Iron requirements of term, breast-fed infants: A study in Sweden and Honduras

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Iron requirements of term, breast-fed infants:  
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Abstract

Background: Iron deficiency anemia (IDA) is a global public health problem, affecting an estimated 51% of children below 4 years of age in developing countries and 12% in developed countries. There is a well-known association between IDA and delayed neurodevelopment in infants. For many reasons, breast milk is important for the infant, and WHO and other organizations recommend breast-feeding for at least one year. However, due to the low iron content of breast milk and high iron requirements for growth, infants who are breast-fed for longer than 4-6 months need iron from additional sources. This is why in many countries iron supplementation, as iron drops, is recommended to breast-fed infants who do not consume sufficient amounts of iron-fortified foods.

Study design: Since the effects of such supplementation are largely unknown, we performed a randomized, controlled, double-blind study of 263 healthy, term infants who received ferrous sulfate drops (starting at 4 or 6 months) or placebo drops. The infants were exclusively breast-fed to 6 months and partially to 9 months. Swedish (n=121) and Honduran (n=142) infants were studied to allow assessment of the effects of iron supplementation across a wide range in iron status. Blood samples were obtained at 4, 6 and 9 months. Iron absorption was studied in 25 infants, using a stable isotope method.

Results: There was a low prevalence of IDA (< 3%) in Swedish infants at 9 months of age. In Honduras, however, 29% of the infants had IDA at 9 months of age, and this proportion was reduced to 9% by giving prophylactic iron drops from 4 or 6 months. Unexpectedly, iron supplementation significantly reduced longitudinal growth and this effect was more pronounced in Swedish infants. Swedish infants, iron supplemented from 4 months, also showed a significant reduction in head growth. At 6 months, fractional iron absorption from human milk was 16%. At 9 months, absorption was still low in iron supplemented infants but had increased to 37% in unsupplemented infants. Dietary iron intake was shown to be an important negative regulator of iron absorption in these infants. This adaptation of iron absorption may explain why we found no effect of complementary food iron intake on iron status. Boys had a 10-fold higher risk for being diagnosed with IDA. The sex difference could not be explained entirely by differences in birth weight, weight gain or complementary food intake. Hemoglobin (Hb) response to iron was shown to be a poor indicator of IDA at 4 months because iron supplemented infants at this age responded with an increase in Hb regardless of initial iron status. New reference values are presented for iron status variables based on iron-replete, breast-fed infants. For some variables, −2 SD cutoffs at 9 months were significantly lower than conventional cutoffs: Hb < 100 g/L and ferritin < 5 μg/L, instead of Hb < 110 g/L and ferritin < 10-12 μg/L.

Conclusions: Iron supplementation effectively prevents IDA in a population with a high prevalence of this condition. In low-risk or mixed populations, routine iron supplementation of breast-fed infants should be avoided because of possible negative effects on growth. Iron requirements of term, breast-fed, Swedish infants are likely to be lower than previously believed. It is necessary to re-evaluate the laboratory criteria for IDA in infants, especially in relation to clinical symptoms such as impaired neurodevelopment. Since iron deficiency is a global public health problem and since the first year of life is a crucial period for growth and development of the central nervous system, this issue deserves high priority.

Keywords: human infant, iron deficiency anemia, iron supplementation, dietary iron, nutritional requirements, randomized controlled trial, iron status, hemoglobin, MCV, zinc protoporphyrin, ferritin, transferrin receptors, international cooperation, growth, morbidity, sex factors, iron absorption, dietary regulator, stable isotopes, human milk, breast-feeding, reference values, infant nutrition
Iron requirements of term, breast-fed infants: 
A study in Sweden and Honduras

by
Magnus Domellöf

Umeå 2001
Iron seems a simple metal but in its nature are many mysteries...
(Joseph Glanvill, 1636-80)

Gold is for the mistress - silver for the maid -
Copper for the craftsman, cunning at his trade
"Good!!" said the baron, sitting in his hall;
"But Iron – Cold Iron – is master of them all."
(Rudyard Kipling, 1865-1936)

To all the Honduran children who were victims of the hurricane Mitch in October 1998.
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ABSTRACT

Background: Iron deficiency anemia (IDA) is a global public health problem, affecting an estimated 51% of children below 4 years of age in developing countries and 12% in developed countries. There is a well-known association between IDA and delayed neurodevelopment in infants. For many reasons, breast milk is important for the infant, and WHO and other organizations recommend breast-feeding for at least one year. However, due to the low iron content of breast milk and high iron requirements for growth, infants who are breast-fed for longer than 4-6 months need iron from additional sources. This is why in many countries iron supplementation, as iron drops, is recommended to breast-fed infants who do not consume sufficient amounts of iron-fortified foods.

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ORIGINAL PAPERS

This thesis is based on the following original papers, which will be referred to by their Roman numerals:


ABBREVIATIONS

AUC Area under the curve
CRP C-reactive protein
DMT1 Divalent metal transporter 1
EDTA Ethylenediamine tetraacetic acid
Fe Iron
fL Femtoliter (10^{-15} L)
Hb Hemoglobin
HbA Adult hemoglobin
HbF Fetal hemoglobin
ID Iron deficiency
IDA Iron deficiency anemia
MCV Erythrocyte mean cell volume
ROC Receiver operating characteristics
SD Standard deviation
SEM Standard error of the mean
TfR Soluble transferrin receptors
TIBC Total iron binding capacity
WHO World Health Organization
ZPP Zinc protoporphyrin
INTRODUCTION

Iron metabolism

Biological significance of iron

Iron is essential for virtually all living organisms. The most important biological property of this transition metal is its ability to alternate between two oxidation states – ferrous (Fe$^{2+}$) and ferric (Fe$^{3+}$) – thereby donating or accepting one electron. This capability of iron to transfer electrons, together with its great abundance in nature, has probably led to its evolutionary selection for a remarkable array of metabolic reactions. However, due to the poor solubility of ferric iron at physiological pH and the ability of ferrous iron to reduce oxygen intermediates to harmful free radicals, all organisms have developed binding molecules (chelators) in order to transport and store iron and to control its reactivity.\(^1\)

Body iron compartments

Iron in the human body can be divided into three compartments: functional, transport and storage iron.\(^2\)

Functional iron

Most of the body iron is bound in functional compounds. About 90% of the functional iron is found in hemoglobin (Hb), the red pigment of blood, which is essential for oxygen transport from the lungs to all tissues. Consequently, anemia (decreased Hb in blood) is the main finding in iron deficiency (ID). The second most abundant functional iron compound is myoglobin, the red pigment of muscle, which is used for oxygen transport and storage for use during muscle contraction. Functional iron is also present in various tissues in heme enzymes (e.g. cytochromes and peroxidases), non-heme iron compounds (e.g. ribonucleotide reductase) and other iron-dependent enzymes. These enzymes are involved in many fundamental metabolic reactions including oxidative phosphorylation and DNA synthesis.\(^3\)

Transport iron

Iron is transported within the body by the plasma glycoprotein transferrin. One transferrin molecule binds two iron atoms. About 0.1% of body iron is found in this transport compartment, which has a high turnover rate (> 10 times the plasma pool daily). Transferrin is recognized by specific cell membrane receptors (transferrin receptors) and the subsequent receptor-mediated endocytosis is crucial for cellular iron acquisition.

Storage iron

Iron is stored intracellularly in ferritin and hemosiderin, which are located primarily in reticuloendothelial macrophages, in hepatocytes and in erythroid precursors of the bone marrow. Ferritin is a large protein shell made up of 24 subunits, which can accommodate up to 4500 atoms of iron in its internal cavity. In iron replete or overloaded cells, ferritin is partially degraded to insoluble hemosiderin. The contribution of storage iron to total body iron can vary widely from less than 5% to more than 30%. In healthy adults, about 10-25% of total body iron is found as storage iron.\(^4\)

Iron stores have an important function as a buffer for events that disturb the balance of iron turnover. When the rate of red cell production exceeds the rate of destruction (e.g. following acute blood loss or during rapid body growth), sufficient iron stores are crucial for mobilizing iron to satisfy the erythropoietic needs, which in the short term cannot be covered by dietary iron alone. On the other hand, when red cell
destruction exceeds production (e.g. in hemolytic states and during the physiological postnatal decrease in erythropoiesis), or when absorbed iron exceeds iron requirements, surplus iron is diverted to stores for later use.

**Erythropoiesis**

Erythropoiesis is the production of erythrocytes (red blood cells), which after birth occurs almost exclusively in the bone marrow. Proerythroblasts divide and mature through various stages via reticulocytes to mature erythrocytes. Protoporphyrin IX is synthesized in erythroid cells by a series of reactions, the rate-limiting step being catalyzed by δ-aminolevulinic acid synthase (ALA synthase). Heme is formed when iron is bound in the center of the protoporphyrin molecule. The common hemoglobin (HbA) is a protein tetramer composed of four globin chains (two α-chains and two β-chains), each with a heme moiety covalently attached. During maturation, Hb accumulates in the red cell precursor and the cell nucleus is eventually lost. The mature erythrocyte enters the blood stream where its sole function during its 120-day life span is to capture oxygen in the lungs and deliver it to all body tissues.

When the concentration of Hb in blood is low (anemia), oxygen delivery is reduced. Tissue hypoxia induces production of erythropoietin in the liver and kidneys and when this hormone reaches the bone marrow, it binds to receptors on erythroid progenitor cells and stimulates erythropoiesis. However, the relatively modest effect of erythropoietin treatment on anemia of prematurity suggests that other, yet unknown factors may also be important in the regulation of erythropoiesis in the newborn.

**Measures of iron status**

**Phlebotomy**

Indirect estimates of iron stores in adults can be made by weekly 500 mL phlebotomies continued until the rate of erythropoiesis is reduced, as a sign of iron depletion. A normal adult man can lose 3 L of blood or approximately half the total blood volume over a period of 3-4 months before signs of depletion appear. Although this remains the gold standard for determining storage iron, it is a very demanding procedure which cannot be used for monitoring iron stores over time.

