Iron and vitamin D deficiency in children living in Western-Europe
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Citation for published version (APA):
Akkermans, M. D. (2017). Iron and vitamin D deficiency in children living in Western-Europe: Diagnostic tests, epidemiological aspects, and prevention
Chapter 1
General introduction
1. GENERAL

1.1 Micronutrient deficiencies and global health
The World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations have declared that “more than two billion people in the world today suffer from micronutrient deficiencies caused largely by a dietary deficiency of vitamins and/or minerals”. Micronutrient deficiencies have important negative health consequences, especially in pregnant women and young children, and the long-ranging health effects can lead to public costs and reduced work capacity caused by high rates of illness and disability.

Micronutrient deficiencies are not uniquely the concern of developing countries. While micronutrient deficiencies occur certainly more frequently and more severe among disadvantaged populations (key factors are poverty, the lack of access to a variety of food products, the lack of knowledge of appropriate dietary practices and a high incidence of infectious diseases), they do cause a public health problem in industrialized countries too. The current lifestyle with increased consumption of highly-processed energy-dense but micronutrient-poor food products in industrialized countries is likely to adversely affect micronutrient intake and subsequently micronutrient status. Therefore, micronutrient deficiencies are also a major impediment for the public health and socioeconomic development of industrialized countries in, for example, Europe.

1.2 World prevalence and global burden of iron and vitamin D deficiency
Iron deficiency (ID) and vitamin D deficiency (VDD) are two of the most common micronutrient deficiencies worldwide. There are no current global prevalence rates of ID, but using anemia as an indirect indicator it can be estimated that most preschool children and pregnant women in developing countries, and at least 30-40% in industrialized countries, are iron deficient. The world prevalence of VDD varies from 0.2% to 98% depending the investigated population (country, age, etc.) and definition used.

ID and VDD contribute substantially to the global burden of disease. For example, according to WHO mortality data, around 0.8 million deaths (1.5% of the global total) can be attributed to ID each year. In terms of the loss of healthy life, expressed in disability-adjusted life years (DALYs), iron deficiency anemia (IDA), the consequence of severe ID, results in 25 million DALYS lost per year (2.4% of
the global total). The scale and impact of VDD is much more difficult to quantify because of the diversity of consequences of VDD. However, it is likely that VDD also substantially contributes to the global burden of disease. From a global health and economical point of view, ID and VDD are two highly relevant health issues that should be prevented.

2. IRON

2.1 Iron-homeostasis: absorption, transport and key regulator hepcidin
Iron is an essential micronutrient crucial to many biologic functions, including oxygen transport, energy metabolism, immune system functioning and DNA synthesis in growing and developing tissues. For example, in young children, iron plays an important role in brain growth and development. On the other hand, iron is required for the survival and virulence of many pathogens. Moreover, unbound iron can catalyze the formation of oxidative radicals that damage proteins, lipids and nucleic acids. The human body has no mechanism for active iron excretion. To prevent both ID and iron overload, iron-homeostasis is tightly regulated by the peptide hormone hepcidin that influences iron release in the systemic circulation.

Iron absorption
Iron absorption takes place primarily in the duodenum and proximal jejunum of the small intestine and highly depends on a child’s iron status; intestinal iron absorption is increased in response to ID and decreased in a state of iron sufficiency. Furthermore, intestinal iron absorption also depends on the type of iron and the presence of enhancing and/or inhibiting factors. Diets contain both heme iron (organic, for example, meat, poultry and fish) and non-heme iron (non-organic, for example, vegetables and fruits). Heme iron usually constitutes only 10% or less of the total iron intake in European mixed diets, whereas its absorption varies from about 10 to 40%. Non-heme iron forms the main part of dietary iron, but its bioavailability is low (1 to 5%). The absorption of non-heme iron is enhanced by meat, vitamin C (ascorbic acid), and inhibited by phytates (from seeds and grains), polyphenols (from plants) and calcium. The composition of a child’s diet can therefore influence non-heme iron absorption.

Heme and non-heme iron are absorbed by two different pathways with specific transporters. Heme iron is absorbed intact by an intestinal heme iron transporter called heme carrier protein 1 (HCP1). Subsequently, heme oxygenase in the enterocyte degradates heme iron to ferrous iron (Fe2+). Absorption of non-heme iron is mediated by the divalent metal iron transporter 1 (DMT1). DMT1 transports
only ferrous iron, but most dietary non-heme iron is in the ferric form (Fe3+).
Therefore, ferric iron must first be reduced to ferrous iron, possibly by the brush border ferric reductase called duodenal cytochrome B (DCYT2) or by dietary components like vitamin C. Once inside the enterocyte, ferrous iron that is not directly transferred into the systemic circulation, is stored as ferritin and ultimately lost when the cell is sloughed at the villus tip (Figure 1).

**Iron transport**

After iron absorption, the transport of iron across the basolateral membrane of the enterocyte into the systemic circulation is facilitated by the transport protein ferroportin 1 (in Figure 1 abbreviated as Ireg-1) and the ferroxidase hephestin. Ferroportin 1 also mediates export of iron from other cells, including macrophages. Ferroportin 1 is upregulated in response to ID. Hephestin is responsible for oxidizing ferrous iron back to ferric iron before it can be incorporated into transferrin. Transferrin is the major transporter for iron trafficking through plasma (Figure 1).

**Figure 1 Iron absorption and transport across the enterocyte**


Abbreviations: Fe3+, ferric iron; DCYT2, duodenal cytochrome B; Fe2+, ferrous iron; DMT-1, divalent metal iron transporter 1; HCP-1, heme carrier protein 1; Ireg-1, ferroportin 1.
Once entered into the systemic circulation, transferrin binds to specific transferrin receptors expressed on all iron-requiring cells and tissues. The majority of iron is transported to the bone marrow where it is used for erythropoiesis. Furthermore, some iron is stored in the liver in hepatocytes as ferritin or hemosiderin. Iron from senescent erythrocytes is recycled by macrophages.4,5

Hepcidin: key regulator of iron-homeostasis
Hepcidin, a peptide hormone synthesized primarily in the liver, is the key regulator of iron-homeostasis. The synthesis of hepcidin is stimulated by iron overload, infection and inflammation, whereas hepcidin synthesis decreases in case of ID, anemia and hypoxia. Hepcidin binds to and induces the degradation of ferroportin 1 on enterocytes and macrophages leading to decreased iron transfer from the basolateral membrane of the enterocyte and less iron release from macrophages into the systemic circulation, respectively. Subsequently, hepcidin increases iron retention in cells of the reticulo-endothelial system and hereby limits the iron-availability in the systemic circulation for iron-requiring cells.4,5

2.2 Iron deficiency: definition and types
ID is defined as a condition in which there are no mobilizable iron stores and in which signs of a compromised supply of iron to tissues, including the erythron, are noted.2 This definition embraces two types of ID: absolute ID and functional ID.

