Iron deficiency in childhood

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Chapter 1
General introduction
Iron is an essential nutrient that is involved in oxygen transport, energy metabolism, immune response, and plays an important role in brain development\textsuperscript{1-3}. Young children are vulnerable to the effects of iron deficiency (ID) because of rapid growth and development of their brain and other organs that occurs from birth to the age of three\textsuperscript{4,5}. In infancy ID negatively affects a variety of neurodevelopmental processes that persist later in life, despite iron supplementation\textsuperscript{6}. Sufficient iron supply during the first years of life is therefore essential for optimal development. On the other hand, iron is required for the growth of bacteria\textsuperscript{7} and unbound iron can catalyze the formation of oxidative radicals that damage proteins, lipids and nucleic acids\textsuperscript{8}. Iron supplementation in iron-replete children is associated with increased risk of infection\textsuperscript{7,9}, impaired growth\textsuperscript{10-12}, impaired cognitive development\textsuperscript{13}, and even increased mortality\textsuperscript{7}. Thus, the therapeutic range of iron is small and it is therefore important to prevent both ID and iron overload.

**Function of iron**

In the biological system, iron exists in three oxidation states; ferrous (Fe\textsuperscript{2+}), ferric (Fe\textsuperscript{3+}) and ferryl (Fe\textsuperscript{4+}). Through conversion of oxidation states, iron participates in electron transport and is able to bind various ligands. Hereby, iron is particularly suited to participate in a large number of biochemical reactions such as oxygen transport and storage, electron transfer, gene regulation, and regulation of cell growth and differentiation.

Four major classes of iron-containing proteins carry out these reactions:

1. iron-containing nonenzymatic proteins,
2. iron-sulfur enzymes,
3. heme-containing enzymes and
4. iron-containing enzymes that are noniron-sulfur, nonheme enzymes\textsuperscript{14}.

In the nonenzymatic proteins, hemoglobin and myoglobin, iron functions as a critical ligand for the binding of oxygen. In iron-sulfur enzymes, iron participates in single-electron transfer reactions, primarily in energy metabolism. In the third category (the heme-containing enzymes), iron is bound to various forms of heme and participates again in electron transfer reactions that are associated with various cofactors (e.g. cytochrome P450 complexes). The final group of iron-containing enzymes (e.g. lipoxygenases) is a catch-all group in which iron is not bound to a porphyrin ring structure or in iron-sulfur complexes. In the brain,
Iron is required for myelination of the spinal cord and white matter of cerebellar folds\textsuperscript{15}, and it is a cofactor for a number of enzymes involved in neurotransmitter synthesis and function.

**Dietary iron intake**
Iron is an essential micronutrient that occurs in the diet as heme (organic) and non-heme iron (non-organic). Heme iron from meat, poultry and fish has a higher bioavailability than non-heme iron (approximately 15-35\% and ≤10\% respectively), which is mostly found in vegetables and grains\textsuperscript{16,17}. Iron absorption takes place primarily in the duodenum and upper jejunum of the small intestines. Heme and non-heme iron are absorbed by distinct pathways (figure 1). Heme iron is absorbed intact by an intestinal heme iron transporter called heme carrier protein 1 (HCP1), whereas non-heme iron has to be reduced by dietary components or by duodenal cytochrome B (DCYTB), before it can be absorbed by the divalent metal ion transporter 1 (DMT1).

**Figure 1: Regulation of intestinal iron uptake**

Haem iron is taken up by the haem iron transporter (HCP), undergoes endocytosis, and Fe\textsubscript{2+} (ferrous iron) is liberated within the endosome or lysosome. Non-haem iron includes Fe\textsubscript{2+} and Fe\textsubscript{3+} (ferric iron) salts. Fe\textsubscript{3+} is reduced to Fe\textsubscript{2+} by ascorbic acid in the lumen or by membrane ferrireductases that include duodenal cytochrome B (DCYTB). At the apical membrane, the acid microclimate provides an H\textsuperscript{+} electrochemical gradient that drives Fe\textsubscript{2+} transport into the enterocyte via the divalent metal-ion transporter (DMT1). At the basolateral membrane, iron transport to transferrin in the circulation is mediated by ferroportin 1, in association with hephaestin.

Hepcidin, produced by the liver, binds to ferroportin 1, causing its internalisation and degradation and decreasing iron transfer into the blood.