**Bone marrow staining**

In bone marrow biopsies or smears, iron granules can be demonstrated in erythropoietic cells and macrophages by staining with Prussian blue. The amount of storage iron in bone marrow can be semiquantitatively graded and this estimation is well correlated with post mortem determinations of iron content in bone marrow and liver. However, like phlebotomy, bone marrow sampling is not ethically acceptable to use for research or screening in healthy infants due to the invasive character of the procedure.

**Ferritin**

More practical than the above two methods for determining iron stores is the measurement of ferritin in serum or plasma. The origin of serum ferritin is uncertain, and its physiologic significance in serum, if any, is unknown. Nevertheless, it is of considerable clinical importance because its concentration closely parallels the size of body iron stores in adults, as measured by bone marrow staining or phlebotomy.
Table 1. Theoretical changes in iron status variables in iron overload and iron deficiency of increasing severity.

<table>
<thead>
<tr>
<th>Compartment</th>
<th>Variable</th>
<th>Iron overload</th>
<th>Mild ID</th>
<th>Moderate ID</th>
<th>Severe ID</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No IDA</td>
<td>Mild IDA</td>
<td>Severe IDA</td>
<td></td>
</tr>
<tr>
<td>BM and RBC</td>
<td>Hb</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MCV</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ZPP</td>
<td>N</td>
<td>N</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Serum</td>
<td>S-Fe</td>
<td>+ +</td>
<td>N</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Serum</td>
<td>TIBC</td>
<td>- -</td>
<td>N</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Serum</td>
<td>Tf Sat</td>
<td>+ +</td>
<td>N</td>
<td>-</td>
<td>- -</td>
</tr>
<tr>
<td>Tissues</td>
<td>TfR</td>
<td>N</td>
<td>N</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Stores</td>
<td>Ferritin</td>
<td>+ +</td>
<td>-</td>
<td>- -</td>
<td>++</td>
</tr>
</tbody>
</table>

ID = Iron deficiency, IDA = Iron deficiency anemia, BM = Bone marrow, RBC = Red blood cells
Hb = Hemoglobin, MCV = Erythrocyte mean cell volume, ZPP = Zinc protoporphyrin
S-Fe = Serum iron, TIBC = Total iron binding capacity in serum (Transferrin)
Tf Sat = Transferrin saturation with iron (calculated from S-Fe and TIBC)
TfR = Soluble transferrin receptors, N = Normal or almost normal.
-/-/- = Lower / much lower than normal, +/+/+ = Higher / much higher than normal.

In adult men, there is an estimated 8-10 mg of storage iron (or 120 µg/kg body weight) for each µg/L of serum ferritin. The serum ferritin concentration often exceeds 1000 µg/L in states of iron overload and a concentration of < 10-12 µg/L reflects depletion of iron stores. However, serum ferritin cannot be used to further assess severity of ID after iron stores are depleted (Table 1). A limitation is that serum ferritin, an acute phase reactant, increases in states of inflammation and infection, and it can also be increased in liver disease and neoplastic disease, without relation to iron stores.

Fe, TIBC and transferrin saturation

Measurement of serum iron (S-Fe) alone provides little useful information because of the considerable hour-to-hour and day-to-day variation. The plasma concentration of transferrin, measured functionally as the total iron binding capacity (TIBC), is increased in ID and decreased in states of iron overload. Saturation of transferrin with iron can be calculated from serum iron and TIBC and is used as a measure of iron status (Table 1). However, for the diagnosis of ID, all of these variables have largely been replaced by the assay of serum ferritin, which is regarded as a more accurate measure of body iron stores.

Transferrin receptors

With the exception of mature erythrocytes, transferrin receptors are probably expressed on all cells, with the highest expression of receptors being found on erythroid precursor cells, placental trophoblasts, neoplastic tissue and rapidly dividing normal cells. Soluble transferrin receptors (TfR) can be demonstrated in serum or plasma using immunological techniques. Most circulating receptors consist of a monomeric form of the extracellular portion of the molecule. The function of TfR in serum, if any, is
unknown. Increased amounts of transferrin receptors are found on surfaces of iron-deficient cells and the concentration of TfR in serum or plasma has been suggested to correlate well with cellular iron needs and may thus be a good indicator of iron status. This notion was reinforced by the finding that, unlike serum ferritin, TfR is not affected by inflammation or infection. Thus, TfR may be used to assess iron status in situations when infections are common. However, since this is the latest addition to the battery of iron status variables, there is currently no international standard for TfR and commercially available assays vary with regard to reference values.

**Erythrocyte variables (Hb, MCV, ZPP)**

The serum concentration of ferritin is useful for detecting depleted iron stores, but is not useful for determining the severity of ID after this stage. When iron stores are nearly depleted, the bone marrow will obtain decreasing amounts of iron for erythropoiesis. Zinc protoporphyrin (ZPP) is regarded as the most sensitive marker of iron-deficient erythropoiesis. ZPP is formed when zinc, instead of iron, is incorporated into protoporphyrin during the final step of heme biosynthesis. Normally, this occurs only in one out of 30,000 heme molecules, but in states of iron-deficient erythropoiesis, it occurs more often. The ratio between ZPP and heme is used clinically to detect states of pre-anemic iron depletion (Table 1). ZPP is also increased in lead poisoning. Some laboratories prefer to measure free erythrocyte protoporphyrin (FEP) which is proportional to ZPP.

When ID increases in the bone marrow, Hb production is reduced which leads to a decreasing concentration of Hb in the blood (Table 1). When each erythrocyte contains less Hb, the mean cell volume (MCV) is reduced as well as the mean cell Hb concentration (MCHC), resulting in the classic microcytic, hypochromic anemia of iron deficiency.

**Criteria for iron deficiency anemia**

From the above, it may seem that the diagnosis of iron deficiency (ID) and iron deficiency anemia (IDA) should be easy, using the available battery of tests for the clinical evaluation of iron status. Indeed, this is most often the case for severe IDA. In milder cases, however, the interpretation of iron status is more challenging since the resulting combination of normal and abnormal values in many cases does not fit into the theoretical model (Table 1). Another problem is that mild or moderate ID, unlike most pathological conditions, often is totally asymptomatic. It is therefore not surprising that there is no consensus about the laboratory criteria for ID and IDA in adults or children.

In the absence of other conditions causing anemia, IDA is usually defined as a low Hb together with other indicators of ID such as either low serum ferritin or a combination of multiple criteria (i.e. abnormal values for any two out of three variables of iron status). Even though the multiple criteria model is most commonly used, there is no consensus on whether to use single or multiple criteria, or which iron status variables to use in the multiple criteria model.

A quite different way of diagnosing IDA in adults is a trial treatment, i.e. to give iron to the patient and to observe the response in Hb. If Hb increases significantly (e.g. 10 g/L) after at least a month of iron supplementation, IDA can retrospectively be confirmed.

**Dietary iron and bioavailability**

As a rule, dietary iron is poorly absorbed compared to many other nutrients. From a mixed adult diet the typical iron bioavailability is about 10%,
whereas the bioavailability from diets in developing countries often is lower (<5%). There are two major forms of dietary iron: 1) **heme** iron, derived from hemoglobin and myoglobin, which is found only in foods of animal origin such as meat, fish, poultry, liver and blood and 2) **non-heme iron**, which is found in foods such as cereals, fruits, vegetables and milk, and also in iron supplements.

**Heme iron**

Heme iron forms a relatively minor part of total iron intake. Even in diets with high meat content, heme iron usually accounts for only 10-15% of the total daily intake. In many developing countries, the intake of heme iron is almost negligible. The absorption of heme iron, however, is usually high and may account for as much as 25% of the iron absorbed from the daily diet. Thus, heme iron is an important source of dietary iron in adults and children. However, since meat is not a major protein source for most infants, heme iron usually does not contribute significantly to dietary iron in this age group.

**Non-heme iron**

Most dietary iron is in the form of non-heme iron: inorganic iron salts or complexes, most often in the ferric form. Non-heme iron absorption is influenced by several dietary factors. Ferrous iron is absorbed better than ferric iron. Ascorbic acid reduces ferric iron to ferrous, and also helps keeping iron in solution by acting as a weak chelator. Citric acid can similarly enhance iron absorption by increasing the solubility of iron. Proteins in meat, fish and poultry enhance absorption by an unknown mechanism known as the "meat factor". Some nutrients inhibit absorption of non-heme iron by chelation or formation of insoluble salts. Examples of such inhibitors are phytates and other inositol phosphates (in cereals, especially those with a high fiber content), calcium, and polyphenols (in tea, coffee and some vegetables).

The bioavailability of non-heme iron is low from most common foods, ranging from 1-2% in rice and spinach to 10-20% in meat and liver. Human milk is a notable exception with a reported iron bioavailability of about 50%.

**Iron absorption**

Iron absorption occurs principally in the duodenum and upper jejunum. Gastric acid production serves to lower the pH in the proximal duodenum, enhancing the solubility and uptake of ferric iron. It has not been studied whether the higher pH in the stomach of the young infant has any negative effect on iron absorption from different foods.

Heme iron is absorbed more efficiently than non-heme iron, and without being affected by inhibitors or enhancers. The molecular mechanisms behind heme iron absorption have not yet been elucidated. The following discussion will focus on non-heme iron absorption.

Iron absorption occurs in two steps: 1) Uptake of iron from the gut lumen into the enterocyte and 2) Transport from the enterocyte through the basolateral surface to portal blood. A variable portion of iron taken into the mucosal cell is released to the blood. The remaining portion is incorporated into ferritin in the enterocyte and either released later or sloughed with the cell at the end of its 3-4-day life span.