Absolute ID
Absolute ID refers to depleted iron stores due to increased demands, insufficient dietary intake, malabsorption and/or chronic blood loss (Table 1). When the iron stores are fully depleted and iron supply is insufficient for Hb synthesis, IDA occurs.4

Functional ID
In functional ID, inflammatory mediators (especially interleukin-6) induce changes in iron-homeostasis by upregulating hepcidin expression. Iron stores can be adequate, but there is limited iron available for the erythropoiesis since export of iron from the enterocytes and macrophages into the systemic circulation is inhibited. Functional ID is therefore also frequently called iron-restricted erythropoiesis. In theory, this is the optimal situation in case of an infection: increased hepcidin levels result in a low plasma iron content and subsequently reduction of microbial growth. On the other hand, this mechanism predisposes patients with chronic infections and/or inflammation to functional ID.4
Furthermore, treatment with erythropoiesis-stimulating agents such as erythropoietin (EPO) (e.g. in patients with renal insufficiency or cancer) can also cause functional ID (Table 1). In treated patients, iron stores may be available but their release into the systemic circulation may not be rapid enough to support the increased erythropoietic rate. Ultimately, functional ID can lead to anemia of chronic disease (ACD).

<table>
<thead>
<tr>
<th>Table 1 Causes of iron deficiency</th>
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<td><strong>Absolute iron deficiency</strong></td>
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<td><strong>Physiological</strong></td>
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<td>Increased demand</td>
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<td>Infancy (especially preterm infants)</td>
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<td>Rapid growth</td>
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<td>Menstrual blood loss</td>
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<td>(Frequent) phlebotomies</td>
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<td><strong>Environmental</strong></td>
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<td>Insufficient intake</td>
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<td>Poverty</td>
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<td>Malnutrition</td>
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<td>Specific diets (vegan, vegetarian)</td>
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<td><strong>Pathological</strong></td>
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<td>Decreased absorption</td>
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<td>Celiac disease</td>
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<td>Inflammatory bowel disease</td>
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<td>Malabsorption syndromes</td>
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<td>Chronic blood loss</td>
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<td><strong>Functional iron deficiency</strong></td>
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<td>Hepcidin production</td>
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<td>Inflammatory conditions</td>
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**2.3 Iron deficiency: diagnostic tests in children**

Diagnosing ID in children is complicated by poorly defined age-specific reference ranges for several iron status biomarkers and the lack of consensus on which biomarkers should be used. Many studies defined ID (in general) as two or more abnormal concentrations in a set of multiple iron status biomarkers including Hb. However, each biomarker represents a different aspect of iron-homeostasis and there is therefore no single standard test to assess ID or to differentiate between absolute ID and functional ID.

The available iron status biomarkers can be divided in subcategories based on their informative capacity regarding iron status: biomarkers reflecting iron stores, biomarkers representing iron availability for the erythropoiesis, and biomarkers representing cellular iron demands. The following paragraphs summarize the available iron status biomarkers by the aforementioned subcategories.

**Biomarkers reflecting iron stores**

**Bone marrow iron staining** is considered to be the ‘gold standard’ for determining iron stores. However, a bone marrow aspiration is an invasive procedure for children that requires general anesthesia and is therefore not recommended for screening. An alternative is to analyze a blood sample to quantify the magnitude of iron stores.²

**Serum ferritin (SF)** is the most specific biomarker in blood reflecting iron stores since it correlates with total body iron stores.² It is important to realize that once iron stores are depleted, SF does not quantitatively reflect further reduction of tissue iron. The generally accepted cut-off levels for SF, established by the WHO, are SF <12 µg/l for children <5 years of age and SF <15 µg/l for children ≥5 years of age.² However, the acute phase response induced by infections and/or inflammation can elevated SF levels, independent of actual iron stores. Consequently, SF levels are only reliable to reflect iron stores in the absence of these conditions. Interpretation of SF levels is therefore problematic in populations in which the incidence of infections or inflammation is high.² Some studies have used higher cut-off levels for SF in patients with infection/inflammation (e.g. 30 or 100µg/l), but these cut-off levels are not evidence-based for the general pediatric population.⁷ Currently, the WHO advises to concurrently measure another acute phase protein to help with the interpretation of SF levels. If the concentration of the additional acute phase protein is higher than the normal threshold, SF should not be considered as a reliable biomarker reflecting iron stores.² Frequently, C-reactive protein (CRP) is chosen as an additional acute phase protein that is easily available in most hospitals.
Instead of SF, some studies measure serum iron, most of which is bound to transport protein transferrin, to investigate iron status. Serum iron concentration is low in absolute ID as well as in functional ID because it depends on both the iron stores and the release of iron from enterocytes and macrophages into the systemic circulation. Serum iron is therefore not a specific biomarker for low iron stores. Furthermore, serum iron has a diurnal pattern, which makes it unsuitable as an iron status indicator, a statement that is underlined by the WHO.2

Another option is to measure transferrin concentrations. Transferrin can be measured directly, or be reported as the total iron binding capacity (TIBC). The transferrin concentration (in mg/dl) can be converted to the TIBC (in µg/dl) by multiplying by 1.389. Subsequently, transferrin saturation is the ratio of serum iron to TIBC (serum iron / TIBC x 100%). In case of absolute and/or functional ID, serum iron is reduced and TIBC is increased, resulting in a lower transferrin saturation. A transferrin saturation below 16% is generally used as an indicator of ID. However, as previously mentioned, serum iron related biomarkers are not recommended by the WHO because of the diurnal variation of serum iron.2