Zimmermann MB, Hurrell RF. Nutritional iron deficiency. The Lancet 2007;370:S11-S20
Ascorbic acid, citric acid, heme iron and breast milk promote non-heme iron absorption, while phytates (from seeds and grains), polyphenols (from plants), calcium and cows' milk inhibited the absorption20-22. Both heme and non-heme iron absorption are highly dependent on the child's iron status; intestinal iron absorption is increased in response to ID and decreased in a state of iron sufficiency23,24.

The enhancing effect of ascorbic acid and citric acid are largely due to their ability to reduce ferric to ferrous iron and to the potential to chelate iron. The nature of the enhancing effect of muscle tissue found in meat on the absorption of non-heme iron is not completely understood, but it is thought to be related to muscle proteins. Similarly to ascorbic acid, cysteine-containing peptides in muscle proteins both reduce ferric to ferrous iron and maintain iron in a soluble complex available for absorption25. Addition of chicken, beef or fish increase the absorption of non-heme iron two to three fold, and 30 g muscle tissue is considered equivalent to 25 mg ascorbic acid26. Iron in breast milk is easily absorbed because it is mainly bound to lactoferrin, which facilitates iron absorption via the lactoferrin receptor into the enterocyte. Although the iron concentration in breast milk starts relatively low (0.6 mg/L), and declines to around 0.2–0.3 mg/L at the age of 5–6 months27-29, the bioavailability of iron in breast milk is high (15–42%)10,30.

Phytates and polyphenols inhibit non-heme iron absorption by forming insoluble complexes, thereby making non-heme iron unavailable for absorption31. The negative effect of cows’ milk consumption on iron status is attributed to the composition of cows’ milk with its low iron content32, and the inhibitory effect of calcium and casein on non-heme iron absorption33. Furthermore, cows’ milk allergy can induce intestinal blood loss that contributes to the development of ID34. ID is more frequently observed in children consuming >400 mL of cows’ milk per day21. Whether cows’ milk is consumed to the exclusion of more iron-rich foods is currently unclear.

The bioavailability of iron in cows’ milk derived formula is much lower compared to iron in human milk, and therefore, significantly greater quantities of iron are necessary to ensure appropriate intake. Infant formula fortification guidelines differ by country. Most commercial European infant formulas subscribe to the European Society for Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) Global Standard and contain 4-8 mg/L of iron35. Follow-on formulas that are used for infants older than 6 months of age usually contain 10-12 mg/L iron. Although lower iron concentrations have been suggested36,37, there is currently insufficient evidence available to determine the appropriate level of iron in infant (follow-on) formulas22.
Iron metabolism

Once inside the enterocyte, iron is stored by ferritin or released into the plasma by the cellular iron exporter ferroportin. After oxidation by ferroxidase hephestin (intestinal cells) or ceruloplasmin (non-intestinal cells), iron is loaded onto transferrin for transport in the plasma. Transferrin binds to specific transferrin receptors, expressed on all iron-requiring cells, but mainly on erythroid precursors in the bone marrow and in the liver. In the liver, iron is stored in hepatocytes as ferritin or haemosiderin. In the bone marrow, iron is used for erythropoiesis. The life span of erythrocytes varies from 60 to 80 days in infants to 120 days in adults. Iron from senescent erythrocytes is recycled by macrophages that take up iron indirectly by phagocytizing the erythrocytes and extracting iron from their hemoglobin. When iron stores decrease, iron supply for erythropoiesis is initially preserved detrimental to other proteins and anemia is therefore a relatively late symptom of ID. Iron is unique among nutrients because the human body has no mechanism for iron excretion. Iron losses from senescent cells in the skin, intestines, urinary tract, and airways are relatively small. To maintain systemic iron homeostasis, effective communication between cells that absorb iron from the diet (duodenal enterocytes), utilize iron (mainly erythroid precursors) and store iron (hepatocytes and macrophages) is crucial.

