families suggest that Iron Refractory Iron Deficiency Anemia is a co-dominant disease

Lectures

2011- present Approximately 15 lectures on benign hematology for pediatric residents

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RESEARCH DATA MANAGEMENT

Science builds upon the discoveries of its antecedents. Therefore, the amount of progress we can make as an academic community is dependent on the information that we make available and reusable for others. Appropriate scientific research data management (RDM) is a prerequisite in this process. RDM includes the organization, storage, preservation, and sharing of data collected and used in a research project, involving the everyday management of research data during the lifetime of a research project, but also decisions about how data will be preserved and shared after the project is completed.

The descriptive data presented in **Chapter 4** and **Chapter 6** were obtained at the Translational Metabolic Laboratory (TML), Department of Laboratory Medicine and at the Radboud Center for Iron Disorders (RCID), Radboud University Medical Center (Radboudumc). The research data presented in **Chapter 7** and **Chapter 8** were obtained at the Department of Pediatrics, Gastro-intestinal Medicine and Emergency Room at the Máxima MC Veldhoven. All data were collected and archived according to the Findable, Accessible, Interoperable and Reusable (FAIR) principles (Wilkinson *et al*, 2016, Scientific Data).

The data of the patients included in the case series (**Chapter 4, Chapter 6**) and in the diagnostic studies (**Chapter 7, Chapter 8**) were recorded in Castor Electronic Data Capture (EDC), a cloud-based application for digital data capture and management. Data were handled confidentially. To guarantee privacy, subjects were labelled with a unique number in the RCID database; name and date of birth were not disclosed in this Castor database. A subject identification code list was used, accessible on the local server of the Radboudumc by the scientific staff members of the RCID. These investigators will be able to access the data and will safeguard the key to the code. The Information and Communications Technology (ICT) Department of the Radboudumc supports the local server of the TML. Data stored on this local server are replicated daily to servers of the Radboud University.

All studies were conducted according to Dutch ethical guidelines and to the principles of the Declaration of Helsinki. All participants included in the diagnostic studies (**Chapter 7, Chapter 8**) gave oral and written consent for participation. In case of minors, the parents or caregivers gave approval. All participants or their parents

or caregivers were asked for consent to store residual blood for 15 years at the TML. In case patients or their parents or caregivers did not agree with storage of residual blood, the leftover material will be destroyed.

The data described in **Chapter 4, Chapter 6** and **Chapter 7** are disclosed in peer-reviewed papers. The data described in **Chapter 8** will be published in the future. All original data generated or analyzed in this thesis are available from the associated corresponding authors on request.

FOR DUTCH DUMMIES

INLEIDING

IJzergebrek is de meest voorkomende oorzaak van bloedarmoede

Bloedarmoede is één van de meest voorkomende redenen waarom mensen een dokter bezoeken. Naar schatting hebben wereldwijd ongeveer 1.62 miljard mensen in meer of mindere mate last van dit probleem, zo'n 25% van de totale bevolking op deze aardbol.

Bij het overgrote deel van de mensen met bloedarmoede is een ijzertekort de oorzaak.

IJzer is een essentieel onderdeel van bloed omdat het onmisbaar is voor de aanmaak van rode bloedcellen. En deze rode bloedcellen zijn cruciaal voor het zuurstoftransport in het lichaam. Te weinig ijzer betekent te weinig rode bloedcellen en dus een tekortschietend zuurstoftransport naar de organen. Het resultaat is bloedarmoede, een aandoening die u vast kent uit uw omgeving of wellicht uit eigen ervaring.

Kenmerkend voor bloedarmoede als gevolg van een ijzertekort is dat de rode bloedcellen kleiner zijn dan gemiddeld; we noemen dit een *microcytaire anemie* (*microcytair*: klein-cellig, *anemie*: bloedarmoede). Het ijzertekort dat deze *microcytaire anemie* veroorzaakt, kan allerlei uiteenlopende oorzaken hebben. Bij jonge kinderen is het probleem meestal dat ze te weinig ijzer binnenkrijgen met de voeding, terwijl ze hard groeien en daardoor juist veel ijzer nodig hebben. Bij pubermeisjes wordt een ijzergebrek en daardoor bloedarmoede vaak veroorzaakt door bloedverlies ten gevolge van de menstruatie in combinatie met de groeispurt. Bij volwassen patiënten is het onderliggende probleem geregeld een maag-darm aandoening, bijvoorbeeld een poliep of een darmtumor, al dan niet in combinatie met antistollingsmedicatie. Verder kan bij iedere leeftijdsgroep het dieet een rol spelen; mensen met een vegetarisch of veganistisch eetpatroon krijgen sneller een ijzertekort dan mensen die wel dierlijke producten eten.