Biomarkers representing iron availability for the erythropoiesis
Zinc protoporphyrin, a normal metabolite that is formed in trace amounts during heme biosynthesis, can be analyzed to investigate iron availability for the erythropoiesis. The final reaction in the biosynthetic pathway of heme is the chelation of iron with protoporphyrin. During periods of less iron availability, zinc becomes an alternative metal substrate for ferrochelatase (i.e. zinc replaces the missing iron during formation of the protoporphyrin IX ring in heme), leading to an increased zinc protoporphyrin/heme ratio (ZnPP/H).8-13 Most of these cited studies have focused on the use of ZnPP/H in adult patients. Limited evidence exists for the use of ZnPP/H in detecting iron availability for the erythropoiesis in children13, although the WHO recommends the following cut-off levels: ZnPP/H > 61 µmol/mol heme for children <5 years of age and ZnPP/H > 70 µmol/mol heme for children ≥ 5 years of age.2

Hematofluorometry is the fastest and easiest method of determining ZnPP/H in blood specimens. Plasma interference, most of which is attributable to bilirubin, can give falsely increased values. Other potentially interfering substances and situations include the use of certain medication, high plasma vitamin B2 concentrations and lead poisoning.12 A common solution to eliminate most of the aforementioned interference requires washing of the plasma12, as performed in the studies presented in the following chapters.
In addition to the measurement of (or some of) the aforementioned traditional iron status biomarkers, it is common practice to concurrently analyze a complete blood cell count, i.a. to investigate the presence of anemia. **Hemoglobin (Hb)** is the iron-containing oxygen transport protein in erythrocytes. The WHO advises gender- and age-specific cut-off levels for Hb because normal Hb distributions vary with age and gender.\(^2\)

**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. It decreases within days after onset of iron-deficient erythropoiesis. However, a recent Dutch study showed a limited value of Ret-Hb in detecting iron depletion following WHO criteria for SF in young children in a high-resource country with a low prevalence of IDA.\(^14\)

**The mean corpuscular volume (MCV)** is an indicator of the average erythrocyte volume and can be used to calculate the red blood cell distribution width.

**Red blood cell distribution width (RDW)** reflects the degree of heterogeneity of erythrocyte volume, conventionally known as anisocytosis. In case of inadequate iron supply, erythrocytes become smaller and show a larger variation in size and subsequently a higher RDW level. On the other hand, the presence of macrocytosis, such as in the case of folate or vitamin B12 deficiency, can also cause an increased RDW level.\(^15\) From a hematological perspective, RDW is nowadays mostly used in anemic adults to differentiate between β-thalassemia and ID. As a general rule, anemia caused by nutritional deficiencies tend to be associated with a greater degree of anisocytosis than anemia caused by genetic defects or primary bone marrow disorders.\(^15\) In children, limited evidence exists for the use of RDW in investigating iron availability for the erythropoiesis.\(^16\)\(^21\) RDW is part of routine red blood cell measurements in laboratories using automated hematology analyzers and could therefore be easily used as a screening tool for ID. Depending on the type of analyzer, RDW can be reported as coefficient of variation (CV) (RDW-CV) or as standard deviation (SD) (RDW-SD). RDW-SD (expressed in femtoliter) is an actual measurement of the width of the erythrocyte size distribution histogram at a certain height level (depending on the type of analyzer, varying from 20 to 50%). In contrast, RDW-CV (expressed in %) is calculated by the following equation: \(1 \text{ SD of erythrocyte volume} / \text{MCV} \times 100\). RDW-CV is mathematically derived from MCV and it is therefore affected by the average erythrocyte size.\(^15\) Studies investigating cut-off levels for both types of RDW to indicate ID in children are scarce, and, furthermore, one should realize that these cut-off levels are instrument-specific.\(^15\)
Biomarkers representing cellular iron demands

Soluble transferrin receptor (sTfR) is a circulating protein derived from cleavage of the membrane transferrin receptor on iron-requiring cells. sTfR reflects cellular iron demands and erythropoietic activity. It can estimate the magnitude of iron deficit once iron stores are depleted and indicate the presence of functional ID. An important note is that sTfR increases only after severe depletion of iron stores and/or high hepcidin concentrations. The diagnostic capacity of sTfR in high-resource countries with moderate ID (minimal IDA) and a low infection pressure is unclear. Previous studies performed in the Netherlands showed that the discriminative value of sTfR for the detection of iron depletion following WHO criteria for SF in young children and pediatric cystic fibrosis (CF) patients is limited.

The sTfR-ferritin index is calculated as the ratio of the sTfR (in mg/l) to the logarithm of SF (in µg/l) (sTfR / log(SF)) and was designed to evaluate changes in both body iron stores and functional iron. It was thought to be more useful than sTfR or SF alone, although an analysis of 4 intervention studies showed no clear advantage of the sTfR-ferritin index over SF alone.

Differentiating between absolute ID and functional ID: why and how

The differentiation between absolute ID and functional ID is important because of therapeutic consequences. Children with absolute ID (with or without anemia) should be treated with iron replacement therapy, whereas in functional ID, underlying infection/inflammation should be treated before considering iron supplementation.

As previously mentioned, SF, in the absence of signs of an acute infection/inflammation, is the recommended and most specific biomarker for absolute ID. In the presence of chronic infections/inflammation, biomarkers such as RDW and ZnPP/H are easily available and can be used to detect iron-restricted erythropoiesis leading to functional ID. However, more studies are necessary to calculate cut-off levels for these biomarkers indicating ID, especially in children with moderate ID living in high-resource countries. Finally, limited evidence exists for the use of hepcidin in differentiating between absolute ID and functional ID. Hepcidin assays are available only in research settings whereas standardization is lacking. Furthermore, age-specific reference ranges for children, and knowledge on the association between hepcidin and other iron status biomarkers in children, are also lacking, limiting the present use of hepcidin as a biomarker for functional ID in the pediatric population.
2.4 Iron deficiency: risk factors in children
In general, risk factors for ID in children differ between populations. For example, a non-Caucasian race and a low socioeconomic status are both associated with an increased risk of absolute ID. More specific risk factors for ID in children can be divided in the following subcategories: perinatal risk factors, growth, diet-related risk factors, day care, blood loss and chronic disorders. The following paragraphs summarize risk factors for ID in children by the aforementioned subcategories.