**Molecular mechanisms**

Only recently, the molecular mysteries of iron absorption have started to become unraveled. The most extensively characterized uptake pathway is via the divalent metal transporter 1 (DMT1; formerly called Nramp2 or DCT1). DMT1 transports ferrous iron (and
possibly some other divalent metal ions) from the intestinal lumen across the apical membrane into the enterocyte through a proton-coupled process. Since DMT1 cannot transport ferric iron, a recently characterized brush-border surface ferric reductase may be important in facilitating iron absorption by reducing ferric to ferrous iron before absorption.29 The quantitative significance of this reductase in humans, however, is uncertain.

A human intestinal lactoferrin receptor has recently been cloned and shown to have higher expression in fetal intestine than in most other tissues.30 Further studies are necessary to determine whether this is a significant pathway for absorption of lactoferrin-bound iron from breast milk.

Ferroportin (also known as IRegl or MTP1) is a transporter of iron across the basolateral membrane. This transporter may require hephaestin (a ceruloplasmin-like ferroxidase) for the transfer of iron to plasma transferrin.31

Regulation of non-heme iron absorption

A unique feature of human iron metabolism is the absence of an excretory pathway.32 Once absorbed, iron is retained in the body, except for the normal small basal losses which cannot be increased even in a state of iron overload. Regulation of iron absorption is therefore critical. Three different regulators of non-heme iron absorption in humans have been suggested: 1) the "stores regulator", 2) the "erythropoietic regulator" and 3) the "dietary regulator" (Fig 1).28 Since iron storage and erythropoiesis occur in tissues remote from the duodenum, the first two of these regulators would need humoral factors to transmit the information to the mature enterocyte or its precursor cell. However, these factors have not yet been characterized.

The stores regulator

Many studies have shown that iron absorption is inversely related to iron stores, a mechanism sometimes referred to as the stores regulator.11 Iron stores are most often assessed by serum or plasma ferritin, the level of which accurately predicts iron absorption in healthy, adult men.33 In an iron-replete adult man, absorption is reduced so that the size of iron stores does not increase further, even if iron supplementation is given for an extended period of time.11 However, the maximal up-regulatory effect of iron stores is relatively small. When iron stores are depleted, iron absorption can only be increased by about 1 mg/day in a human adult.11

The erythropoietic regulator

When the iron needs of the erythropoietic marrow are not met, iron absorption is increased substantially, up to 4-5 mg/day in adults. Furthermore, when erythropoiesis was stimulated by injection of erythropoietin, iron absorption increased 2.5-fold, when controlling statistically for serum ferritin concentrations.23 This indicates a regulating effect of erythropoietic activity, independent of iron stores. However, in chronic hemolytic states e.g. hereditary spherocytosis, iron absorption is usually normal despite increased erythropoiesis.34 In contrast, iron absorption is inappropriately increased in

![Fig 1. Regulators of iron absorption. Schematic drawing of an enterocyte.](image)
thalassemia major and other anemias associated with markedly increased but ineffective erythropoiesis. As a result, iron overload is a major complication of such anemias. These different iron absorption patterns in different hemolytic states indicate that it is not the erythropoietic rate per se that affects iron absorption, but rather some other component of erythropoietic activity.

The dietary regulator

Iron absorption is also regulated by recent dietary iron intake, independent on size of iron stores and rate of erythropoiesis. An enteral bolus of iron renders enterocytes resistant to absorbing additional iron for several days, a phenomenon referred to as "mucosal block". This dietary regulator of iron absorption (also known as mucosal adaptation) has been less emphasized than the other two regulators, possibly because habitual dietary iron intake and size of iron stores often are correlated in the non-experimental setting. A recent study suggests that changes in non-heme iron absorption in adult men are affected by recent bioavailable iron intake rather than by iron stores as measured by ferritin. An increased dietary iron load is likely to increase the enterocyte iron content, believed to be pivotal in the regulation of iron absorption, and may thus inhibit the expression of iron transporters such as DMT1 or ferroportin. Adaptation of iron absorption due to the dietary regulator may explain why dietary interventions to increase iron intake not always result in the expected improvement in iron status, even in iron deficient populations.

Iron toxicity

Excessive iron intake can lead to poisoning and death. In the absence of a pathway for iron excretion, the ability to increase iron stores in the liver is essential to protect the body from iron toxicity in case of overload of exogenous iron. Due to its pro-oxidant effects, excess iron has been indicated as a potential risk factor for cancer as well as coronary heart disease. Several recent studies have shown a correlation between dietary iron intake and increased risk for colorectal cancer in the adult population. However, a recent meta-analysis did not find any support for the theory that iron intake is correlated to coronary heart disease. Hereditary hemochromatosis is a common genetic disorder, especially in individuals of European origin, affecting as many as 1/300. In these individuals, iron supplementation is contraindicated since a progressive increase in body iron stores results in the deposition of excessive amounts of iron in the liver, pancreas, heart and other organs. Younger individuals with this disorder most often have not yet been diagnosed, and iron supplementation or fortification might theoretically aggravate their iron overload.

Iron requirements and iron deficiency in infants

History

As reported by Guggenheim, the first published description of a disorder that may be identified as IDA appeared in 1554 when the German physician Johann Lange gave the following description of a girl: "weak... sadly paled, the heart trembles... and she is seized with dyspnea in dancing and climbing the stairs". In 1615, the name "chlorosis" (green sickness) was coined to describe a similar condition, due to the supposed greenish tint of the paleness. Chlorosis was classified among the "hysterical" diseases by Sydenham (1624-89), who suggested treatment with iron-rich mineral water. Already the ancient Greeks associated iron with blood,
possibly because of its red color and its use for weapons, but not until 1713 did Lemery and Geoffroy discover that iron is a constituent of blood.\textsuperscript{48} In 1832, it was noted that blood iron content was decreased in chlorosis. Blaud showed the same year that treatment with ferrous sulfate pills was effective,\textsuperscript{50} but this treatment was not generally accepted. Bunge discovered in 1892 that the milk from some animals contains very little iron and he believed that animals which depended entirely upon it for some time after birth were born with stores of iron to last over the suckling period.\textsuperscript{51}

In the early 20\textsuperscript{th} century, anemia was prevalent and often severe among infants in Europe and the USA, especially in poor families, and often in combination with general malnutrition and chronic infections, a situation much resembling current conditions in many developing countries.\textsuperscript{52} However, the cause for anemia in these infants was not known, and treatment was controversial. At this time, a vast majority of infants were breast-fed during most of their first year of life (by their mother or a wet nurse), but the use of cow's milk was gaining in popularity since technical advances in milk handling such as pasteurization, condensation, evaporation and refrigeration facilitated its use for infant feeding.\textsuperscript{53} It was recognized that anemia was more common in infants who were fed cow's milk than in those who were not.\textsuperscript{52} In 1928, the British pediatrician Helen Mackay showed that anemia in infants could be prevented through iron fortification of evaporated cow's milk.\textsuperscript{54} Unfortunately, it would take several decades before iron fortification of infant formulas came into more widespread use.

As diagnostic methods improved, ill-defined terms such as chlorosis eventually disappeared from medical textbooks, and in the 1940s, it was generally accepted that IDA is the most common anemia during infancy.\textsuperscript{52} At this time, most infant formulas were home-prepared from evaporated milk, water and sugar (in Sweden also flour). In the 1950s, the popularity of commercially prepared infant formulas increased dramatically. During the 1950s and 1960s, IDA in infancy was still common, with a prevalence of >40\% in some urban populations.\textsuperscript{55} Iron fortification of commercial formula was introduced after the studies of Marsh (1959) and Andelman (1966), who essentially confirmed the results of Mackay.\textsuperscript{56, 57} However, the use of unfortified formula and whole cow’s milk was still common and breast-feeding continued to decline until about 1970. During the 1970s and 1980s, the prevalence of IDA among children diminished in developed countries, a decline attributed to the increased use of iron-fortification of infant formulas and other infant foods.\textsuperscript{58} At the same time, there was a marked increase in breast-feeding in the Western world, leading to new research questions concerning iron requirements of breast-fed infants. Thus, despite enormous research efforts during more than a century, many mysteries remain about the role of the "simple metal" iron in infant nutrition.

### Symptoms of ID in infants

Nutritional IDA seldom causes any overt symptoms in infancy. Fatigue and tachypnea only occur in severe anemia. Pallor is a consistent finding in cases of moderate and severe anemia, but often passes unnoticed by the parents. However, several studies have shown impaired neurodevelopment and behavioral disturbances in infants with IDA\textsuperscript{59, 60} and it has been shown in infants as well as in school children that ID can have adverse effects on cognition, which are reversible with iron therapy.\textsuperscript{61, 62} From a public health perspective, this must be regarded as the most concerning manifestation of ID.
in infancy, even if the causal relationship between IDA and poor neurodevelopment has not been conclusively proven.\textsuperscript{63,64}

**Developmental aspects**

**Fetal iron metabolism**

Fetal erythropoiesis can be detected by the 14\textsuperscript{th} day of gestation. This is essential, because diffusion is no longer a sufficient mechanism for oxygen transport when the size of the embryo exceeds a few mm. Blood formation occurs mainly in the mesenchyme during the first trimester, in the liver during the second trimester and in the bone marrow during the last trimester. Fetal erythropoiesis results in the orderly evolution of a series of different hemoglobins. In late pregnancy, the predominant tetramers are fetal Hb (HbF, two α-chains and two γ-chains) and adult Hb (HbA, two α-chains and two β-chains). HbF has a higher affinity for oxygen than HbA, facilitating the transport of oxygen from maternal to fetal blood in the placenta.