Bloedarmoede door een genetische aandoening van de ijzerstofwisseling is zeldzaam, maar belangrijk om op te sporen

Soms zien we in het ziekenhuis patiënten met een *microcytaire* vorm van bloedarmoede in combinatie met een afwijkend ijzergehalte in het bloed, terwijl de onderliggende oorzaak onduidelijk is en de gebruikelijke behandeling met ijzermedicatie niet (genoeg) helpt. Dus de patiënt(e) eet gewoon vlees en vis en heeft geen last van bloedverlies dat er niet hoort te zijn. IJzertherapie, in de vorm van pillen of een infuus, is niet of onvoldoende effectief. Zulke patiënten zijn vaak een moeilijke puzzel voor de dokter. Maar vooral voor de patiënten zelf is het erg frustrerend om een onbehandelbare aandoening te hebben die veel klachten geeft en waarvan de oorzaak niet is opgehelderd, ondanks vele en vaak vervelende onderzoeken, zoals een scopie van de darm of een beenmergpunctie.

Bij dergelijke patiënten kan er sprake zijn van een erfelijke afwijking in de genen die belangrijk zijn voor de ijzerstofwisseling. Denk aan genen die zorgen dat ijzer vanuit de darm naar het bloed wordt gesluisd of aan genen die de ijzeropname in het beenmerg regelen, de fabriek waar het bloed wordt gemaakt. Of aan genen die een rol spelen bij het inbouwen van het ijzer in de rode bloedcel, dus bij het productieproces zelf. Over deze genetische aandoeningen gaat dit proefschrift. We willen daarmee aandacht vragen voor deze zeldzame vormen van bloedarmoede, zodat ze tijdig worden opgespoord. We hopen daarmee te bereiken dat de patiënten niet onnodig allerlei vervelende onderzoeken hoeven te ondergaan of bloot worden gesteld aan behandelingen zoals ijzertabletten of ijzerinjecties die niet (voldoende) helpen maar mogelijk wel bijwerkingen hebben.

Verder weten we dat sommige patiënten met bloedarmoede door een genetische afwijking in de ijzerstofwisseling kans hebben op ijzer*stapeling*, dus het tegenovergestelde van een ijzer*tekort*. Een overschot aan ijzer in het bloed leidt tot oxidatieve stress, een situatie waarbij er meer reactieve zuurstofverbindingen vrijkomen dan gebruikelijk en dat is schadelijk voor het menselijk lichaam. Vooral de lever en het hart zijn gevoelig voor ijzerstapeling. Eén van de meest beruchte complicaties van ijzerstapeling is dan ook het ontwikkelen van leverkanker. Het verraderlijke van ijzerstapeling is dat de patiënt dit aanvankelijk niet voelt. Het probleem kan dus lang onder de radar blijven, soms zelfs totdat er al onherstelbare schade aan de organen is opgetreden. Ook daarom is het erg belangrijk om bloedarmoede door een genetische afwijking in de ijzerstofwisseling tijdig op te sporen.

Maar hoe zit het dan precies met de ijzerstofwisseling en hoe regelt het lichaam dat de ijzerhuishouding in balans is? Dat leggen we uit in **hoofdstuk 1**, de inleiding van dit proefschrift.

Hepcidine: de thermostaat van de ijzerhuishouding

Een volwassen man heeft in totaal ongeveer 4 tot 5 gram (4000 tot 5000 mg) ijzer in zijn lichaam. Daarvan bevindt zich het grootste gedeelte, zo'n 2500 mg, in de rode bloedcellen. Zoals we hierboven uitleggen, zijn de rode bloedcellen van levensbelang omdat ze verantwoordelijk zijn voor het zuurstoftransport door het lichaam. Belangrijk om te weten: een rode bloedcel gaat gemiddeld 120 dagen mee. Daarna wordt de rode bloedcel afgebroken, voornamelijk in de milt. Dit betekent dat het lichaam steeds opnieuw heel veel rode bloedcellen moet maken, zo'n 200 x 10^9 per dag. Hiervoor is per dag ongeveer 20 mg ijzer nodig, oftewel 2 x 10^{15} ijzeratomen per seconde. Waar komt al dit ijzer vandaan? Het grootste gedeelte is gerecycled ijzer dat vrijkomt bij het opruimen van oude rode bloedcellen en dat wordt opgeslagen in de macrofagen. Macrofagen zijn witte bloedcellen die een belangrijke rol spelen bij het opruimen van beschadigde of dode lichaamscellen. Slechts een klein gedeelte van het benodigde ijzer komt uit de voeding. Het lichaam gaat dus heel efficiënt met ijzer om; het ijzer wordt voortdurend hergebruikt en niet uitgescheiden via de urine of de ontlasting, zoals dat met veel andere vitamines en mineralen wel gebeurt. Belangrijk om te realiseren: het lichaam kán ijzer ook niet actief uitscheiden. En dat houdt een risico in; namelijk het risico op ijzerstapeling met alle gevolgen van dien, zoals we hierboven uitleggen.