Perinatal risk factors
Fetal iron accretion via the placenta, depending on maternal iron status, predominantly occurs in the third trimester of the pregnancy. Therefore, preterm infants and infants born to iron deficient mothers are prone to develop absolute ID. Furthermore, placental iron transport can also be negatively influenced by maternal hypertension, maternal smoking or pregnancy-induced diabetes. In healthy term infants born to healthy and iron sufficient mothers, iron stores at birth are considered to be sufficient to cover the iron demands for growth during the first 4 to 6 months of life. After this age, next to breast milk and/or formula, complementary food containing sufficient amounts of iron is necessary to cover the iron demands for further growth and development of young children.

Growth
In periods of rapid growth, such as after birth, the toddler period, and the growth spurt in teenagers, iron demands are increased due to high growth velocities and a high rate of development. For example, there is growth of bone and muscle and, consequently, expansion of total blood volume requiring use of stored iron.

Diet-related risk factors
The European Food Safety Authority (EFSA) has established iron requirements for children based on their age and gender. The Estimated Average Requirement (EAR) for iron, defined as the average daily iron intake level estimated to meet the requirement of half the healthy individuals in a particular life stage and gender group, varies from 5 mg/day in infants aged 7-11 months to 8 mg/day in boys aged 12-17 years. For girls aged 12-17 years, the Population Reference Intake (PRI), calculated as the requirement at the 97.5th percentile, is estimated at 13 mg/day. However, a recent study revealed that most young European children do not reach the aforementioned requirements and are therefore at risk to develop absolute ID. The use or non-use of iron-rich food products or food products containing iron absorption inhibiting and/or enhancing components influence iron status of children.
In infants and young children, the received type of milk influences their iron intake and subsequent iron status. For example, breast milk has a relatively low iron content that declines with increasing age from 0.5-0.6 mg/l at two weeks of lactation to approximately 0.3 mg/l at the age of 5 months. However, iron in breast milk is easily absorbed because it is mainly bound to lactoferrin, which facilitates intestinal iron absorption via the lactoferrin receptor into the enterocyte. In European preterm infants receiving breast milk, adding breast milk fortifier is recommended although these do not always contain iron. In contrast, cow’s milk (CM) derived formula, either specific for preterm infants or standard formula for term infants, has a higher iron content than breast milk. In Europe, infant formula fortification guidelines differ per country but most commercially available standard infant formulas for term infants subscribe to the European Society for Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) standard and contain 4-8 mg iron per liter. Follow-on formulas for infants older than 6 months of age usually contain even more iron. However, the iron bioavailability in CM derived formula is much lower than that of iron in breast milk. After the age of 1 year, the use of regular CM, instead of, for example, young child formula (YCF) (that contains more iron than CM), is broadly accepted. The negative effect of CM consumption on iron status is attributed to the composition of CM with its low iron content, and the inhibitory effect of calcium on non-heme iron absorption.

Besides type of milk, it is well known that an unbalanced diet can contribute to absolute ID. For example, it has been suggested that vegetarians are more prone to develop absolute ID than omnivores, although some research shows that eliminating meat leads to a higher intake of whole-grain and fortified cereals, legumes, nuts, seeds and dried fruit, and subsequently a higher iron intake than in omnivores. The poorer iron status observed in vegetarians may be due, at least partly, to the lower bioavailability of non-heme iron in the aforementioned food products.

Day care
Young children staying at home, instead of attending day care, may be at a higher risk to develop absolute ID. It is thought that children maybe eat more (or food of better nutritional quality) at preschool/day care because of meals that are collectively used on structural moments with more time and attention for eating.
Blood loss
Specific pediatric populations encounter regular blood losses that make them prone to absolute ID. For example, infants born prematurely are susceptible to absolute ID because of frequent phlebotomies during the first weeks of life, e.g. to analyze blood glucose levels and bilirubin levels.\textsuperscript{26} Another example is an adolescent girl whose menstrual blood loss predisposes her to absolute ID. It is estimated that 30-40 ml of blood is lost in each menstruation leading to a loss of approximately 15-30 mg iron per cycle.\textsuperscript{27} Other examples are gastro-intestinal (GI) blood loss in patients with GI-tract disorders and infections such as inflammatory bowel disease (IBD) and Helicobacter Pylori and hookworm infection.\textsuperscript{2}

Chronic disorders
Risk factors for functional ID include chronic disorders that evolve from the activation of the immune system leading to hepcidin expression. These disorders include mainly chronic infections, (hematological) malignancies and chronic inflammatory (auto-immune) disorders (Figure 2).\textsuperscript{4,35} Currently, it is not known to what extent certain chronic disorders like IBD and diabetes mellitus (DM) type 1 influence functional iron status in pediatric patients. Furthermore, obesity may also be associated with mild functional ID because of subclinical inflammation with subsequent increased hepcidin levels. It is thought that adipose tissue-derived cytokines stimulate hepcidin expression in the liver, and, furthermore, also direct expression of hepcidin in adipose tissue can lead to functional ID.\textsuperscript{36,37} All these and the aforementioned risk factors for absolute ID and functional ID in children are summarized in Figure 2.
2.5 Iron deficiency: consequences in children

ID in childhood is associated with many adverse effects depending on the age of the child and the severity and progress of the deficiency. In general, a fast progressive deprived iron status, like acute blood loss, causes short-term symptoms while a more slowly developing ID can remain asymptomatic for some time. ID can cause symptoms of fatigue and paleness which can worsen to a reduced exercise capacity, cardiac palpitations and dizziness in the case of anemia.\(^{38}\) Furthermore, the development and maturing of the central nervous system (CNS) 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, especially when ID occurs during the brain growth spurt in early infancy. Animal and human studies supporting this are summarized below.