**Iron status at birth**

It has been estimated that the term newborn has a total body iron content averaging about 75 mg/kg body weight,\textsuperscript{22,65} which can be compared with 55 mg/kg for an adult man. Although placental iron transport is negligible during the first two trimesters, it rises progressively to 4 mg daily towards the end of the third trimester.\textsuperscript{66} Consequently, birth weight as well as gestational age are major determinants of the total body iron content at birth, even though interindividual variation is considerable.

In the newborn, the largest proportion of iron is in the circulating Hb mass (about 50 mg/kg). Another 5 mg/kg is believed to be present as tissue iron, including myoglobin.\textsuperscript{66} The amount of storage iron in liver, spleen and bone marrow is about 20 mg/kg, adding up to a total body iron of 75 mg/kg.

The amount of circulating Hb iron is a function of the concentration of Hb and the blood volume. Average Hb concentration of cord blood from normal term infants is 170 g/L with a range from 135 to 210 g/L.\textsuperscript{66} Average blood volume at birth is about 85 mL/kg,\textsuperscript{67} thus depending mostly on birth weight, but to some extent also on the timing of umbilical cord clamping. For about 3 minutes after delivery, uterine contractions send placental blood through the umbilical cord to increase the proportion of blood in the infant. Late clamping (after cessation of pulsations in the umbilical cord), compared to early clamping (directly after delivery) may increase infant blood volume by as much as 20 mL/kg.\textsuperscript{66,67} Thus, for infants with similar birth weight, individual variations in Hb and blood volume at birth is a major cause of differences in body iron content.

Iron supplements are commonly recommended to pregnant women but many studies have failed to show any correlation between maternal Hb and cord blood Hb. It is therefore generally assumed that the iron status of the fetus, and subsequently the infant, is rather independent of maternal iron status during pregnancy.\textsuperscript{66} In studies investigating the possible association between maternal ferritin and cord blood ferritin, some have shown a positive correlation while others have not.\textsuperscript{68} However, there is substantial evidence that maternal IDA increases the risk of preterm delivery and subsequent low birth weight.\textsuperscript{68} Furthermore, one follow-up study of infants at 12 months\textsuperscript{59} and one study on aborted fetuses\textsuperscript{70} have also suggested that poor maternal iron status may be associated with low total body iron and anemia in the infant, even when adjusting for birth weight. A direct effect of maternal iron status on infant iron stores can thus not be excluded, and
further studies are needed to investigate this possibility.\(^2\), \(^68\)

**Postnatal changes in iron metabolism**

*Physiologic changes*

The rates of Hb synthesis and erythropoiesis decrease dramatically during the first few days after delivery.\(^67\) At one week of age, the production has been reduced by a factor of 10. This is undoubtedly initiated by the equally sudden increase in tissue oxygen tension that takes place at birth, which is reflected by a marked decrease in plasma erythropoietin. Thus, during the first 6-8 weeks of extraterine life, there is a virtual cessation of erythropoiesis.\(^66\) Coincident with the ensuing decline in Hb is a shift of iron from the circulating Hb mass to storage sites. It is not until the Hb concentration falls from the umbilical cord level of 170 g/L to about 120 g/L that erythropoiesis resumes.

After birth, the production of fetal HbF is gradually switched to adult HbA, but at 4 months of age, more than 5% of the newly synthesized Hb is still HbF.\(^67\)

*Changes in Hb and MCV*

After the rapid decrease in Hb during the first 2-3 months of life, there is a slow decrease until a nadir is reached at about 8-18 months of age, when mean Hb usually is 110-120 g/L. After 2 years of age, mean Hb slowly increases to 135-140 g/L at 12 years of age.\(^71,\)\(^72\)

Fetal and neonatal erythrocytes have considerably larger size compared to those of older children and adults. Mean MCV is 135 fl at 24-25 weeks of gestation, 119 fl at term, reaches a nadir of 72-77 fl at about 6-18 months of age and thereafter steadily increases to 85-90 fl in adult age.\(^67,\)\(^71-\)\(^73\)

From the above can be concluded that infants between the ages of 6 months and 2 years have lower Hb and lower MCV than at any other age. In unselected populations of seemingly well-nourished infants in this age interval, many studies have reported a high prevalence of mild anemia.\(^71,\)\(^74-\)\(^76\) Large doses of intramuscular iron given to 9-month-old well-nourished infants (three doses over 1 month, totaling 250 mg of iron), resulted in significantly higher mean Hb at 12 months of age (123 g/L compared to 116 g/L in controls), significantly lower free erythrocyte protoporphyrin (corresponding to ZPP), but no significant difference in mean MCV (79 fl in treated compared to 77 fl in controls).\(^77\)

This suggests that at least part of this so called "anemia of late infancy" is caused by ID but a sizeable proportion of the low Hb, and especially the low MCV, at 6-24 months of age, probably reflects normal physiologic changes in the developing infant.

*Changes in other iron status variables*

Mean serum ferritin is high at birth in term infants (100-200 \(\mu g/L\)) and increases further to 200-400 \(\mu g/L\) during the first weeks of life. After erythropoiesis resumes there is a progressive decline in serum ferritin to about 30 \(\mu g/L\) at 1 year of age.\(^78\) Since these changes parallel the theoretical early increase and later decrease of body iron stores (see above), ferritin is considered a valid measure of iron stores in infants, even though it is noteworthy that this has never been validated by other methods.\(^66\)

To our knowledge, age-related changes in ZPP have not been studied in infants. Several studies have confirmed that mean levels of soluble TfR are considerably higher in infants than in adults.\(^79\) The reason for this is not yet known, even though one might speculate that it may be due to a large proportion of rapidly dividing cells in the growing infant.
High iron requirements in infancy

Low iron content of breast milk

Human milk has unique properties, making exclusive breast-feeding the optimal nutritional strategy during the first 4-6 months of life. However, the iron concentration of human milk is low (0.2-0.4 mg/L), which is thought to be partly compensated for by its high bioavailability. Assuming a daily intake of 800 mL breast milk and an absorption of 50%, an exclusively breast-fed 4-month-old infant would absorb 0.12 mg of iron daily (18 μg/kg/d assuming a weight of 6.7 kg). This is approximately sufficient for covering the estimated basal iron losses (20 μg/kg/d), but does not allow for net gain of iron. Thus, additional sources of iron are needed after about 4-6 months when neonatal iron stores have been depleted (Fig 2).

Iron requirements for growth

In the most studied primate, the adult male medical student, there is little exchange of iron between the body and the environment, since body iron is recycled. In the infant, however, the recycling yields a deficit since a substantial part of iron turnover is diverted to growing tissues. Fig 3 shows that in an infant aged 6 months, about 0.5 mg/d is needed for "blood growth" and 0.1 mg/d for growth of muscle and other tissues. Since iron requirements without growth would equal basal iron losses (0.15 mg/d), infant growth increases iron requirements 5-fold to approximately 0.75 mg/d. (Fig 3).

As a consequence of the reduced Hb concentration and the redistribution of iron to storage sites, the infant is able to double its birth weight without exhausting iron reserves, independent of external iron. For term infants this occurs at about 5 months of age and for preterm infants as early as 2-3 months of age. At some time soon thereafter, iron absorption from the diet becomes critical to the maintenance of iron balance.

In proportion to body weight, the need for dietary iron is greater during late infancy than during any other period of life. Based on assumptions about certain factors such as total body iron at birth (71 mg/kg) and basal iron losses (20 μg/kg/d), Oski estimated the average requirement for absorbed iron during the first year of life to be 280 mg, averaging 0.8 mg/d. From this follows that if an infant is exclusively breast-fed until 6 months of age, thus gaining very little iron, then the need for dietary iron from other sources than breast milk during the second half of the first year of life, assuming 10% absorption, would be 2800 mg or 15 mg/d. This is an intake which is virtually impossible to achieve with unmodified complementary foods, suggesting that additional iron is needed, either as food fortification or as separate iron supplements, to cover the estimated needs.
Increased iron losses

Physiologic iron losses from the body are small and relatively constant in the healthy infant. However, it is important to note that iron losses in infants are substantially increased in case of overt or occult intestinal bleeding which may occur during episodes of diarrhea, parasitic infestations (hookworm, giardia) or when feeding whole cow’s milk to infants.

Problems with cow’s milk

A milk-based diet is convenient for the infant as well as for the mother, and some infants have difficulties in the transition to a solid diet. It has long been known that infants fed whole cow’s milk are at high risk for IDA. In exclusively cow’s milk fed infants ("milkoholics"), this is easily explained by the low concentration of iron in cow’s milk (about 0.3 mg/L), its poor bioavailability (about 10%), and — above all — the lack of any other dietary source of iron.

In some susceptible individuals, whole cow’s milk induces gastrointestinal bleeding and, in these, a clear dose-response relationship has been shown between the quantity of cow’s milk consumed and the amount of blood lost in the feces. The susceptibility to cow’s milk induced gastrointestinal bleeding seems to decrease with age and this condition is rarely seen after 2 years of age. The factor that induces blood loss has not been identified, but several studies
have shown that cow’s milk formula reduces intestinal bleeding significantly compared with cow’s milk.\textsuperscript{82, 83}

Consumption of unfortified cow’s milk formula gives a slightly higher risk for IDA, compared with breast-feeding.\textsuperscript{84} This is most likely due to the lower bioavailability of iron in formula but, to our knowledge, it has never been studied whether cow’s milk formula causes increased intestinal blood loss compared with breast milk. Increased blood loss is likely at least in some individuals with cow’s milk allergy, a common condition in infancy.

**Prevention of IDA**

IDA is the most common micronutrient deficiency in the world, with about 600 million individuals affected.\textsuperscript{85} Rapid growth makes infants and young children a particular risk group for IDA. In 1980, the World Health Organization estimated the worldwide prevalence of anemia in children below the age of 4 years to be 43\%, with a higher prevalence in developing regions (51\%) than in developed regions (12\%).\textsuperscript{86}

**Iron fortification & supplementation**

Strategies for prevention of IDA in infants include dietary recommendations, iron fortification of common foods, and supplementation in the form of iron drops.