Omdat zowel een tekort aan ijzer als een teveel aan ijzer negatieve gevolgen heeft voor de gezondheid, is het essentieel dat het lichaam over een regelsysteem beschikt. Dat regelsysteem moet ervoor zorgen dat het ijzergehalte niet te laag maar ook niet te hoog wordt. Voor zo'n regelsysteem is een goede thermostaat nodig, vergelijkbaar met de thermostaat bij u thuis die de temperatuur in uw huis regelt en die zorgt dat u een warme douche kunt nemen op ieder gewenst moment. Gelukkig beschikt het lichaam over zo'n regelsysteem en zo'n thermostaat voor de ijzerhuishouding in de vorm van het hormoon hepcidine. Dit hormoon, dat door de lever gemaakt wordt, detecteert de hoeveelheid ijzer in het menselijk lichaam en stuurt vervolgens de opname van ijzer door de darm en de afgifte van gerecycled en opgeslagen ijzer. Een hoog hepcidine gehalte zorgt ervoor dat de ijzerpoorten

in de darm en de macrofagen op slot gaan, een laag hepcidine gehalte zorgt er juist voor dat ze opengaan. Dus bij een (dreigend) ijzertekort schroeft de lever de hepcidine productie omlaag, andersom bij een (dreigend) ijzeroverschot juist omhoog.

Overigens bestaat er nog een tweede regelsysteem dat de hoeveelheid ijzer op het niveau van de cel regelt. Dit is een ingenieus systeem dat werkt via het meer of minder af laten lezen van genen die bepalend zijn voor instroom en uitstroom van ijzer op celniveau (het lichaam als geheel kan dus geen ijzer uitscheiden, maar individuele cellen wel). Om het nog ingewikkelder te maken; beide regelsystemen werken op verschillende punten met elkaar samen.

Tot zover de gezonde situatie. Bij een fors en aanhoudend ijzergebrek, bijvoorbeeld doordat iemand onvoldoende ijzer binnenkrijgt met de voeding of omdat iemand bloed verliest via een poliep in de darm, wordt het hepcidine regelsysteem overvraagd. Het lichaam is dan niet meer in staat het ijzergehalte op peil te houden en de patiënt ontwikkelt bloedarmoede. Vergelijk het met een oud, tochtig huis met enkel-glas dat u probeert warm te stoken terwijl het buiten vriest dat het kraakt.

Typische klachten van bloedarmoede zijn bleek-zien, vermoeidheid en kortademigheid bij inspanning, zoals de trap oplopen of een eindje fietsen. Een ijzertekort leidt overigens niet alleen tot bloedarmoede. Ieder orgaansysteem in het lichaam heeft ijzer nodig, ook het brein bijvoorbeeld. Dit weten we uit studies met muizen en ratten, maar we zien hier ook aanwijzingen voor in de praktijk. Zo zien we bij peuters en kleuters met een ijzergebrek soms zogenaamde *pica* klachten. Dit houdt in dat het kind een onbedwingbare neiging ontwikkelt tot het eten van zaken die niet voor consumptie bestemd zijn, zoals zand of papier. Onlangs zag ik dit nog bij een patiëntje van mij, een kleuter van vijf jaar oud. In een onbewaakt ogenblik had hij zijn op papier meegebrachte laboratorium uitslagen helaas al opgegeten voordat hij aan mijn bureau zat....We denken dat dit fascinerende verschijnsel het gevolg is van een ijzertekort van de hersenen. Overigens liggen er nog veel onbeantwoorde vragen op dit terrein, maar daarover straks meer.