Neurodevelopmental effects of ID in animal models

Induction of early ID in rats has been shown to directly affect oligodendrocytes.
These cells form myelin, the fatty acid sheath around axons that enables fast neural signal transmission. Insufficient myelinisation can impair neural signal transmission speed and subsequent several neurologic functions. Furthermore, early ID also alters brain metabolism and morphology as has been studied in the hippocampal formation. The hippocampus is involved in recognition memory and other important cognitive and emotional functions. It has been observed that early ID decreases neuronal metabolism, dendritic growth and arborization, and synapse formation. Finally, animal studies also show short- and long-term effects of ID on gene and protein profiles and several neurotransmitter systems. In summary, ID in animal models has shown multiple and varied effects on the CNS of animals.39

Neurodevelopmental effects of ID in children
The presence of ID or IDA in infants and young children is associated with impaired neuro-cognitive development indicated by disturbed cognitive, motor, and/or social-emotional functioning at a later age.40-74 For example, compared with non-anemic infants, those with IDA show deprived auditory brainstem responses, altered rapid eye movement density in active sleep, poorer recognition memory with event-related potentials and altered electroencephalographic frontal asymmetry.39 Most information on outcome beyond early childhood comes from a longitudinal study performed in Costa Rica. By early adolescence, Costa Rican children with chronic, severe ID in infancy had repeated a grade in school and they did not catch up in motor performance to their iron sufficient peers. Moreover, their mothers and teachers reported them to have more anxiety/depression, social problems and inattention. At 19 years, they did worse on neuro-cognitive tests of executive function and recognition memory. Recently, functional outcomes at 25 years of age related to education, health and close relationships were reported. Previously iron deficient adults showed a lower educational level and they report poorer emotional health and negative emotions and feelings of dissociation/detachment.73 Studies focusing on the long-term neuro-cognitive functioning of iron deficient children in developed countries like in Western-Europe with mostly moderate deficiencies are scarce. However, overall, it can be concluded that the presence of ID or IDA in both animals and humans has been linked with several negative effects on the CNS.

Other effects of ID in children
Besides the previously mentioned general symptoms of ID, and the diverse effects of ID on the CNS, ID and IDA in children are also associated with growth retardation2, breath-holding spells75, febrile convulsions75, restless legs75 and an impaired immune response2,75.
The presence of ID or anemia may also influence Hb-related biomarkers like hemoglobin A1c (HbA1c). HbA1c reflects the amount of glycated Hb and is used in the diagnostic process and treatment of patients with diabetes. An altered erythrocyte lifespan, in particular due to anemia, is thought to be an important confounder in the use of HbA1c in patients with diabetes. Studies in adult patients with diabetes suggest that ID per se may cause elevated HbA1c levels, irrespective of the presence of anemia.76 In pediatric DM type 1 patients, information about the influence of iron status on HbA1c levels is scarce and incomplete. Only two small studies with children with DM type 1 revealed higher HbA1c levels in patients with IDA than in those without IDA. These HbA1c levels decreased significantly after iron replacement therapy.77,78

2.6 Iron deficiency: prevention and treatment in children
The aim of treatment of absolute ID and IDA is to replenish iron stores and to supply enough iron to normalize Hb concentrations.38 In Europe, ferrous fumarate is the most frequently prescribed oral preparation. The Dutch pediatric medication standard advises ferrous fumarate 9 mg/kg/day (= 3 mg Fe) during at least two months until the iron status (and the Hb level) have normalized.79 At risk-populations, such as preterm infants and patients with IBD, are frequently screened for ID and subsequently treated. In contrast, and as explained before, patients with functional ID or ACD should not be treated with iron replacement therapy.4

Besides screening at-risk populations and active iron replacement therapy in patients with confirmed absolute ID or IDA, several nutritional prevention strategies exist. On a global level, the WHO recommends the promotion of access to, and consumption of, iron-rich food products such as meat and organs from cattle, fowl, fish, and poultry, and non-animal food products such as legumes and green leafy vegetables.38 These approaches, however, should also take into account that the bioavailability of iron depends on the composition of the diet as explained before. Advice about the composition of the diet, for example, drinking orange juice instead of milk with a meal, can help with maintaining or improving iron status. Furthermore, fortification of commonly used food products such as formula or cereals is another nutritional prevention strategy to improve the iron status of populations.38 Several international trials have shown beneficial effects of milk fortification on iron status in children.80-89 Unfortunately, adequate studies regarding this strategy in West-European children are lacking. Finally, there is no general recommendation regarding iron supplementation since supplementation in iron-replete children may have adverse effects such as increased risk of infection.90
3. VITAMIN D

3.1 Vitamin D-homeostasis: absorption, conversion, transport and key regulators

Vitamin D is a micronutrient, but can also be synthesized in the human skin by sunlight exposure. The main functions of vitamin D are the regulation of calcium, phosphate, and parathyroid hormone (PTH) metabolism and subsequent bone health.91-93

Vitamin D absorption, conversion and transport

There are three sources of vitamin D: ultraviolet B (UVB) radiation-dependent endogenous production, dietary supplements and other nutritional sources such as fatty fish, liver and organ meats, egg yolk, certain fungi and cod liver oil. UVB radiation-dependent vitamin D production starts with the non-enzymatically conversion of 7-dehydrocholesterol to bio-inactive previtamin D called cholecalciferol (vitamin D3) in the epidermal layer of the skin. Dietary vitamin D, either from supplements or from other food products, usually contains cholecalciferol or ergocalciferol (vitamin D2). These two forms of so-called previtamin D are incorporated into micelles in the intestine before absorption by enterocytes occurs. Subsequently, they are packed into chylomicrons entering the systemic circulation.

To become bioactive, cholecalciferol and ergocalciferol bound to vitamin D-binding protein undergo two modifications. Firstly, they are converted enzymatically in the liver to calcidiol (25-hydroxyvitamin D) (25(OH)D). Secondly, calcidiol is enzymatically converted to bioactive calcitriol (1,25-dihydroxyvitamin D) (1,25(OH)2D) in the kidney. In the systemic circulation, calcitriol binds to a vitamin D receptor (VDR) on nucleated cells of target tissues where it regulates mineral metabolism, bone resorption, gene transcription, and immune system functioning (Figure 3).
Figure 3 Pathways of vitamin D synthesis and its effects on minerals and bone

Abbreviations: UVB, ultraviolet B; Ca\(^{2+}\), calcium; PO\(_4\)^{3-}, phosphate; PTH, parathyroid hormone; FGF-23, fibroblast growth factor-23.