It has long been recognized that iron fortification of infant formula significantly reduces the risk for IDA,\textsuperscript{56} even though the optimal level of iron fortification still remains to be determined.\textsuperscript{87, 88} However, the preventive effects of iron-fortified formula only apply to formula-fed infants.

The World Health Assembly (WHA) as well as the American Academy of Pediatrics (AAP) recommends exclusive breast-feeding until about 6 months of age.\textsuperscript{89, 90} Furthermore, it is recommended that partial breast-feeding should be continued until at least 1 year of age.\textsuperscript{89, 90}

Iron drops at a dose corresponding to 1-2 mg Fe /kg/d have been recommended for term infants who are exclusively breast-fed between 4 and 6 months\textsuperscript{91} and for infants after 6 months of age if they do not consume adequate amounts of iron-fortified foods (e.g. at least 400 mL of iron-fortified formula daily).\textsuperscript{92-94}

**Potential side effects of iron**

Iron is a known trophic factor for several pathogenic bacteria and there has also been concern that iron supplementation of breast-fed infants might saturate human milk lactoferrin, thus diminishing its anti-infective properties.\textsuperscript{95} Parenteral iron therapy has been associated to exacerbation of malaria and neonatal sepsis.\textsuperscript{96} For these reasons, it has been suggested that iron fortification and supplementation might increase the incidence of gastroenteritis and other infections in infants. However, several studies have shown that this is not the case for iron-fortified formula and one study even showed that iron fortification provided a small, but significant protection against diarrhea.\textsuperscript{97}

Several studies have addressed the issue whether iron supplementation affects growth, but results have been contradictory. Iron supplementation to anemic\textsuperscript{98} or malnourished children\textsuperscript{99, 100} resulted in improved growth, in the latter case possibly due to reduced morbidity. Some studies have shown no effect of iron on growth,\textsuperscript{101-103} whereas a single study has shown less weight gain in iron supplemented children.\textsuperscript{104}

There has been concern that iron supplements or iron fortification would compete with other divalent ions e.g. zinc and copper, and thus decrease the absorption of these other minerals. Studies have suggested that this may be the case for copper,\textsuperscript{105, 106} but not for zinc.\textsuperscript{107}
Considering the possible long-term effects of iron overload (see iron toxicity above), short time iron supplementation or fortification in infancy is unlikely to have any major influence on iron status in adulthood.

Some remaining questions

In spite of the widespread recommendations for supplementation of breast-fed infants with iron drops,9 the effects on iron status have not been evaluated adequately. Effects of iron supplementation have been studied in preterm infants, anemic infants and older or mixed age groups, but to our knowledge, no published study to date has investigated the effect of prophylactic iron supplementation of healthy, full term breast-fed infants starting at 4-6 months of age. Since there are potential adverse effects of iron supplementation on morbidity and growth, the efficacy and safety of this approach should be evaluated before it is implemented.

Although there is a large interindividual variation in iron absorption, estimates of iron requirements in infancy and recommendations of iron fortification levels in infant foods are often based on absorption data.87, 108 In healthy adults, absorption of iron increases in states of iron depletion, but it is not known whether the same regulating mechanism applies during infancy, a period characterized by dramatic changes in the size of iron stores as well as in the rate of erythropoiesis. Some authors have suggested differences in iron absorption depending on infant age,109 but longitudinal studies are lacking.

It is well known that there are differences in iron status between males and females in adolescence and adulthood, largely due to menstrual losses of iron in women of fertile age.110, 111 To date, little has been reported about sex-related differences in indicators of iron status in infants, even though ID in infants and toddlers is a significant public health problem.

Finally, conventional cutoff levels for iron status variables in infants are poorly documented and have recently been challenged.76 Commonly used cutoffs at 6-12 months of age for the definition of anemia and ID are Hb < 110 g/L92, 93 and serum ferritin <10-12 µg/L,112, 113 but these values are in fact extrapolated from older age groups and there is evidence that they may not be appropriate for infants.74 Cutoffs for other measures of iron status such as erythrocyte mean cell volume (MCV), erythrocyte zinc protoporphyrin (ZPP) and soluble transferrin receptors (TfR) are even less well documented in infants.
The current randomized, controlled double-blind study of iron supplementation of term, breast-fed infants was conducted at two sites where extended breast-feeding is common: San Pedro Sula, Honduras and Umeå, Sweden. The dual site design permitted assessment of the effects of iron supplementation across a wide range of iron status.

The aims were to:

1. Investigate the effects of daily iron supplementation, given to breast-fed infants (from 4-9 or from 6-9 months of age), on Hb and other measures of iron status.

2. Investigate the effects of iron supplementation on growth and morbidity.

3. Investigate the influence of iron supplementation, infant age, iron status and complementary food intake on iron absorption from human milk.

4. Investigate whether there were differences between male and female infants in Hb and other measures of iron status.

5. Define useful cutoff levels for Hb, ferritin, MCV, ZPP and TfR for diagnosing ID in infants.
MATERIALS & METHODS

Subjects

Papers I-II and IV-V were based on data from 263 infants participating in an iron supplementation trial (the "main study") conducted at two sites: San Pedro Sula, Honduras and Umeå, Sweden. Paper III, the "absorption study", was based on a subsample of 25 Swedish infants from the main study.

San Pedro Sula (population ~350 000) is an industrial city in Honduras. Umeå (population ~100 000) is a university town in northern Sweden. For the sake of simplicity, San Pedro Sula will be referred to as "Honduras" and Umeå as "Sweden".

These sites were chosen because both populations have a high proportion of infants breast-fed for 9 months or longer. Including two very different sites permitted assessment of the effects of iron supplementation across a wide range of iron status and complementary feeding patterns. Average dietary iron intake of 6-12 months old Swedish infants is relatively high (10-12 mg/d) and the main sources are iron-fortified formula or cereals. In contrast, the complementary diet in Honduras typically does not include iron-fortified products and is low in meat, poultry and fish. Non-nutritional anemias, caused by malaria, thalassemia or sickle cell anemia, are rare in both populations. Parasitic infestations (e.g. hookworm and Giardia) are very rare in Sweden and more common in Honduras, although prevalence as well as parasite load is likely to be low in breast-fed infants.

Exclusively breast-fed infants were recruited as described in Paper I. Eligibility criteria were as follows:

- Gestational age ≥ 37 weeks
- Birth weight > 2500 g
- No chronic illness
- Maternal age ≥ 16 years
- Exclusively breast-fed until 6 mo
- Breast-fed until at least 9 mo

Infants with Hb < 90 g/L at any time were to have been referred to a pediatrician for iron treatment, but no such cases occurred at 4 or 6 months.

The study was approved by the Ethical Committee of the Faculty of Medicine and Odontology at Umeå University, Sweden and the Human Subjects Review Committee of the University of California, Davis, USA. All participating mothers/parents gave written, informed consent.

Study design

Intervention

Since the optimal timing of iron supplementation of breast-fed infants is not known, we chose to study iron supplementation starting at either 4 or 6 months of age. Subjects were stratified by study site and sex, and randomized to three intervention groups:

1) Iron drops at 4-9 mo
2) Placebo at 4-6 mo, Iron at 6-9 mo
3) Placebo at 4-9 mo

Fe 4-9
Fe 6-9
P 4-9

4 6 9 Age (mo)

Iron supplement, 1 mg/kg/d
Placebo

Fig 4. The three intervention groups.
The iron supplement was a commercially available liquid formulation of ferrous sulfate (Fer-In-Sol, Mead Johnson, Evansville, IN) containing 25 mg/mL of elemental iron. The placebo solution was prepared as described in Papers I and III. The investigators as well as the parents were blinded to the intervention. The supplement was given at a dose corresponding to 1 mg of elemental iron per kg per day, which is a commonly recommended supplemental dose for prophylactic purposes, and the dose was adjusted monthly according to weight. The supplement or placebo was given by the mother each morning, just before or after breast-feeding and at least 1 hour before or after any other food intake.

Compliance with the intervention was monitored by asking the mothers to keep a daily checklist indicating whether the drops were given, and by collecting the used bottles each month and measuring the amount of fluid remaining. Subjects who received the study drops less than 75% of the days, according to either mother’s checklist or measurement of returned bottles, during either of the age intervals (4-6 months or 6-9 months) were considered “non-compliant”.

Infant diet

All infants were exclusively breast fed from birth to at least 6 months of age. Between 4 and 6 months, the mothers were discouraged from giving any other foods or fluids, the only exception being “taste portions”. Those mothers who wished to feed their babies taste portions were allowed to give ≤ 1 tablespoon per day of commercial baby food (strained fruits or vegetables) containing little or no iron, which was provided by the investigators. Between 6 and 9 months, the mothers continued breast-feeding, but were allowed to give complementary food at their own discretion. Apart from the taste portions, no attempt was made by the investigators to influence the choice of foods or the extent of breast-feeding.

Data collection & analysis

Blood samples

Venous blood (~ 5 ml) was obtained at 4, 6 and 9 months of age. In case of fever (> 38.0 °C), blood sampling was postponed one week. EDTA blood was analyzed immediately on site in duplicate for Hb and ZPP. Erythrocyte MCV was measured in the remainder of the EDTA blood, using automated blood counters at each site. Heparin plasma was frozen and transported to the Department of Nutrition, University of California, Davis, USA for measurement of ferritin, TiR and C-reactive protein (CRP) as described in Paper I.

Absorption measurements

Since the 1970s, it has not been considered ethical to use radioactive isotopes for research in healthy infants. Stable isotopes are non-radioactive and are therefore regarded as the preferred alternative for metabolic research, including mineral absorption studies.

Ferrous sulfate solutions of the stable isotopes $^{57}$Fe and $^{58}$Fe were prepared as described in Paper III.