DEEL 1: LITERATUUR STUDIES EN KLINISCHE STUDIES

Kind en ijzer

De zeldzame vormen van bloedarmoede door een genetisch probleem in de ijzerstofwisseling, waar dit proefschrift over gaat, zien we op alle leeftijden. Dus zowel huisartsen, kinderartsen als internisten kunnen er mee te maken krijgen. Daarom is het cruciaal dat artsen alert zijn op de symptomen van deze aandoeningen en dat ze weten hoe ze de diagnose moeten stellen. Om vast te stellen dat een patiënt zo'n aandoening heeft, moet je eerst heel goed weten hoe de ijzerstofwisseling werkt bij iemand die gezond is, en dat kan afhangen van de leeftijd van de persoon. Een kind is namelijk geen mini-volwassene, dat was de eerste les die ik leerde tijdens mijn opleiding tot kinderarts. De anatomie en fysiologie bij een kind zijn echt anders dan bij een volwassene. Daarom hebben we een uitgebreide literatuurstudie gedaan om in kaart te brengen wat er bekend is over de ijzerstofwisseling tijdens de verschillende fasen van het leven; in de baarmoeder, op de kinderleeftijd en tijdens de adolescentie, tot aan de volwassenheid. En voor welke verschillende orgaansystemen ijzer belangrijk is, vooral tijdens de groei en ontwikkeling. Dit beschrijven we in **hoofdstuk 2**. Uiteraard is er veel ijzer nodig voor de aanmaak van bloed, net als bij volwassenen. Het grote verschil is echter dat een volwassene alleen de afgebroken en gerecyclede oude rode bloedcellen moet vervangen door nieuwe, terwijl een kind ook extra rode bloedcellen moet maken vanwege de groei. Het circulerend bloedvolume bij een pasgeboren baby van 3 kg bedraagt ongeveer 250 ml, bij een volwassen man van 70 kg ongeveer 5 l; dat betekent een toename met een factor 20. U kunt zich voorstellen dat hier grote hoeveelheden ijzer voor nodig zijn. IJzer is echter ook van groot belang voor de groei van de spieren en botten, voor de rijping van het immuunsysteem, voor de vorming van de darmflora en voor de groei en ontwikkeling van het brein. Denk aan het voorbeeld van de kleuter met *pica,* die zijn papieren uitslag opat. De ontwikkeling van het brein is overigens een erg ingewikkeld proces, waar nog veel vragen en onduidelijkheden over zijn. Wel weten we dat het proces in verschillende, achtereenvolgende stappen verloopt en dat ijzer er een rol bij speelt. Proefdieronderzoek met muizen en ratten wijst uit dat de hoeveelheid ijzer die nodig is voor een optimale breinontwikkeling, per stap verschillend is. Dit impliceert dat de gevoeligheid van het hersenen voor schade door een ijzertekort (of juist een overschot) afhangt van het moment waarop dit optreedt. Het moeilijke van dergelijk onderzoek met muizen en ratten is altijd dat de resultaten ervan niet één op één vertaald kunnen worden naar kinderen. Er zijn echter klinische studies die suggereren dat blootstelling aan een ijzertekort bij

kinderen in de baarmoeder of op de zuigelingenleeftijd een blijvende negatieve invloed kan hebben op de psychomotore ontwikkeling. En dat je die negatieve invloed niet meer ongedaan kunt maken door het kind ijzer te geven als het al wat ouder is, bijvoorbeeld op de peuterleeftijd. De theorie is dat dit komt omdat het zogenaamde *window of opportunity*, waarbij ijzer een cruciale rol speelt in een specifiek stukje van de breinontwikkeling, inmiddels is gepasseerd. Nu zou je kunnen denken dat het dan wellicht verstandig is om extra ijzer te geven aan alle jonge kinderen. Vooral aan kinderen die extra gevoelig zijn voor een ijzertekort zoals prematuren of kinderen met een laag geboortegewicht voor de zwangerschapsduur. Echter, blootstelling aan te veel ijzer op jonge leeftijd kan eveneens schadelijke effecten hebben, bijvoorbeeld op de darmflora en op het afweersysteem. Verder zijn veel ziekmakende bacteriën voor hun overleving afhankelijk van ijzer. Dus eigenlijk is er een soort competitie; het kind heeft ijzer nodig, maar de bacterie ook. Dit wordt in de literatuur *the battle for iron* genoemd. In de tropen, waar de infectiedruk hoog is, is dit een reëel thema. Zo hebben meerdere klinische studies uitgewezen dat het willekeurig geven van ijzertabletten of ijzerdrank aan kinderen in de tropen leidt tot een toename van het aantal malaria gevallen. Dit geeft aan dat, meer nog dan bij volwassenen, de balans tussen enerzijds niet te weinig maar anderzijds niet te veel ijzer, van groot belang is op de kinderleeftijd. Hoe het lichaam de ijzerbalans dan regelt bij kinderen en of dit hetzelfde gaat als bij volwassenen, is nog grotendeels onbekend. (In hoofdstuk 7 gaan we hier verder op in.)