Hepcidin, a peptide hormone produced mainly in the liver, is the key regulator of systemic iron homeostasis. Hepcidin binds to ferroportin, causing its internalization and degradation and thereby decreasing iron transfer to the blood. As a result, dietary iron absorption and circulating iron in the blood decrease, whereas intracellular iron stores increase. Furthermore, hepcidin has a bacteriostatic effect since it reduces the plasma iron content, that is essential for microbial growth. The synthesis of hepcidin is stimulated by iron overload, infection and inflammation, whereas hepcidin synthesis decreases in situations that require increased concentrations of circulating iron, such as ID, hypoxia, anemia and conditions characterized by increased erythropoietic activity. Furthermore, ID and hypoxia stimulate duodenal expression of DMT1, DCYTB, and ferroportin, and thereby increase iron absorption.

Iron requirements

To maintain iron homeostasis, the sum of iron losses plus the iron required for growth must be provided by the diet. In infants, about 30% of required iron comes from the diet and 70% comes from recycled erythrocytes, while in adults 5% of required iron is obtained from the diet and 95% from recycled erythrocytes.
Term infants
Healthy, term infants with adequate iron stores at birth are able to cover the iron demands for growth during the first 4 to 6 months of life. However, after that age, dietary iron requirements increase rapidly to amounts higher than in any other period of life.

Preterm infants
Fetal iron accretion predominantly occurs in the third trimester of pregnancy. In preterm infants iron accretion ends before it has been completed since iron supply via the placenta stops at birth. Furthermore, placental iron transport may be limited in placental insufficiency, due to maternal hypertension, maternal smoking, or pregnancy-induced diabetes. Compromised iron stores combined with rapid growth, accelerated erythropoiesis and frequent blood sampling after birth, render preterm infants at high risk for ID and iron deficiency anemia (IDA). The ESPGHAN recommends an iron intake of 1 – 2 and 2 – 3 mg/kg per day during the first 6 months of life for preterm infants with birth weight <2500 g and <1800 g respectively. Iron requirements in preterm infants with a higher birth weight are poorly defined, since studies on iron status in preterm infants have mainly focused on very low birth weight infants, those with a BW birth weight <1500 g. Results of a study in infants with a birth weight of 2000 – 2500 g (both preterm and term infants born small for gestational age) suggest that iron supplementation at a dose of 1 – 2 mg/kg per day, is necessary to prevent ID at the age of 6 months. However, the potential risk of iron overload and the poorly developed anti-oxidant measures in the preterm infant argues against indiscriminate iron supplementation in this population. Unfortunately, no data are available on criteria to discriminate those infants who may benefit from iron supplementation, from infants in whom iron supplementation is not necessary and even may be harmful.

Children aged 0.5 to 3 years
After the age of about 6 months, children become exclusively dependent on dietary iron intake. Exact iron requirements are difficult to determine, since actual iron absorption depends on form of iron (heme/non-heme), presence of enhancing and inhibiting factors, infection or inflammation and the child’s iron status. The recommended daily allowance, defined as the amount that will meet the daily requirement of almost all (97.5%) individuals, varies from 7.0 to 11.0 mg per day and 5.8 to 9.0 mg per day for children aged 0.5 to 1 year and 1 to 3 years, respectively.
Children older than 3 years
Recommended iron intakes in school-age children vary from 4 to 10 mg per day\textsuperscript{55,56}. Adolescents have higher iron requirements, especially during the growth spurt phase. Moreover, increased muscular growth in boys leads to increased demand for iron, while in girls the onset of menstruation leads to iron losses\textsuperscript{57}. Recommended iron intakes for pubertal boys and girls are 10 to 13 mg and 15 to 22 mg per day respectively\textsuperscript{55,56}.

Children with a chronic inflammatory disease
A special group of children at risk for ID are children with a chronic inflammatory disease, such as cystic fibrosis (CF), inflammatory bowel disease (IBD), rheumatologic diseases, infections, diabetes mellitus, and malignancies\textsuperscript{58-63}. Furthermore it has been shown that obesity, characterized by a chronic low-grade inflammation, is associated with decreased iron absorption, independent of iron status\textsuperscript{64}. The inflammatory response may induce an increase in hepcidin concentration and thereby inhibit the enteral iron absorption (figure 1). Despite sufficient iron stores, decreased iron content available for erythropoiesis may cause a functional ID. Moreover, children with a chronic inflammatory disease have an increased risk of under nutrition in general\textsuperscript{65}, which contribute to the development of absolute ID. Disease specific factors associated with of ID and IDA are gastrointestinal blood loss in patients with IBD, and the ability of Pseudomonas aeruginosa (PA) to obtain extracellular iron directly from host tissues in patients with CF\textsuperscript{66}. Functional ID is commonly described in adult patients\textsuperscript{67,68}. Data on prevalence and causes of ID in children with a chronic inflammatory disease are scarce\textsuperscript{58-60,63}.