Iron absorption was measured at two ages in each subject by giving a test meal 2 weeks before 6 months of age and an identical test meal 2 weeks before 9 months of age. For the sake of simplicity, these ages are referred to as 6 and 9 months, respectively.

For each test meal (Fig 5), a portion of human milk (approximately 100 mL) from each child’s own mother was mixed with 150 µg $^{58}$Fe and equilibrated overnight at 4°C. The $^{58}$Fe labeled milk was fed to the infant by the investigators as described in Paper III.
In order to increase the amount of information from each absorption measurement, a reference dose of 2.85 mg $^{57}$Fe was given orally to the infant using a 2 ml syringe at least 6 h after the completion of the test meal. In effect, this was a second measurement of iron absorption in the same infant at the same time. To make the reference dose more palatable, it was mixed with a small amount of apple juice in the syringe. No food or fluid was given for a period of at least 2 h before and after the reference dose.

No iron drops (or placebo drops) were given on the day of the test meal. No other food than human milk was given from 6 h before the test meal until 2 h after the reference dose.

A venous blood sample was obtained 2 weeks after each test meal, and erythrocytes were analyzed for isotope ratios using mass spectrometry as described in Paper III. The same blood samples were also analyzed for iron status variables. Just prior to the second test meal, a blood sample was obtained in order to determine the baseline isotope ratio.

Fractional absorption was calculated as described in Paper III, assuming that 90% of the absorbed isotope was incorporated into erythrocytes after 2 weeks.

**Dietary intake**

Intake of complementary food (defined as all food, fluid or solid, except human milk) between 6 and 9 months was estimated in Honduras by a bi-weekly 24 h recall and a food frequency questionnaire and in Sweden by a monthly 5-day food diary.

Intakes of specific nutrients from complementary food were calculated as described in Papers I and III. When total dietary iron intake was calculated in Paper III, supplemental iron intake (0 or 1 mg/kg/day, respectively) was added to complementary food iron intake. Breast milk intake was not measured, so the contribution of iron from this source was not included in the calculation, but it
should, theoretically, be less than 0.04 mg/kg/day.

**Anthropometry**

Birth weight was measured by the study team in Honduras and excerpted from medical charts in Sweden. Each month from 4 to 9 months, infant weight was measured on an electronic scale (to the nearest 10 g), length was measured on a recumbent length board (to the nearest mm) and head circumference was measured using a tape measure (to the nearest mm). In Honduras, one individual (RJC) performed all of the measurements; in Sweden, two pediatric research nurses (MB and MH) performed measurements after completing a standardization procedure to ensure that the inter-observer variability was within tolerable limits.

**Morbidity data**

Morbidity data were collected by providing a daily calendar for mothers to record the infant's stool frequency and consistency and any symptoms of illness (cough, fever, nasal congestion or discharge, diarrhea, vomiting or skin rash) or diagnoses made by a health care provider (e.g. otitis media). The records were reviewed with the mothers every two weeks. Diarrhea was defined as > 3 abnormally loose stools per day.

**Statistics and definitions**

**Power analysis**

For the main study, a pre-study analysis showed that a sample size of 39 infants per intervention group was required ($\alpha=0.05$, 80% power) to detect a difference in Hb of 5 g/L among treatment groups. To compensate for possible differences in the effects of iron supplementation in Honduras and Sweden, the sample size was increased by 50% to 60 infants per group, or 30 infants per group per site.

For the absorption study (Paper III), using previous variability data, a pre-study analysis showed that a group size of 10 would give a power of 80% at a significance level of 0.05 to find a 20% difference in iron absorption between groups. From each of the randomization groups, 12-14 families were asked to participate in the absorption study and a total of 25 families agreed (6 in group 1, 8 in group 2, and 11 in group 3). Since the actual number of recruited infants was slightly lower than the target number, this could limit the statistical power. However, since absorption from the reference dose proved to be strongly correlated with absorption from breast milk, we had two independent measurements of iron absorption in each infant at each point in time, which increased the reliability of the data.

**Conventional definition of IDA**

In Paper I and Paper IV, IDA was defined using multiple criteria and conventional cutoffs. Hence, anemia was defined as Hb <110 g/L. ID was defined as 2 of 3 iron status indices (ferritin, MCV and ZPP) being abnormal, using the following cutoff values: ferritin <12 µg/L, MCV <70 fl and ZPP >80 µmol/mol heme. IDA was defined as anemia in combination with ID.

**Statistical analyses**

**Excluded cases**

In Papers I, II and IV, non-compliers with the intervention (as defined above) were included in all statistical analyses according to the "intention to treat" principle. In Paper III, there was no non-complier and in Paper V, non-compliers were excluded in iron supplemented
groups, since we specifically wanted to study iron replete infants.

In all papers, infants who dropped out or became ineligible before 6 months of age were excluded from the statistical analyses.

Ongoing or recent infections may influence certain iron status variables: ferritin may be elevated whereas Hb may be decreased. Therefore, in all cases of ongoing infection where the infant was febrile, blood sampling was postponed one week. To detect recent infections, CRP was analyzed and this indicator was elevated (>10 mg/L) in 39 of 671 blood samples (5.8%). However, iron status in samples with elevated CRP levels was not obviously different from other samples. This was further demonstrated by the following two findings: In Paper I, when samples with elevated CRP were excluded, all the significant intervention effects of iron supplementation on ferritin remained unchanged. In Paper V, when samples with elevated CRP were excluded, the cutoff values for Hb and ferritin in the “unselected” and “iron replete” groups as well as in both iron supplemented groups at 9 months were unchanged. Thus, to maximize statistical power, samples with elevated CRP were not excluded from statistical analyses in any of the papers.

Skewed variables

Since the distributions of ferritin and ZPP values were skewed, they were log transformed for all statistical calculations in Paper I-II and Papers IV-V. In the presentations, values were converted back to the original units as geometric means and standard deviations. In Paper III, ferritin and ZPP passed a check for normality, most likely due to the smaller number of infants, whereas one other variable (absorption from the reference dose) was slightly skewed. Log transforming ferritin or absorption data did not change the results, so we preferred to use the original values in that paper for ease of presentation.

Because the morbidity data were highly skewed (many cases with no illness), dichotomous variables for presence or absence of illness in each age interval were created and used in Paper II.

Cutoffs

In Paper V, some special statistical methods were used in an attempt to establish cutoffs for iron status variables:

Normative population methods

Several different methods have been used to establish cutoffs for iron status variables. The most common approach is the normative population method whereby the reference range is calculated for a “healthy” population sample. The basic approach is to sample a population likely to have a low prevalence of ID, but without further selection of iron sufficient subjects within the population sample. Below, we will call this the “unselected” normative population approach. Beyond this, there are at least two possible approaches to minimize the risk of including iron deficient individuals in the normative population. One is to exclude individuals with possible ID according to conventional cutoffs for iron status variables other than the one studied. We will call this the “iron replete” normative population approach. Finally, ID can be excluded by prophylactic iron supplementation of the population sample. We will call this the “iron supplemented” normative population approach.

We applied the unselected, iron replete and iron supplemented normative population methods to our population sample. To illustrate the differences between the approaches, they were not combined. Therefore, iron supplemented infants were excluded when using the
"unselected" and "iron replete" approaches. To avoid using adult reference values in infants, we modified the iron replete normative population method as described in Paper V.

Hb response to iron

A quite different approach to establish cutoffs is the "Hb response" method, whereby individuals with IDA are identified by studying Hb response after iron treatment.

Sensitivity and specificity for different cutoff levels for various iron status variables can then be tested against this "gold standard". Since the optimal level of Hb response in infants is not known, we tested three response levels: >5, >10 and >15 g/L. Non-compliers and infants with any missing iron status variable during the time period (4-6 or 6-9 months) were excluded from the analyses. Receiver operating characteristics (ROC) curves were constructed and area under the curve (AUC) calculated as an estimate of the performance of each variable as a diagnostic test for IDA. Theoretically, AUC varies between 0.5 and 1. An AUC of 1 means 100% sensitivity and specificity. The null hypothesis (AUC = 0.5) is that the diagnostic test is no better than chance.

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RESULTS

Subjects
At 4 months of age, 263 infants were randomized into the three intervention groups. Of these, 232 infants (88%) remained in the study at 6 months and 214 (81%) at 9 months (Fig 6). There were no significant differences between intervention groups in maternal height, weight, age or parity, infant weight or length at birth or at 4 months, or any of the iron status variables (Hb, MCV, ZPP, ferritin and TfR) at 4 months of age. There was no significant interaction between infant sex and randomization group for any of the iron status variables at 4 months.

Total dropout rate was not significantly different between Honduras and Sweden or between male and female infants. There were no significant differences between dropouts and non-dropouts with respect to intervention group, maternal characteristics, infant anthropometrics or iron status at 4 months, within each study site (Paper I).

Compliance with giving the iron or placebo drops was 92% at 4-6 months (89% in Honduras and 95% in Sweden) and 95% at 6-9 months (95% in Honduras and 96% in Sweden).

Iron absorption was measured in 25 Swedish infants at 6 months of age, and the measurement was repeated in 18 of them at 9 months of age. The reason for these 7 dropouts was in all cases parental refusal based on the demanding study procedure. In the absorption study, there was no significant difference between groups in baseline Hb, ferritin, ZPP, TfR, body weight or infant sex distribution. Furthermore, there was no significant difference in these baseline variables between dropouts and non-dropouts.

Background information and baseline data

Site differences
There were large socioeconomic differences between sites. As an example, 91% of the Honduran mothers had an education of ≤ 9 years, while 97% of the Swedish mothers had an education of > 9 years.