Richtlijn voor het opsporen en behandelen van zeldzame vormen van bloedarmoede door een genetisch defect van de ijzerstofwisseling

In hoofdstuk 3 van dit proefschrift geven we aan de hand van een systematische analyse van de literatuur een overzicht van de verschillende bekende genetische aandoeningen van de ijzerstofwisseling die leiden tot een *microcytaire anemie*, dus bloedarmoede met de kenmerkende kleine rode bloedcellen. Zoals weergegeven in de inleiding betreft het ziekten waarbij ofwel het ijzer vanuit de darm niet goed naar het bloed wordt gesluisd, ofwel niet goed wordt afgeleverd en opgenomen in het beenmerg ofwel niet goed wordt ingebouwd in de rode bloedcel. We beschrijven de symptomen van de verschillende aandoeningen en doen aanbevelingen voor de klinische en genetische diagnostiek en ook voor de therapie op basis van het bewijs dat daarvoor is in de literatuur. Verder geven we advies over het al dan niet screenen van de familieleden van de patiënt waarbij een dergelijke afwijking in de ijzerstofwisseling is vastgesteld.

Iron Refractory Iron Deficiency Anemia: een defecte ijzerthermostaat met een te hoog hepcidine

In hoofdstuk 4-6 *zoomen* we verder *in* op twee vormen van bloedarmoede waarbij de ijzerstofwisseling verstoord is. De eerste aandoening betreft Iron Refractory Iron Deficiency Anemia, afgekort IRIDA. Bij IRIDA is er sprake van een afwijking in het *TMPRSS6* gen, wat een rol speelt bij de hepcidine-productie van de lever, de thermostaat van de ijzerhuishouding. Het gevolg van een defect in dit gen is dus een niet goed functionerende ijzerthermostaat. Bij IRIDA kan de lever de hepcidine-productie onvoldoende bijsturen en afremmen als de situatie daarom vraagt. Zoals we in de inleiding uitleggen, hoort het hepcidine gehalte omlaag te gaan bij een (dreigend) ijzertekort, waardoor de ijzerpoorten in de darm en op de macrofagen opengaan en er meer ijzer beschikbaar komt in de bloedbaan. Bij IRIDA blijft het hepcidine gehalte hoog, ondanks een lage hoeveelheid ijzer in het lichaam. De ijzerpoorten in de darm en op de macrofaag blijven dan ten onrechte op slot en de patiënt ontwikkelt een ijzergebrek en bloedarmoede. Typerend voor IRIDA is het niet goed reageren op ijzertabletten, wat we kunnen verklaren aan de hand van het te hoge hepcidine gehalte en daardoor de gesloten ijzerpoorten in de darm. De meeste IRIDA patiënten hebben dan ook ijzerinfusen nodig ter behandeling van het ijzergebrek en de bloedarmoede. Overigens wordt het probleem daarmee niet volledig opgelost. Het ijzer dat via een infuus wordt toegediend, verdwijnt namelijk voor een belangrijk deel in de macrofagen, waar het dan vervolgens vast komt te zitten omdat de ijzerpoorten daar ook grotendeels dicht zitten bij een IRIDA patiënt als gevolg van het hoge hepcidine gehalte.

In hoofdstuk 4 beschrijven we een serie van 21 Nederlandse IRIDA patiënten waarbij we zowel het defect in het *TMPRSS6* gen in kaart brengen (het *genotype*) als het klinisch beeld, dus de mate van de bloedarmoede en het ijzergebrek, de reactie op ijzertabletten en ijzerinfusen en de hoogte van het hepcidine gehalte in het bloed (het *fenotype*). Op basis van onze bevindingen concluderen we dat er grote verschillen zijn tussen de ene IRIDA patiënt en de andere, zowel wat betreft het *genotype* als het *fenotype*. Ook concluderen we dat er geen eenduidige aanpak is bij de verschillende behandelaren wat betreft de diagnostiek en therapie. Verder onderzoek is nodig om dit ziektebeeld verder te ontrafelen, zodat er vervolgens een goed onderbouwde behandelrichtlijn opgesteld kan worden. In hoofdstuk 5 geven we een reactie op een casus beschrijving in de literatuur van een vermeende IRIDA patiënte.