Iron intake and feeding practices
Preterm infants
Although uniquely well absorbed, the low iron content in breastfeeding may be insufficient to meet the increased iron demands in preterm infants during the first 6 months of life. Exclusively breastfeeding and no formula in preterm infants is associated with an increased risk of ID\textsuperscript{50}. Breast milk fortifiers do not contain iron. When breast milk is insufficiently available, common practice in the Netherlands is to provide formula for preterm infants to infants up to a weight of 3500 g. These formulas contain 9.0 mg/L to 16.0 mg/L iron. With an average intake of 150 mL/kg per day, formulas for preterm infants provide 1.4 to 2.4 mg iron per kg per day. The lower iron content in standard formulas (5.3 to 7.8 mg/L) provides 0.8 to 1.2 mg/kg iron per day.
Since the iron content in breast milk and/or (preterm) formulas are often too low to meet iron requirements, iron supplementation is recommended for infants with a birth weight of <2500 g from 2 to 6 weeks until 6 months of age\textsuperscript{51,69}. However, in clinical practice, large variations exist between with respect to indications, doses, times of initiation, and duration of iron supplement administration, especially in preterm infants born after 32 week GA.

**Children aged 0.5 to 1 years**
Most young children do not consume large quantities of iron-rich foods such as red meat and green leafy vegetables. Even in high-income countries in Europe, a low dietary iron intake is therefore frequently reported in young children. In children aged 0.5 to 1 year, average iron intakes were around 8 to 9 mg per day in most European countries\textsuperscript{70}. Lower iron intakes were reported in Iceland (5.8 to 6.8 mg per day), Germany (6.1 mg per day) and in the UK (5.2 mg per day)\textsuperscript{70,71}. No data are available on iron intake and prevalence of ID and IDA in children aged 0.5 to 1 year in the Netherlands.

**Children aged 1 to 3 years**
For European children aged 1 to 3 years, average iron intakes were around the recommended daily allowance of 7 mg per day\textsuperscript{70}. Lower iron intakes were reported in Iceland and Germany. In the Netherlands, a large food consumption survey in 2005-2006 showed that the mean iron intake in children aged 2 to 3 years was 6.1 mg/day\textsuperscript{72}. Furthermore, the intake of cows' milk products in young children in the Netherlands was high\textsuperscript{72}. Whether the low iron intake and high consumption of cows' milk products result in a low iron status in these children is unknown since no data are available on the prevalence of ID and IDA in healthy children aged 1 to 3 years in the Netherlands.

**Defining iron deficiency**
Iron deficiency (ID) is defined as a reduction in total body iron to an extent that iron stores are fully exhausted and some degree of tissue ID is present\textsuperscript{73}. ID is conventionally considered to develop in three stages:
1. iron depletion,
2. iron-deficient erythropoiesis (IDE) and
3. iron deficiency anemia (IDA).

In the first stage body iron stores are reduced. IDE refers to laboratory evidence of an impaired supply of iron to the erythroid marrow for hemoglobin (Hb) synthesis. IDA is defined as the combination of ID and anemia.
Absolute ID refers to the depletion of iron stores due to an insufficient dietary iron intake or increased iron loss. In functional ID, there is a discrepancy between iron export to the plasma and iron requirements for erythropoiesis. Despite adequate iron stores, increased hepcidin concentrations due to chronic infection or inflammation inhibit iron export from the enterocytes and macrophages. This will ultimately lead to the so-called anemia of chronic disease. Suggested reference ranges for ID and IDA will be discussed later.

**Effects of iron deficiency**

Iron is essential for DNA synthesis, electron transport, myelin formation and synthesis and function of neurotransmitters. IDA in childhood is associated with many adverse effects, such as an increased risk or poor neurodevelopment, growth retardation and impaired immune response. Since it has been suggested that these effects may be irreversible, many studies have focused on the outcomes of children with IDA, and the effect of iron supplementation.