Regulators of vitamin D-homeostasis

The aforementioned and illustrated effects of calcitriol require a tight regulation of vitamin D-homeostasis that occurs through a series of negative and positive feedback loops resulting in changes in the expression of renal hydroxylase enzymes involved in converting calcidiol to bioactive calcitriol. A low calcium, phosphate and/or PTH concentration results in increased conversion of calcidiol into calcitriol. In turn, calcitriol suppresses PTH production and negatively
regulates its own production to protect against hypercalcemia. Furthermore, fibroblast growth factor 23 (FGF-23) produced in bone is also a regulator of vitamin D-homeostasis. FGF-23, stimulated by calcitriol, down-regulates specific transporters in the kidney leading to reduced renal phosphate reabsorption. Moreover, FGF-23 down-regulates renal hydroxylase enzymes leading to a decreased production of calcitriol forming a negative feedback circuit between the FGF-23 and the vitamin D endocrine system. Finally, FGF-23 directly inhibits PTH synthesis and hereby also causes a decreased production of calcitriol (Figure 3).

3.2 Vitamin D deficiency: definition
The terminology for “low vitamin D status” is various and includes deficiency, insufficiency, inadequacy, and hypovitaminosis of vitamin D. In this chapter, and in the following chapters, we use the terminology “vitamin D deficiency” (VDD) that embraces a spectrum of low vitamin D status with different severity regarding bone health and other consequences. Classically, overt VDD is characterized by hypocalcemia and/or hypophosphatemia and rickets and/or osteomalacia in children. However, not every VDD leads to the aforementioned clinical and laboratory findings in children.

3.3 Vitamin D deficiency: diagnostic tests in children
Among the various forms of (pre)vitamin D described before, the level of bio-inactive calcidiol is considered to be the best indicator of vitamin D status. Calcidiol is the major circulating form of vitamin D and has a half-life of two to three weeks. In contrast, bioactive calcitriol has a much shorter half-life of only four hours and it circulates in much lower concentrations than calcidiol. Furthermore, calcitriol is also susceptible to fluctuations induced by PTH in response to subtle changes in calcium concentration thus limiting the reliable use of calcitriol as a biomarker for vitamin D status.

Reference ranges and subsequent cut-off levels for calcidiol indicating deficiency are difficult to establish because of assay variability and lack of agreement regarding the definition of a ‘normal’ population. Furthermore, defining reference ranges and cut-off levels for calcidiol may also be complicated by the need for different levels for various tissues and endpoints. For example, the cut-off level for non-classical targets of vitamin D such as the immune system might vary from that for bone. Moreover, it is also likely that the optimal calcidiol level varies between individuals. Despite the aforementioned arguments, most vitamin D experts and health organizations and societies such as The American Academy of...
Pediatrics, the Institute of Medicine and the ESPGHAN suggest 50 nmol/l (20 ng/ml) as the best cut-off level for calcidiol indicating VDD.\textsuperscript{91-93,96} This cut-off level is based on data that considered clinical, radiological and laboratory findings such as bone density, calcium absorption and the presence of rickets.\textsuperscript{93}

### 3.4 Vitamin D deficiency: risk factors in children

Aging negatively influences the amount of 7-dehydrocholesterol in humans and subsequently limits UVB-dependent cutaneous production of cholecalciferol.\textsuperscript{96} More specific risk factors for VDD in children can be divided in the following subcategories: perinatal risk factors, sun exposure, diet-related risk factors including vitamin D supplementation, and pathological conditions and medication. The following paragraphs summarize risk factors for VDD in children by the aforementioned subcategories.

#### Perinatal risk factors

VDD in (future) mothers can cause fetal VDD and subsequent fetal rickets because of limited transplacental transfer of vitamin D from the mother to the unborn fetus.\textsuperscript{96} Furthermore, maternal VDD has been linked to pre-eclampsia (new onset hypertension and proteinuria during pregnancy) that can lead to the premature birth of a fetus.\textsuperscript{97} These preterm born infants are more prone to develop VDD than term infants because they have less time to accumulate vitamin D from their mother.\textsuperscript{96}

#### Sun exposure

The angle of the sun (including the factors that influence this angle such as altitude and local date and time), the intensity of the sun (i.a. influenced by the weather), and skin pigmentation (melanin absorbs UVB radiation) and/or protection (by sunscreen or clothing) all influence the UVB-dependent cutaneous production of cholecalciferol.\textsuperscript{96,98}

#### Diet-related risk factors including vitamin D supplementation

EFSA has established vitamin D requirements for children based on their age. The adequate intake (AI) for vitamin D is set at 10 µg/day and 15 µg/day for infants aged 7-11 months and children aged 1-17 years, respectively.\textsuperscript{29} However, most young European children do not reach these intakes and are therefore at risk to develop VDD.\textsuperscript{29} The use or non-use of vitamin D-rich food products, vitamin D fortified products and/or vitamin D supplementation influence dietary intake of vitamin D and subsequent status of children.
In infants and young children, the received type of milk influences their vitamin D intake and subsequent vitamin D status. For example, the vitamin D content of breast milk, even of a healthy and vitamin D sufficient mother, is relatively low ranging from 0.25 to 2.0 µg/l. In contrast, CM derived infant formulas have a higher vitamin D content although not sufficient to prevent VDD in every infant. After the age of 1 year, the use of regular CM, instead of, for example, YCF (that contains more vitamin D than CM), is accepted. Its use in Europe, where regular CM is not fortified with vitamin D, in contrast to the United States and Canada, can lead to VDD.

Vitamin D-rich food products are products not typically consumed by infants and young children consistently such as fatty fish, cod liver oil, and organ meats. Therefore, some dairy products like butter and certain cereals are fortified with vitamin D although not every child consumes these products.

To achieve an adequate vitamin D intake and subsequent status in infants and toddlers, the governments of most European countries advice the use of vitamin D supplementation. The ‘protective effect’ of vitamin D supplementation on VDD (rickets) has been extensively reported. Guidelines regarding specific indications for vitamin D supplementation (like age and skin pigmentation), the exact dosage, and time differ between countries. Unfortunately, compliance to these guidelines seems to be low.