X-linked sideroblastaire anemie door een defect in het *ALAS2* gen: een defecte hemoglobine productie en een ontregelde ijzerthermostaat met een te laag hepcidine gehalte

De tweede aandoening waar we verder op in gaan in dit proefschrift is *X-linked* sideroblastaire anemie ten gevolge van een defect in het *ALAS2* gen, afgekort XLSA. *X-linked* betekent dat het gendefect dat verantwoordelijk is voor deze aandoening, in dit geval het *ALAS2* gen, gelegen is op het X-chromosoom. Dit geeft een typerend overervingspatroon waarbij de ziekte in principe alleen voorkomt bij mannen en niet bij vrouwen, uitzonderingen daargelaten. Dat komt omdat vrouwen twee X-chromosomen hebben en mannen maar één; mannen hebben een X- en een Y-chromosoom. Een vrouw met een defect *ALAS2* gen op het ene chromosoom, heeft in het algemeen een intact en goed functionerend *ALAS2* gen op het andere chromosoom, wat voorkomt dat ze in de problemen raakt. Mannen met een *ALAS2* defect hebben daarentegen maar één X-chromosoom en dus ook maar één *ALAS2* gen. Wat gebeurt er als dit *ALAS2* gen niet (goed) functioneert? Het ALAS2 eiwit dat wordt gemaakt als het *ALAS2* gen wordt afgelezen, speelt een belangrijke rol bij de aanmaak van hemoglobine, het onderdeel van de rode bloedcel dat ervoor zorgt dat zuurstof aan de rode bloedcel kan binden. Een defect ALAS2 eiwit resulteert dan ook in een verminderde productie van hemoglobine. Daardoor ontstaat er een tekort aan rode bloedcellen en zijn de rode bloedcellen die wel geproduceerd worden, kwetsbaar en van matige kwaliteit. Het gevolg is een *microcytaire anemie*, de al eerdergenoemde bloedarmoede met de kenmerkende kleine rode bloedcellen.

Een ander opvallend kenmerk van XLSA patiënten door een defect in het *ALAS2* gen is ijzerstapeling in de organen, met name in de lever. We denken dat het hepcidine regelsysteem hier een rol in speelt. Om dit uit te leggen, gaan we weer terug naar het voorbeeld van de ketel, en dan een combi-ketel, zo'n ketel die zowel het huis verwarmt als het water (als het goed is tenminste...) Zelf heb ik een keer meegemaakt dat mijn combi-ketel kapot was; het gevolg was een ijskoude douche, een loeiende verwarming en een tropische temperatuur in de woonkamer... We denken dat er iets vergelijkbaars gebeurt bij XLSA patiënten door een defect in het *ALAS2* gen. Door het kapotte ALAS2 eiwit kan het eindproduct, namelijk de rode bloedcel, onvoldoende worden aangemaakt en gaan de rode bloedcellen die wel geproduceerd worden, snel stuk. Als reactie hierop zal het beenmerg proberen de productie van rode bloedcellen op te schroeven (wat niet goed lukt door het defecte ALAS2 eiwit). Voor deze poging tot compensatie is veel ijzer nodig en het beenmerg

zal daarom communiceren met de lever dat de hepcidine-productie omlaag moet, zodat de ijzerpoorten in de darm en op de macrofagen wijd open komen te staan. Het gevolg hiervan is een forse toename van de ijzer opname door de darm en van de ijzerafgifte door de macrofagen, waardoor het ijzergehalte in het bloed gaat stijgen. Dit leidt echter niet tot een verbetering van de bloedaanmaak vanwege het defecte ALAS2 eiwit, maar wel tot ijzerstapeling. We noemen dit verschijnsel *ineffectieve erytropoëse*. Het is de oorzaak van ijzerstapeling bij bijvoorbeeld *bèta-thalassemie*, een vorm van erfelijke bloedarmoede die o.a. veel voorkomt bij mensen die afkomstig zijn uit het Middellandse zeegebied (*thalassa* is het Griekse woord voor zee). Of dit mechanisme van *ineffectieve erytropoëse* ook verklaart waarom XLSA patiënten ijzer stapelen is overigens nog niet bevestigd voor zover wij weten, maar het is wel aannemelijk.

In **hoofdstuk 6** beschrijven we een serie van 15 Nederlands XLSA patiënten door een defect in het *ALAS2* gen. Net als bij de IRIDA serie brengen we het *genotype* en het *fenotype* in kaart. Verder doen we aanbevelingen voor de diagnostiek en behandeling, zowel gericht op het behandelen van de bloedarmoede als op het voorkomen en eventueel behandelen van ijzerstapeling.