**Neurodevelopmental effects of iron deficiency**

Developing central nervous system processes are highly dependent on iron-containing enzymes and proteins. Neurodevelopmental effects of ID and their reversibility with iron repletion vary, depending on the timing and severity of ID, and the timing of iron treatment.

**Evidence from animal models**

Iron depletion of the brain occurs in rats within several weeks of feeding a low-iron diet. When ID occurred after the weaning period, refeeding increased the brain iron content. On the other hand, brain iron content was irreversible after intrauterine ID. Dopaminergic tracts appear to be sensitive to regional brain ID. Reduced dopamine receptor densities in the striatum, increased extracellular dopamine concentrations, and reduced densities of dopamine and other monoamine transporters have been found in iron deficient rodents and monkeys. The ability to process environmental information is highly dependent on appropriate rates of dopamine clearance from the interstitial space; thus alterations in dopamine metabolism in the mesolimbic and the nigrostriatal tracts could be related to altered perception and motivation. Other monoaminergic systems that appear to be sensitive to ID are the serotonergic and noradrenergic systems.

ID in utero and post weaning is associated with significant decreases in glutamate decarboxylase, glutamate dehydrogenase, and gamma amino butyric acid (GABA) transaminase activities. These latter two enzymes are shunt enzymes.
responsible for the synthesis and degradation of GABA. ID is associated with increased concentrations of GABA in the hippocampus, striatum, and globus pallidus86. Furthermore, ID affects oligodendrocytes, which form myelin, essential for neural transmission91. Iron uptake in brain is highest in postnatal period of rapid brain development and this increase coincides with the onset of myelination. Hypomyelination could be causally related to delayed motor and behavioral development.

**Neurodevelopmental effects of iron deficiency in children**

Case-control studies in children have shown an association between IDA in infancy and long-lasting negative effects on cognitive, behavioral74,76,92-94 and motor development95-97. The observed longer latencies for auditory brainstem responses and visual evoked potentials in 4-year old children with former IDA, support the hypothesis that IDA in infancy alters myelination94,98. The associations between ID and neurodevelopmental outcome have been observed in low-income countries with a high prevalence of malnutrition96 as well as in high-income countries with well-nourished population cohorts21,99,100. Most information on long-term outcome originates from a longitudinal study in 191 children in Costa Rica. Children aged 12 to 23 months with IDA (defined as Hb <100 g/L and serum ferritin <12 μg/L, and either a zinc protoporphyrin ≥100 μmol/L red blood cells or transferrin saturation <10%), scored lower on motor, cognitive and behavioral performances compared to non-anemic controls at the age of 5101 and 11 to 14 years102, despite early iron therapy. At the age of 19, lower scores on neurocognitive tests of executive function and recognition memory were observed103,104, and more negative emotions via behavioral problems were present even up to 25 years of age75. Although it is difficult to control for all potential confounders, well-designed studies showed that ID was associated with neurodevelopment, after adjustment for several socio-economic factors75,99,104.

The effect of iron supplementation on neurodevelopmental outcome was described in a meta-analysis of 17 randomized clinical trials105. Iron supplementation had a modest positive effect on mental development, especially in children older than 7 years who were initially anemic or had IDA105. In accordance with an earlier systematic review106, no beneficial effect of iron supplementation on motor and cognitive development was found in children younger than 3 years105. This lack of effect in the youngest infants may be due to (a) irreversible structural effects of IDA on the developing brain, due to (b) the fact that cognitive development is more difficult to measure in young children,
or (c) because the duration of iron supplementation may have been too short to correct IDA (8 of 13 studies evaluating Bayley indices intervened for less than 1 month)\textsuperscript{22,105}.

Whether these negative neurodevelopmental consequences also apply to ID without anemia is controversial, but there is certain evidence supporting that possibility\textsuperscript{99,107,108}. During ID, available iron is prioritized to red blood cells over other organs, including the brain\textsuperscript{109}, suggesting that negative neurodevelopmental effects may develop before anemia occurs\textsuperscript{14}. Infants who had ID without anemia showed effects that were intermediate between infants with ID and iron sufficiency\textsuperscript{108}. However, the severity of ID that may impact the development of children remains unknown\textsuperscript{110} and there is a lack of dose-response studies linking indicators of iron status as continuous risk factors with later cognitive outcomes\textsuperscript{52}.