In Honduras (vs Sweden), mothers were significantly younger (26 vs 31 years), shorter (151 vs 167 cm), lighter (56 vs 65 kg) and more multiparous (2.8 vs 1.9) mothers, and infants had lower weight (3.2 vs 3.6 kg) and length (49 vs 51 cm) at birth (Paper I). However, there were no significant differences in any of these variables between intervention groups within either site.

At baseline (4 months of age), Honduran infants (vs Swedish) had significantly lower Hb (112 vs 119 g/L), MCV (74.5 vs 79.6 fL) and ferritin (68 vs 110 μg/L) and higher ZPP (51 vs 45 μmol/mol heme) while TfR was not significantly different between sites (Paper I). Lower birth weight and larger weight gain explained part of the difference between sites in baseline iron status during the first 4 months of life in the Honduran infants. When adjusting for these two
factors, the difference in baseline ferritin between sites was reduced by 47% and the difference in ZPP was reduced by 45%; however, the differences in Hb and MCV between sites were not appreciably reduced. Eighty-five percent of the Honduran and 40% of the Swedish mothers received iron (or iron and multivitamin) supplements during pregnancy. There was no correlation between maternal iron supplementation and infant iron status at 4 months.

Sex differences

Boys (vs girls) had non-significantly higher birth weight (3.4 vs 3.3 kg, p=0.065) and significantly larger postnatal weight gain. There were no significant differences between sexes in energy or iron intake from complementary food (Paper IV).

At 4 months, boys had 2 g/L lower mean Hb (p=0.013), 2.7 fL lower MCV (p<0.001), 11% higher ZPP (p=0.007), 40% lower ferritin (p<0.001) and 1.1 mg/L higher TfR (p<0.001). When adjusting for site, birth weight and postnatal weight gain from birth to 4 months, all these sex differences remained significant except for Hb (p=0.095).

Effects of iron drops

Iron status and IDA

Iron status

Since in unsupplemented infants, a decrease in mean value for Hb, MCV and ferritin, and an increase for ZPP and TfR, were observed from 4 to 9 months (Paper I), iron status in iron supplemented infants had to be considered in relation to iron status in unsupplemented infants.

The effect of iron supplements from 4 to 9 months was similar at the two sites for MCV, ferritin, ZPP and TfR (Table 2). Iron supplementation from either 4 or 6 months had similar and significant effects on MCV (+2.7 fL) and TfR (-1.5 mg/L) compared to placebo. There was a significantly more pronounced effect of iron supplementation from 4-9 months, compared to 6-9 months, on ferritin (+32 vs +20 pg/L, p=0.044) and ZPP (-15 vs -9 μmol/mol heme, p=0.016).

Table 2. Effect of iron supplementation from 4-9 or 6-9 mo on iron status at 9 mo.

<table>
<thead>
<tr>
<th>Variable</th>
<th>4 mo</th>
<th></th>
<th></th>
<th>9 mo 1</th>
<th>Intervention</th>
<th>Site</th>
<th>Interv x Site</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Placebo</td>
<td>Fe 6-9 mo</td>
<td>Fe 4-9 mo</td>
<td>Intervention</td>
<td>Site</td>
<td>Interv x Site</td>
</tr>
<tr>
<td></td>
<td>n=214 2</td>
<td>n=77</td>
<td>n=67</td>
<td>n=70</td>
<td>p^3</td>
<td>p^4</td>
<td>p^5</td>
</tr>
<tr>
<td>Hb (g/L)</td>
<td>115</td>
<td>(111)</td>
<td>(116)</td>
<td>(117)</td>
<td>&lt;0.001</td>
<td>0.942</td>
<td>0.017</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>76.8</td>
<td>71.9</td>
<td>74.2*</td>
<td>74.9*</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.097</td>
</tr>
<tr>
<td>Ferritin (µg/L)</td>
<td>83</td>
<td>19</td>
<td>38*</td>
<td>51**</td>
<td>&lt;0.001</td>
<td>0.316</td>
<td>0.693</td>
</tr>
<tr>
<td>ZPP (µmol/mol heme)</td>
<td>49</td>
<td>61</td>
<td>52*</td>
<td>46**</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.354</td>
</tr>
<tr>
<td>TfR (mg/L)</td>
<td>6.7</td>
<td>8.6</td>
<td>7.2*</td>
<td>6.9*</td>
<td>&lt;0.001</td>
<td>0.300</td>
<td>0.060</td>
</tr>
</tbody>
</table>

1 Controlling for baseline value, 2 All dropouts excluded
3 p-value for intervention effect (all three groups) 4 p-value for site (Honduras vs Sweden)
5 p-value for interaction between intervention and site (different intervention effect at the two sites)
Numbers in parentheses are not interpretable due to significant interaction between intervention and site.
* Significantly different from placebo, ** Significantly different from placebo and from Fe 6-9 mo.
The effect of iron supplementation on Hb was different at the two sites. In Honduras, supplementation had a significant effect on Hb (+9 g/L, Fe 4-9 and Fe 6-9 combined) and there was no difference in effect depending on whether supplementation was started at 4 or 6 months. In Sweden, supplementation from 4-9 months had a significant effect on Hb (+5 g/L, p=0.002) whereas the effect of iron from 6-9 months was not significant (+2 g/L, p=0.132).

Furthermore, the effect of iron supplementation on Hb was different at the two time intervals. At 4-6 months, iron supplementation increased Hb by 5 g/L compared to placebo, with no significant site difference (Fig 7). At 6-9 months, however, there was no significant effect of iron supplementation on Hb in Sweden, but a significant increase of 7 g/L in Honduras compared to placebo (Fig 7). Controlling for iron intake from complementary food did not significantly change these results. The effect of iron supplementation on Hb from 6-9 months was significantly modulated by the 6-month levels of MCV, ferritin, ZPP and TfR. In contrast, the effect of iron supplementation from 4-6 months was not significantly modulated by any of the indices of iron status at 4 months (Paper I).

**IDA**

Using a strict, multiple criteria definition of IDA (see above) there was a low proportion of IDA at 4 months (<3%), in all groups at both sites (Fig 8). The proportion of IDA in Honduras at 6 months was 9% in the supplemented compared with 19% in the two unsupplemented groups combined (p=0.135). The reduction in IDA at 9 months by supplementation from either 4 or 6 months was significant (p=0.006) and at 9 months there was no significant difference between the two supplemented Honduran groups (p=0.70). In Sweden, the proportion of IDA was still < 3% in the placebo group at 9 months, and there was no significant effect of iron supplementation on the prevalence of IDA at 6 or 9 months.

Neither in Sweden nor in Honduras did iron intake from complementary foods, which in Sweden on the average amounted to 44% of the iron supplement dose, have any effect on any of the main study variables or on the proportion of IDA at 9 months.

**Growth and morbidity**

There was no significant effect of iron supplementation on weight gain at either site (Paper II). Length gain did not differ significantly among groups in Honduras during any of the intervals. In Sweden, however, length gain from 6-9 months was significantly lower in the iron-supplemented groups than in the placebo group. Furthermore, length gain from 4-9
months in the two combined iron supplemented Swedish groups was significantly lower than in the placebo group (7.9 vs 7.2 cm, p=0.014, Fig 9) and this difference remained significant when controlling for baseline length or BMI at 4 months and/or maternal height. Similarly, with both sites combined, length gain from 4 to 9 months was significantly lower in the two iron-supplemented groups than in the placebo group, controlling for possible confounders (Paper II).

Change in head circumference from 4-9 months did not differ significantly among groups in Honduras, but in Sweden it was lower in the group given iron beginning at 4 months than in the placebo group (3.6 vs 3.9 cm, p=0.010, Fig 10).

There was no significant main effect of treatment group on the likelihood of illness during any of the age intervals, but there was a significant interaction of iron with initial Hb concentration: among infants with low Hb (<110 g/L) at 4 months, diarrhea was less common among those given Fe than in those given placebo at 4-9 months, but the opposite was true among those with Hb ≥110 g/L (Paper II). However, the difference in length gain among treatment groups in Sweden was not explained by differences in diarrhea incidence: when the diarrhea variable was included in the model for length gain, the results did not change.
Iron absorption

At 6 months, the mean fractional absorption of $^{58}$Fe from human milk was 16%, with no significant difference between iron-supplemented (12%) and unsupplemented (18%) infants (Fig 11). At 9 months, mean fractional iron absorption was significantly higher in unsupplemented infants (37%) as compared with iron-supplemented infants (17%, Fig 11). There was a significant increase in fractional iron absorption from 6 to 9 months in the placebo group ($p = 0.009$), but not in iron-supplemented infants (Paper III).

Similar changes were observed in iron absorption from the reference dose at 6 and 9 months. Fractional iron absorption from the reference dose was strongly correlated to the simultaneously measured iron absorption from human milk in the same individual ($r = 0.63$, $p < 0.001$, Paper III).

In order to determine whether the increase in iron absorption seen in unsupplemented infants was induced by decreased iron stores, correlations between different indices of iron status and iron absorption were calculated, but none of these were significant at 6 or 9 months of age (Paper III).

In contrast, dietary iron intake at 9 months was negatively correlated to iron absorption from human milk ($p = 0.031$) as well as from the reference dose ($p=0.001$, Paper III). In the subgroup of unsupplemented infants ($n=8$), there was a significant inverse correlation between complementary food iron intake and iron absorption from the reference dose (Fig 12).
Sex differences

The significant sex differences in iron status observed already at 4 months, remained throughout the study. In all infants at 9 months of age (n=214), a considerably larger proportion of boys, compared to girls were classified as having IDA (17% vs 2%, p < 0.001, Fig 13), using a multiple criteria definition based on conventional cutoff levels (defined above). At 9 months, boys (compared to girls) had 4 g/L lower mean Hb (p < 0.001), 3.5 fL lower MCV (p < 0.001), 20% higher ZPP (p = 0.007), 30% lower ferritin (p = 0.007) and 1.1 mg/L higher TfR (p = 0.002, Paper IV).