DEEL II: DIAGNOSTISCHE STUDIES: FOCUS OP IRON REFRACTORY IRON DEFICIENCY ANEMIA

Referentiewaarden van hepcidine bij Nederlandse kinderen: de afstelling van de hepcidine thermostaat is afhankelijk van de leeftijd

Zoals we al aangeven in **hoofdstuk 2**, is het essentieel om te weten hoe de ijzerhuishouding werkt bij kinderen en of dit anders gaat dan bij volwassenen. Dat is namelijk het vertrekpunt om als kinderarts vast te kunnen stellen of een kind wel of niet een aandoening heeft van de ijzerstofwisseling. Daarom hebben we bij 266 gezonde kinderen tussen de 4 maanden en 17 jaar, die voor een kleine ingreep het ziekenhuis bezochten (bijvoorbeeld voor een flapoorcorrectie of een liesbreukoperatie) het hepcidine gehalte in het bloed bepaald. De resultaten hiervan beschrijven we in **hoofdstuk 7**. We zien dat het hepcidine gehalte bij kinderen erg afhangt van het ijzergehalte in het lichaam, net als bij volwassenen. Dat is ook logisch gezien de thermostaatfunctie van hepcidine; bij een grote hoeveelheid lichaamsijzer hoort een hoog hepcidine gehalte met als gevolg gesloten ijzerpoorten. Bij een kleine hoeveelheid lichaamsijzer verwacht je het tegenovergestelde; een laag hepcidine gehalte met als gevolg openstaande ijzerpoorten. Om het effect van de

hoeveelheid lichaamsijzer weg te filteren, hebben we daarom ratio's berekend tussen de hoeveelheid hepcidine en de hoeveelheid lichaamsijzer. Na deze correctie zien we dat het hepcidine gehalte ten opzichte van de hoeveelheid ijzer in het lichaam duidelijk lager is bij kinderen ouder dan 12 jaar. Het lijkt er dus op dat de afstelling van de ijzerthermostaat afhangt van de leeftijd van het kind. Mogelijk is het relatief lage hepcidine gehalte bij oudere kinderen het gevolg van de remmende invloed van de geslachtshormonen op de productie van het hormoon. Het gevolg is in ieder geval dat bij een puber de ijzerpoorten wijd open staan en dat is natuurlijk nuttig met het oog op de enorme groeispurt die een adolescent doormaakt. Opvallend genoeg vinden we bij jonge kinderen relatief hoge hepcidine waarden ten opzichte van de hoeveelheid lichaamsijzer, vergelijkbaar met de waarden die we vinden bij volwassenen. Terwijl een jong kind relatief nog harder groeit dan een adolescent; in het eerste levensjaar neemt het gewicht toe met een factor 3, van 3 kg tot 10 kg, en de lengte met een factor 1,5, van 50 cm tot 75 cm.

Waarom zou het hepcidine gehalte ten opzichte van de hoeveelheid lichaamsijzer relatief hoog zijn bij jonge kinderen? We kunnen hier natuurlijk alleen maar over speculeren, maar mogelijk heeft het iets te maken met de afweer en de kans op infecties. Zoals we uitleggen in de inleiding zorgt een hoog hepcidine gehalte ervoor dat het ijzer in de macrofagen blijft zitten en niet in de bloedbaan terecht komt. Dit is nadelig voor bacteriën die binnendringen in de bloedbaan en die voor hun overleving afhankelijk zijn van ijzer. Een relatief hoog hepcidine gehalte heeft dus mogelijk een functie bij de bescherming tegen infecties, waar met name jonge kinderen erg vatbaar voor zijn omdat het afweersysteem nog onrijp is aan het begin van het leven. Hier komt dus weer de *battle for iron* om de hoek kijken, waar we eerder al over schreven. Een belangrijke kanttekening is overigens dat we in onze studie relatief weinig jonge kinderen hebben kunnen includeren. Vervolgonderzoek is dan ook zeker nodig om bovenstaande bevinding te bevestigen dan wel te ontkrachten.

De ratio tussen ijzer in het bloed en het hepcidine als diagnostische test voor IRIDA

Zoals we schreven in de inleiding komt bloedarmoede door een ijzergebrek ontzettend veel voor. Bij de meeste mensen is daar een voor de hand liggende verklaring voor, zoals een vegetarisch of veganistisch dieet of bloedverlies door een poliep in de darm. Sommige mensen hebben een IRIDA door een defect in het *TMPRSS6* gen waardoor de ijzerthermostaat niet goed functioneert en het