\textit{Other effects of iron deficiency}

IDA and ID are associated with decreased motor activity\textsuperscript{97,111}, breath-holding spells\textsuperscript{112-115}, febrile convulsions\textsuperscript{116} and restless legs\textsuperscript{117}.

The relation between ID and immunity and infectious diseases remains controversial\textsuperscript{77}. ID depresses certain aspects of cell-mediated immunity, including, lymphocyte, neutrophil and macrophage function\textsuperscript{77} and hereby increase the susceptibility for infections. On the other hand, unbound iron may be a source of iron for bacterial growth. Increased malaria morbidity and mortality have been reported in children receiving iron supplementation, especially in iron replete children\textsuperscript{7}. However, when insecticide-treated bed nets were provided and appropriate malaria treatment was available, daily use of a micronutrient powder fortified with iron did not result in an increased incidence of malaria among young children\textsuperscript{118}. Results of a systematic review showed that iron supplementation had no harmful effect on the overall incidence of infectious diseases in children\textsuperscript{119}.

\textbf{Iron status biomarkers}

\textbf{Ferritin} is the most specific biomarker of ID since ferritin concentrations are in direct proportion to body iron stores\textsuperscript{120-122}. However, the acute phase response induced by infection or inflammation can elevate ferritin independent of iron stores\textsuperscript{123}. Furthermore, once iron stores are depleted, ferritin does not quantitatively reflect further reduction of tissue iron\textsuperscript{124}.

The \textbf{soluble transferrin receptor (sTfR)} is expressed by iron-requiring cells, mainly erythroid precursors, and reflects cellular iron demands and erythropoietic activity\textsuperscript{125}. The advantage of sTfR as an indicator of ID is the possibility of
estimating the magnitude of iron deficit once iron stores are depleted\textsuperscript{124}. Furthermore, the sTfR is less affected by infection or inflammation than ferritin and is therefore used to discriminate IDA from the anemia of chronic disease\textsuperscript{123,126}. The value in the sTfR in populations with a low prevalence of IDA is unclear since the sTfR begins to change only after severe depletion of iron stores\textsuperscript{127,128}. The ratio of sTfR to ferritin (sTfR/ferritin) was designed to evaluate changes in both body iron stores and functional iron, and was thought to be more useful than either sTfR or ferritin alone\textsuperscript{129}. sTfR/ferritin has been used to calculate body iron as follows: body iron (mg/kg)=−[\log(sTfR/ferritin)−2.8229]/0.1207\textsuperscript{128}. Although only validated for adults\textsuperscript{130}, sTfR/ferritin has also been used in children\textsuperscript{131,132}. However, in an analysis of pooled data from iron intervention studies, calculation of body iron from the sTfR/ferritin showed no clear advantage over ferritin alone\textsuperscript{122}. 

**Hemoglobin (Hb)** is the iron-containing oxygen transport protein in the erythrocytes. The **mean cell volume (MCV)** is an indicator of the average erythrocyte volume. The MCV is used to calculate the red cell distribution width (RDW), an indicator of the variation in erythrocyte volume. ID is characterized by a small **red blood cell volume** (low MCV), together with a high variation in red blood cell size (high RDW). When iron stores decrease, red blood cell indicators as Hb, MCV and RDW are initially preserved detrimental to other proteins and microcytic anemia is therefore a relatively late symptom of ID\textsuperscript{39}. Increased concentrations of **zinc protoporphyrin (ZPP)** have been found in children with IDE since zinc replaces the missing iron during formation of the protoporphyrin ring\textsuperscript{133}. The ZPP is described as a suitable biomarker to measure IDE. A limitation of the ZPP is that it increases with lead toxicity, and even the normal range varies with environmental lead exposure\textsuperscript{134}. 

**Reticulocyte hemoglobin content (Ret–Hb)** reflects the Hb content in reticulocytes. With a lifespan of reticulocytes of only 24 to 48 hours, Ret-Hb provides a real-time view of bone marrow iron status in adults\textsuperscript{135} and decreases within days after onset of iron deficient erythropoiesis\textsuperscript{136}. Therefore, Ret-Hb has been described as a more sensitive biomarker than Hb in the detection of ID\textsuperscript{135,137,138}. 