Pathological conditions and medication
Several medical conditions increase the risk of VDD such as obesity leading to the sequestration of vitamin D in adipose tissue, and conditions that impair fat absorption like celiac disease (CD), IBD and CF. Furthermore, children with certain genetic disorders, liver and/or kidney disease may be prone to develop VDD when the disease is accompanied with deficient converting enzymes.

The use of certain medication (e.g. anticonvulsants, antifungal agents, antiretroviral drugs, and glucocorticosteroids) increases the risk of VDD because these drugs precipitate the conversion of previtamin D into bioactive vitamin D. All these and the aforementioned risk factors for VDD in children are summarized in Figure 4.
**3.5 Vitamin D deficiency: consequences in children**

VDD in childhood is associated with many adverse effects although not all reveal clear symptoms at an early stage. The adverse effects of VDD can be divided in skeletal and non-skeletal effects and are summarized below by subcategories of human tissue containing a VDR.

**Bone: rickets, osteomalacia and fractures**

The most frequently reported and widely known consequences of VDD are the development of rickets in growing children and osteomalacia in adolescents and adults. Rickets refers to a failure of mineralization of growing bone and cartilage and, depending on the severity, a child may be asymptomatic, or present with varying degrees of pain, irritability, motor delays, poor growth, and increased susceptibility to infections. Younger children may manifest with delayed closure of fontanelles, craniotabes (thinning of the skull), frontal bossing, prominence of costochondral junctions, widening of wrists and ankles, and bow legs or knock knees (genu valgum or varum). In older adolescents and adults, growth is complete, epiphyseal plates are fused, and there is usually some degree of mineralization, all of which help prevent bony deformities. Therefore, mildly impaired mineralization in older children and adults causes osteomalacia, which may be asymptomatic or manifest as isolated or generalized muscle and bone...
pain. Furthermore, several studies observed that VDD or vitamin D insufficiency (depending on the chosen cut-off level for 25(OH)D) in children and adolescents was associated with lower bone mineral density (BMD) which can lead to an increased risk for fractures.96

Muscle: disturbed function and osteoporosis
Vitamin D may have a direct and indirect effect on muscle tissue. VDR knockout mice and vitamin D deficient animals show significant defects in muscle function and development. Adequate muscle mass accrual is essential for the attainment of peak bone mass that may be associated with a lower risk of osteoporosis in later life.96

Immune system cells: impaired function, infections and auto-immune diseases
Vitamin D has various effects on the innate and adaptive immune system. For example, vitamin D enhances chemotaxis and phagocytic capabilities of cells of the innate immune system (i.e. macrophages and monocytes). Furthermore, the calcitriol-VDR complex has proven to directly activate the transcription of antimicrobial peptides such as defensin β2 and cathelicidin that cause destabilization of microbial membranes.105 These mechanisms can predispose vitamin D deficient children to infections.96

Antigen presenting cells such as dendritic cells (DCs) are responsible for the initiation of the adaptive immune response. They present antigens to T-cells and B-cells and are able to modulate them by either immunogenic or tolerogenic signals such as cytokines and expression of co-stimulatory molecules. Vitamin D can alter the function and morphology of DCs to induce a more tolerogenic, immature state. Subsequently, in case of VDD, there is increased production of pro-inflammatory cytokines that can eventually stimulate auto-reactivity that can lead to the development of auto-immune diseases. Furthermore, vitamin D also directly effects T-cells and B-cells. For example, vitamin D influences B-cell homeostasis and controls B-cell activation. This is clinically relevant for the pathogenesis of auto-immune diseases since B-cells can produce auto-reactive antibodies.105 This explains why observational studies have linked improved vitamin D status with a reduced risk for DM type 1, multiple sclerosis, Crohn’s disease, and rheumatoid arthritis.96

Other effects of VDD
Although rare in children, VDD has been linked to cardiovascular disease (i.a. hypertension, myocardial infarction, stroke and cardiac mortality), several cancers (including pancreatic, colon and breast), and psychiatric and neurological disorders (i.a. neurocognitive decline, schizophrenia and depression).96
3.6 Vitamin D deficiency: prevention and treatment in children

The aim of treatment of VDD is to normalize 25(OH)D levels. Several preparations (cholecalciferol and ergocalciferol) with different dosages depending on (gestational) age and possible underlying conditions and medication use exist. At risk-populations, such as preterm infants and patients with CF and IBD, are frequently screened for VDD and subsequently treated.

Besides screening at-risk populations and active vitamin D replacement therapy in patients with confirmed VDD, several prevention strategies, mainly to increase dietary vitamin D intake, exist. Directly after birth and during the first years of life, international guidelines recommend the use of vitamin D supplementation. As previously mentioned, these guidelines differ between countries on several aspects. In Western-Europe, supplementation policies recommend vitamin D varying from 7 to 20 µg per day for children until the age of 5 years. Low compliance to vitamin D supplementation has led to the recommendation of fortification of commonly used food products such as milk. Several international trials have shown beneficial effects of food fortification (i.e. milk, bread and margarine) on vitamin D status. Unfortunately, adequate studies regarding this strategy in West-European children are lacking.

Finally, since the cutaneous production of cholecalciferol depends on several factors that differ during the year, throughout the world and per person, there are no universal recommendations regarding how much sun exposure is required to maintain an adequate vitamin D status. Furthermore, the avoidance of intense sun exposure and the use of sunscreen should be promoted and stimulated, particular in young children, to prevent sun exposure-associated skin cancer.

4. IRON AND VITAMIN D DEFICIENCY

4.1 Multiple micronutrient deficiencies: poor diet, limited absorption and micronutrient interaction

Multiple micronutrient deficiencies often occur simultaneously as a result of a poor-quality diet. Furthermore, GI-tract infections and/or disorders can limit the intestinal absorption of many micronutrients. Finally, micronutrients can also interact with each other in such a way that inadequate intake of one micronutrient can negatively influence the absorption, conversion, and homeostasis of other micronutrients.
The co-existence of both ID and VDD in children has only been previously described in Korean, Indian and Jordanian infants, toddlers, older children and adolescents.\textsuperscript{114-118} Several specific mechanisms for the co-existence of ID and VDD have been proposed and are summarized in the following two paragraphs.