hepcidine te hoog staat afgesteld ten opzichte van de hoeveelheid lichaamsijzer. Voor de behandeld arts is het belangrijk om in de spreekkamer het onderscheid te kunnen maken tussen een 'gewone' bloedarmoede door een ijzertekort en tussen een IRIDA. Daarom hebben we een studie gedaan waarbij we de IRIDA patiënten uit **hoofdstuk 4** vergelijken met 39 volwassen patiënten die op de spoedeisende hulp of bij de maag-darm-lever arts kwamen met zo'n 'gewone' bloedarmoede. De resultaten van deze studie beschrijven we in **hoofdstuk 8**. We hebben de ratio tussen het circulerend ijzer in het bloed en de concentratie hepcidine (de zogenaamde TSAT/hepcidine ratio) vergeleken tussen deze twee groepen en uitgerekend waar de grenswaarde ligt die onderscheid maakt tussen enerzijds IRIDA en anderzijds ijzergebreks-bloedarmoede door bijvoorbeeld een bloedende darmpoliep. We concluderen op grond van onze resultaten dat de TSAT/hepcidine ratio een bruikbaar en gemakkelijk toepasbaar instrument is in de spreekkamer om een IRIDA op te sporen of (nagenoeg) uit te sluiten. Ook voor deze studie geldt dat vervolgonderzoek nodig is; we weten bijvoorbeeld nog niet wat er gebeurt met de ratio als de patiënt recent ijzertabletten heeft geslikt of als de patiënt behalve een ijzergebrek ook een andere aandoening onder de leden heeft. Deze patiënten waren in onze studie namelijk uitgesloten van deelname.

DEEL III: DISCUSSIE, SAMENVATTING EN BIJLAGEN

De toekomst: hoe nu verder?

In **hoofdstuk 9**, de discussie van dit proefschrift, houden we onze bevindingen ten aanzien van de diagnostiek en behandeling van zeldzame vormen van bloedarmoede door een probleem in de ijzerstofwisseling kritisch tegen het licht en bespreken we de implicaties ervan voor de klinische praktijk. Verder brengen we de lacunes in kaart wat betreft onze kennis en formuleren we doelen voor toekomstig onderzoek. We richten ons hierbij met name op IRIDA.

Zoals we al eerder schreven, zijn de aandoeningen die we bestuderen in dit proefschrift zeldzaam. Daarom is onze belangrijkste aanbeveling om op nationaal en internationaal niveau meer samen te werken en de krachten te bundelen. Een goed middel om dit te bewerkstelligen is het opzetten van een (inter) nationale ijzer-biobank, waarin zowel biologisch materiaal (bijvoorbeeld buisjes bloed) als demografische gegevens (leeftijd, geslacht, afkomst) als klinische data (laboratoriumuitslagen, ziektebeloop, behandeling) van patiënten met bloedarmoede door een probleem in de ijzerstofwisseling zijn opgeslagen. Zo'n

biobank leidt tot schaalvergroting en geeft ook de mogelijkheid om patiënten in de tijd te vervolgen, dit noemen we *prospectief* onderzoek. Dit in tegenstelling tot *retrospectief* onderzoek, waarbij je als onderzoeker terugkijkt, zoals wij ook gedaan hebben bij onze studies.

In het Radboudumc te Nijmegen is er al een ijzer-biobank in ontwikkeling. Het is onze ambitie deze ijzer-biobank verder uit te rollen, eerst in Nederland en vervolgens ook in het buitenland. Overigens is de wet- en regelgeving rondom biobanken erg complex. Enerzijds dient de privacy, autonomie en zeggenschap van de patiënten die materiaal en data afstaan aan de biobank geborgd te worden, anderzijds is het van groot belang dat de voortgang van het wetenschappelijk onderzoek niet belemmerd wordt. Omdat er steeds meer biobanken komen, is deze wet- en regelgeving nog volop in ontwikkeling.

Verder is ons plan om zowel de genetica als het klinisch beeld van IRIDA verder in kaart te brengen. We willen bijvoorbeeld onderzoeken of er behalve het *TMPRSS6* gen nog andere genen betrokken zijn die ervoor zorgen dat de ijzerthermostaat ontregelt en het hepcidine gehalte te hoog wordt ten opzichte van de hoeveelheid lichaamsijzer. Verder denken we dat mogelijk andere orgaansystemen, behalve het beenmerg, te lijden hebben onder het ijzergebrek dat bij IRIDA optreedt, denk aan het brein of de spieren. Dit is echter een hypothese, die we nog moeten toetsen door de verschillende orgaanfuncties van IRIDA patiënten goed in kaart te brengen. Uiteindelijk hopen we dat een beter inzicht in het ontstaan en het beloop van IRIDA zal leiden tot een effectieve therapie. Naast ijzerinfusen, die we nu geven, zal het in de toekomst wellicht mogelijk zijn om medicijnen te geven die het hepcidine remmen. Maar voor we zover zijn, is er nog veel onderzoek nodig....

In **hoofdstuk 10** vatten we dit proefschrift samen. **Hoofdstuk 11** bevat de bijlagen van dit proefschrift.

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