Studies reporting **hepcidin** concentrations in children are scarce. Limited evidence suggests that hepcidin might contribute to the diagnosis of ID in preterm infants\textsuperscript{139} and low birth weight infants\textsuperscript{24} and could help to distinguish between iron deficiency anemia (IDA) and anemia of chronic disease\textsuperscript{140-143}. Furthermore, hepcidin could be used in children to guide the effect of iron supplementation therapy and to screen for primary defects in hepcidin regulation such as iron refractory IDA\textsuperscript{40}. 

General introduction
Transferrin saturation (Tfsat) is a widely used screening test for ID, calculated as the ratio of serum iron to total iron binding capacity. However, its use is limited by diurnal variation in serum iron and the many clinical disorders that influence transferrin levels.

Diagnosis of ID
Considering the negative consequences of ID, early detection of ID is essential for optimal development. Since most children with ID or IDA are asymptomatic, ID is mainly a laboratory diagnosis. However, there is no consensus on the laboratory criteria for ID in children. Suggested age-specific reference values for Hb, MCV, Ret-Hb, ZPP, ferritin and sTfR for children in western countries are summarized in addendum 1. Pediatric reference ranges for serum hepcidin are available only for a small group of non-anemic, iron replete, young, Kenyan infants with a CRP<5 mg/L, showing a mean of 2.3 nmol/L (P2.5 P97.5 <0.5 – 18.1). The large variations in reported reference ranges are, at least partly, due to differences in laboratory assays. The agreement between different assays for most biomarkers, but especially for sTfR, ZPP and hepcidin is poor, which hinders the ability to compare reference ranges between studies. Another difficulty is that different methods have been used to calculate reference ranges, which are often defined as 2 SD values in a ‘healthy population’. However, in some studies this ‘healthy population’ consists of an unselected population likely to have a low prevalence of ID, while in other studies individuals with possible infection or ID were excluded, according to cut-off values for iron status indicators other than one under study. Although excluding children with possible ID remains problematic due to the absence of a golden standard, this seems to be the most useful method to define reference ranges for iron status biomarkers.

The World Health Organization (WHO) defines ID as a ferritin <12 μg/L or <15 μg/L in children aged <5 years or ≥5 years respectively. IDA is defined as ID in combination with a Hb SD >2 below the mean of similarly aged children. Another frequently used method is to define ID as two or more abnormal concentrations in a set of multiple iron status biomarkers. However, there is no agreement on the best combination of biomarkers. As described in this introduction, different biomarkers represent different aspects of iron status. By defining ID as two or more abnormal concentrations in a set of multiple iron status indicators, only children with severe, longstanding IDA will be classified as ID. The use of this method in a population with a low prevalence of severe IDA is therefore questionable.
Aims of the studies described in this thesis
The aim of this thesis was to investigate the prevalence and risk factors of ID in children in a high-income country. Furthermore, we aimed to analyze the value of different iron status biomarkers in the diagnosis of ID. The studies described in this thesis focused on preterm infants (part I), healthy children aged 0.5 to 3 years (part II), and children with cystic fibrosis (CF) (part III).

Part 1 Iron deficiency in preterm infants born after 32 to 37 weeks of gestational age
Studies on iron status in preterm infants have mainly focused on very low birth weight infants. Data on iron status in late preterm infants are scarce. In the Netherlands, infants born after 32 to 37 weeks GA do not receive iron supplementation on a structural basis. We therefore hypothesize that dietary iron intake in these infants might be insufficient to meet the high iron requirements during the first 6 months of life. To investigate the prevalence and risk factors of ID in the first 6 months of life in infants born after 32 to 37 weeks GA who do not receive iron supplementation, we conducted a prospective cohort study, named ‘Iron status in Preterm Infants’ (IPI study). Iron status was assessed in the first week after birth during hospital stay, and at the postnatal age of 1.5, 4 and 6 months respectively. Results of this study are presented in chapter 2. Hepcidin has been suggested as a promising indicator of ID in preterm infants. Lower hepcidin concentrations have been observed in infants with ID compared to infants with no ID at the age of 6 months respectively. However, in that study the majority of the iron-replete infants received iron supplementation. Whether higher hepcidin concentrations in iron-replete infants represent a physiologic increase, or an upregulation in response to iron supplementation is unclear. We therefore aim to investigate changes in hepcidin concentrations during the first 4 months of life in a population of late-preterm infants who received no iron supplementation. We hypothesized that hepcidin concentrations are lower in infants with ID compared to those without ID at the age of 4 months. Furthermore, we aimed to investigate associations between hepcidin and the type of feeding, other indicators of iron status, and erythropoiesis. This was investigated in a study described in chapter 3.