4.2 Iron deficiency leading to vitamin D deficiency

It is known that ID impairs the intestinal absorption of fat and fat-soluble vitamin A and thereby maybe also the absorption of fat-soluble vitamin D. Furthermore, iron is a co-factor for the enzyme 1α-hydroxylase which is responsible for the conversion of calcidiol into calcitriol.\textsuperscript{119} These mechanisms can (partially) explain how ID can lead to VDD in children.

4.3 Vitamin D deficiency leading to iron deficiency

As previously explained, VDD can impair immune system functioning and subsequently increase the likelihood of an infection. As a consequence, microorganisms could ‘consume’ iron and hereby cause absolute ID. Furthermore, VDD can also lead to the development of functional ID. The underlying mechanism may be the lack of direct suppression of hepcidin transcription and the lack of reduction of pro-inflammatory cytokines by vitamin D.\textsuperscript{120} Moreover, vitamin D has been shown to support erythropoiesis by increasing burst-forming unit-erythroid proliferation and having a synergistic effect with EPO to further enhance erythroid progenitor cell proliferation.\textsuperscript{120}

In addition to vitamin D, other ‘hormones’ involved in the bone-mineral axis, including FGF-23 and PTH that are also influenced by vitamin D status, have been shown to be involved in iron-homeostasis and erythropoiesis. In contrast to vitamin D, FGF-23 and PTH are negative regulators of iron-homeostasis and erythropoiesis. FGF-23 decreases red blood cell counts and EPO concentrations in mice.\textsuperscript{120} Furthermore, PTH causes unfavorable alterations in erythropoiesis and fibrosis of the bone marrow leading to the development of anemia.\textsuperscript{120} The aforementioned mechanisms influencing iron and vitamin D status are illustrated in Figure 5.
**Figure 5 Pathways leading from ID to VDD and vice versa**

This figure illustrates the proposed mechanisms for the co-existence of both iron deficiency and vitamin D deficiency in children.

**Abbreviations:** ID, iron deficiency; IDA, iron deficiency anemia; VDD, vitamin D deficiency; 25(OH)D, 25-hydroxyvitamin D; 1,25(OH)2D, 1,25-dihydroxyvitamin D3; EPO, erythropoietin; FGF-23, fibroblast growth factor-23; PTH, parathyroid hormone.

### 5. THIS THESIS

#### 5.1 Aim

The aim of this thesis was to investigate the iron and vitamin D status of children living in Western-Europe. The studies described in this thesis focused on three aspects of iron and vitamin D status: diagnostic tests for assessing iron status (part I), epidemiological aspects of iron and vitamin D deficiency in young children and different groups of pediatric patients at risk for deficiency (part II), and finally the effect of a micronutrient-fortified YCF as a strategy to prevent ID and VDD in young children living in Western-Europe (part III).

#### 5.2 Part I: Diagnostic tests for assessing iron status

The first step of investigating the iron status of children living in Western-Europe is to define ID in this group. Using SF as a reliable estimate of body iron stores is a widely accepted and recommended alternative by the WHO for an invasive bone marrow aspiration to diagnose absolute ID in children. Diagnosing functional ID in children is more complicated because of the lack of age-specific reference
ranges for several biomarkers, the moderate availability and expensive costs of some biomarkers, and the lack of knowledge on the association between different iron status biomarkers. As previously mentioned, RDW and ZnPP/H are two less expensive and easily available biomarkers although limited evidence exists for their use in West-European children. Therefore, in chapter 2, 3 and 4, we analyzed the diagnostic capacity of RDW and/or ZnPP/H to detect an impaired iron-homeostasis in three different pediatric populations living in the Netherlands: (1) healthy 0.5-3 year old children (chapter 2), (2) children with CF (chapter 3) and (3) moderately preterm infants with a gestational age of 32 to 37 weeks (chapter 4). These populations were specifically chosen since, as previously described, they are at a higher risk for ID with subsequent health consequences and it is therefore highly important to adequately investigate and assess their iron status.

5.3 Part II: Epidemiological aspects of iron and vitamin D deficiency in young children and different groups of pediatric patients at risk for deficiency

As previously described, risk factors for ID and VDD differ between populations and, as a consequence, different strategies may be needed to prevent ID and VDD in different children. It is therefore important to know the prevalence of and specific risk factors for ID and VDD in different children/pediatric patients with an assumed risk of deficiency. With the definitions and biomarkers of part I of this thesis we investigated the iron and/or vitamin D status of several moderately researched children/pediatric patients with an increasing age. Firstly, in chapter 5, we assessed the prevalence of iron depletion (low iron stores) and the corresponding predictive factors in moderately preterm infants at the postnatal age of 6 weeks. The predictive factors can be used in an algorithm for individualized iron supplementation in moderately preterm infants. Secondly, in chapter 6, we determined the prevalence of and risk factors for ID and VDD in healthy 12-36 month old children living in Western-Europe. Besides the two aforementioned primarily healthy children, we also investigated the iron status of two pediatric patient groups: children with IBD (chapter 7) and children with DM type 1 (chapter 8). It is often reported that chronic diseases/inflammatory conditions are frequently accompanied with impaired iron-homeostasis although adequate studies in pediatric patients are scarce. We used the WHO guidelines to define ID while we also differentiated between absolute and functional ID. Possible risk factors for ID such as a high disease activity score or certain disease-related symptoms and/or biomarkers can help a clinician in identifying those patients at risk for ID.
5.4 Part III: Prevention of iron and vitamin D deficiency in young children living in Western-Europe

As previously described, compliance to current nutritional recommendations regarding iron and vitamin D intake, including the use of vitamin D supplements, in young European children is low. This emphasizes the need for a more strict focus on the use of supplements or new policies. A new policy could be the promotion of the use of fortified commonly used food products like milk although this has scarcely been studied in Western-Europe. We therefore performed a study in which healthy young European children were randomly allocated to receive either a micronutrient-fortified YCF or a non-fortified CM during 20 weeks to determine the effect of this strategy on their iron and vitamin D status. The results of the study are presented in chapter 9 and chapter 10.
CHAPTER 1

REFERENCE LIST


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CHAPTER 1


General introduction