Part 2 Iron deficiency in healthy, young children
A large food consumption survey in the Netherlands in 2005 – 2006 showed that the mean iron intake in children aged 2 to 3 years was 6.1 mg/day, which was below the advised intake of 7 mg/day. Therefore we hypothesized that ID would be a common problem in these children. In the second study, presented
in chapter 4, we investigated the prevalence and risk factors for ID in healthy children aged 0.5 to 3 years. In this study, we defined ID according to the criteria of the WHO (ferritin <12 μg/L). Another frequently used method is to define ID as two or more abnormal concentrations in a set of multiple biomarkers, e.g. ferritin, Hb, MCV, Ret-Hb and sTfR. However, the value of Ret-Hb and sTfR in the detection of ID in children in countries with a low prevalence of severe IDA is still unclear. We hypothesized that children with a ferritin <12 μg/L would have lower Ret-Hb and higher sTfR compared to children with a ferritin ≥12 μg/L. We aimed to establish the extent to which iron stores must be depleted to cause a significant change in Ret-Hb and sTfR. We also analyzed the associations between ferritin and Ret-Hb, sTfR, MCV and Hb. These results are presented in chapter 5.

Furthermore, we were interested in the role of hepcidin in the diagnosis of ID. However, the diagnostic use of hepcidin is limited by the absence of standardization in general, age specific reference ranges of hepcidin and knowledge on the association between hepcidin and other iron status indicators in children in particular. Therefore, we aimed to determine reference ranges of serum hepcidin in healthy children aged 0.5 to 3 years using Weak Cation eXchange Time of Flight mass-spectometry and a commercial immunochemical assay, and to investigate its association with other indicators of iron status and inflammation. We present these results in chapter 6.

In maternal conditions such as pregnancy-induced diabetes and placental insufficiency, iron transfer is limited with subsequently a higher risk of compromised iron stores in infants up to 1 year of age. However, it remains unclear whether iron status in children older than 1 year is affected by these conditions. Moreover, conflicting data exists on the influence of maternal iron status during pregnancy on iron status in children. In chapter 7 we present the results of a study that describes the association between ID in healthy young children, and maternal conditions that are associated with decreased placental iron transport.

Dietary iron absorption depends on the form of ingested iron (haem/non-haem), the presence of enhancers and inhibitors and the child’s iron status. Consumption of foods such as meat, fruits, cereals and fortified formulas seem to protect against ID while a high consumption of cows’ milk products is associated with a ID. We used the results of a cross-sectional survey on feeding practices in children visiting daycare institutions to investigate the effect of a high intake of cows’ milk products on the intake of more iron-rich foods. We hypothesized that a high consumption of cows’ milk products results in a lower iron intake not only because of the low iron content of cows’ milk, but also because cows’ milk
products might be consumed to the exclusion of more iron-rich foods. Results of this study are presented in chapter 8.

**Part 3 Iron deficiency in children with cystic fibrosis**

In adult CF patients ID is common and primarily functional due to chronic inflammation. No recent data are available on the cause of ID and IDA in children with CF. In the last section of this thesis we present the results of a retrospective study on prevalence and risk factors of ID in children with CF (chapter 9). In this study, ID was defined according to the criteria of the WHO (ferritin <12 μg/L and ferritin <15 μg/L in children <5 years and ≥5 years of age respectively).

However, ferritin acts as an acute phase reactant, and is therefore no reliable indicator of ID in the presence of infection or inflammation. Since CF is characterized by continuous inflammation from an early age, the results of this study might therefore underestimate the prevalence of absolute ID in children with CF. The sTfR and hepcidin might provide more information than ferritin in assessing iron status in patients with CF. We hypothesized that hepcidin concentrations are increased in children with CF, which may contribute to the development of ID. Furthermore, we hypothesized that sTfR may be a more useful indicator of ID than ferritin in children with CF. We therefore assessed sTfR and hepcidin in addition to conventional iron status indicators in children with CF. These results are presented in chapter 10.

**REFERENCE LIST**


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