To determine whether or not these sex differences were iron-dependent, the interaction between sex and iron supplementation was calculated for the change in each variable between 4 and 9 months of age. This interaction was significant for Hb and TfR, even when controlling for birth weight, weight gain from 4-9 months, complementary food energy intake, study site, compliance and baseline value. In contrast, for MCV, ZPP and ferritin, the interaction between sex and iron supplementation was not significant, suggesting that the sex differences in these variables may be independent on iron (Paper IV).

At 9 months, the sex differences for MCV and ZPP remained when controlling for possible confounders e.g. iron supplementation, duration of iron supplementation, compliance, birth weight, postnatal weight gain, complementary food energy intake and study site (Paper IV).

For Hb at 9 months in unsupplemented infants, there was a significant interaction between sex and birth weight. Among unsupplemented infants with a birth weight of < 3500 g, boys (vs girls) had 9 g/L lower Hb (105 vs 114 g/L), the difference being significant even when controlling for possible confounders, whereas among unsupplemented infants with a birth weight of ≥ 3500 g, boys and girls did not have significantly different Hb (113 vs 114 g/L, Paper IV).

For ferritin at 9 months, there was a significant interaction between sex and site, indicating that the sex difference in ferritin was different in Honduras and Sweden. When studying the two sites separately, we found no significant sex difference in Honduras, while in Sweden there was a significant interaction between sex and birth weight, reflecting a larger sex difference in Swedish infants with lower birth weight (Paper IV).

For TfR, in iron-supplemented, compliant infants, there was no significant sex difference. Among unsupplemented infants with a birth weight of < 3500 g, boys had 3.3 mg/L higher TfR, and among those with a birth weight of ≥ 3500 g, boys had 1.6 mg/L higher TfR, the differences in both weight groups being significant even when controlling for other possible confounders (Paper IV).

Fig 13. Proportion of boys and girls classified as having IDA at 9 months. Error bars are standard errors of proportions. Conventional definition of IDA (see methods).
Reference ranges

At each of the three ages, the following sub-populations were defined:

1) "Unselected", defined as all unsupplemented infants.
2) "Iron replete", defined as those of the unsupplemented infants who fulfilled certain criteria for iron sufficiency (Paper V).
3) "Iron supplemented", defined as all iron supplemented, compliant infants. Within these, the Fe 4-9 and Fe 6-9 groups were considered separately.

Fig 14 shows mean values and 95% reference ranges (± 2 SD) for Hb in unselected, iron replete and iron supplemented infants at 4, 6 and 9 months. The coefficient of variation was relatively small in all groups, suggesting a tight homeostatic regulation of this variable. In unselected as well as iron replete infants, mean Hb decreased slightly from 4 to 9 months, probably reflecting a physiologic decrease. The reference range for unselected infants at all ages was wider than for iron replete infants, suggesting that there might exist a certain proportion of anemic infants in the unselected group.

The range for iron supplemented infants was also wider than for iron replete infants, possibly reflecting a more complete exclusion of anemic infants in the iron replete group. At 6 and 9 months, mean Hb was higher in the Fe 4-9 group than in any other group, consistent with the uniform Hb response to iron at 4-6 months (Fig 7). Despite different mean values, the –2 SD cutoffs at 9 months were very close to 100 g/L in iron replete and both groups of iron supplemented infants.

Similar graphs as Fig 14 were constructed for MCV, ZPP, ferritin and TfR (Paper V) and 2 SD cutoffs were derived, based on iron replete infants (Table 3). Due to an iron-independent site difference in MCV, this variable was treated separately at the two sites, and cutoffs were reported for Swedish infants (Table 3).

Using 2 SD cutoffs based on iron replete, breast-fed, unsupplemented infants, an alternative definition for IDA was tested: Low Hb in combination with at least one of the following: high ZPP, low ferritin or high TfR, based on the 2 SD cutoffs from Table 3. MCV was not included in this definition due to the site difference in this variable. Using this definition, < 3% of unsupplemented Swedish infants were classified as having IDA at 4, 6 and 9 months (Fig 15). There was no significant difference between intervention groups in Honduras at 4 or 6 months, but at 9 months, a larger proportion of unsupplemented infants (vs iron supplemented) had IDA (14% vs 3%, p=0.023, Fig 15).

Hb response

To evaluate the usefulness of Hb response to iron as a definition of IDA in infants, the response in Hb was studied in iron supplemented infants at two ages.

At 4-6 months, 48% of iron supplemented infants responded by an increase in Hb of > 5 g/L, with no significant difference between study sites (p=0.788). ROC analyses showed that Hb, MCV, ZPP, ferritin and TfR at 4 months did not have any significant predictive value for Hb response to iron, nor did birth weight or postnatal weight gain (Paper V).

At 6-9 months, the proportion of iron supplemented infants (group Fe 6-9) showing Hb response (> 5 g/L) was different in Sweden and Honduras (11% vs 63%, p<0.001). ROC analyses showed that Hb, MCV and ZPP at 6 months as well as birth weight, and postnatal weight gain were significant predictors of Hb response, whereas ferritin and TfR at 6 months were not (Paper V). Sensitivities of iron replete 2 SD cutoffs at 6 months (Hb < 100 g/L, MCV < 71 fL and ZPP > 75 μmol/mol heme) to predict Hb response > 5 g/L were low (44%, 60% and 36%, respectively) whereas specificities of the same cutoffs were higher (95%, 76% and 97%, respectively).
Fig 14. Reference ranges for Hb in different subgroups of infants at 4, 6 and 9 months. Means (±2 SD) "Unselected", defined as all unsupplemented infants. "Iron replete", defined as those of the unsupplemented infants who fulfilled certain criteria for iron sufficiency (Paper V). "Iron supplemented", defined as all iron supplemented, compliant infants. Within these, the Fe 4-9 and Fe 6-9 groups were considered separately.
Table 3. Suggested 2 SD cutoffs for iron status variables at 4, 6 and 9 months of age, based on iron-replete, breast-fed infants.

<table>
<thead>
<tr>
<th></th>
<th>4 mo</th>
<th>6 mo</th>
<th>9 mo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb</td>
<td>&lt;105</td>
<td>&lt;105</td>
<td>&lt;100</td>
</tr>
<tr>
<td>MCV</td>
<td>&lt;73*</td>
<td>&lt;71*</td>
<td>&lt;71*</td>
</tr>
<tr>
<td>ZPP</td>
<td>&gt;75</td>
<td>&gt;75</td>
<td>&gt;90</td>
</tr>
<tr>
<td>Ferritin</td>
<td>&lt;20</td>
<td>&lt;9</td>
<td>&lt;5</td>
</tr>
<tr>
<td>TfR</td>
<td>&gt;11</td>
<td>&gt;11</td>
<td>&gt;11</td>
</tr>
</tbody>
</table>

* Based on Swedish infants.

Fig 15. Proportion of infants with IDA in Sweden and Honduras at 4, 6 and 9 months of age, using an alternative definition of IDA.
Error bars are standard errors of proportions. IDA was defined as low Hb in combination with at least one of the following: high ZPP, low ferritin or high TfR, based on the 2 SD cutoffs from Table 3.
DISCUSSION
This randomized, placebo-controlled trial is the first study of iron supplementation of exclusively breast-fed infants starting at 4 or 6 months of age. The dual site design is unique, permitting assessment of iron status, growth and morbidity in two populations with large socioeconomic differences.

Iron deficiency anemia
Using a strict, multiple criteria definition of IDA, and conventional cutoffs, the proportion of IDA in unsupplemented infants at 9 months of age was 10-fold higher in Honduras (29%) than in Sweden (3%).

The high prevalence of IDA in Honduran infants supports the view that this condition is a significant public health problem in developing countries. Iron supplementation, starting at either 4 or 6 months, significantly reduced the incidence of IDA in Honduran infants. Considering the IDA outcome at 9 months, there was no significant advantage of giving the iron supplement from 4-9 months compared to 6-9 months, but our power to find a significant difference in the proportion of IDA between these two groups was low.

One possible explanation for the low prevalence of IDA in Swedish 9-month-olds is that Swedish infants might receive more iron from complementary foods. Indeed, mean complementary food iron intake of the Swedish infants in our study was significantly higher compared to the Honduran infants (0.44 vs 0.14 mg/kg/d), but we found no correlation between complementary food iron intake and various iron status variables at 9 months (Paper I). Furthermore, significant differences in iron status between Swedish and Honduran infants were seen already at 4 months of age. This observation is not likely explained by differences in dietary intake of iron, since all infants were exclusively breast-fed and breast milk iron concentration has been shown to be unaffected by maternal iron status.12

Even though only term infants with birth weights > 2500 g were included, the Honduran infants had significantly lower birth weight and larger weight gain from birth to 4 months, suggesting catch-up growth. Both of these factors had a negative impact on ferritin and ZPP at 4 months, placing the Honduran infants at higher risk for ID. We do not have information on maternal nutritional status during pregnancy, maternal smoking, timing of umbilical cord clamping or infections during the first 4 months of life, but these factors, possibly in combination with genetic factors, may also be important in explaining the differences in baseline iron status between the Honduran and Swedish infants.

Yet, the observed prevalence of IDA in unsupplemented, breast-fed Swedish infants at 9 months of age was lower than might have been expected. Assuming an average breast milk intake of 800 mL/d from birth to 9 months1 2 3 (with a generously assumed 50% bioavailability of iron26) and an average complementary food iron intake of 3.7 mg/d from 6-9 months (our data) with 10% bioavailability25, the amount of absorbed iron from birth to 9 months would be 67 mg, which is very low compared to the estimated need for 280 mg of absorbed iron during the first year of life.22 We suggest that iron requirements of healthy, Swedish infants are lower than previously assumed.

Growth and morbidity
Our results indicate that low-dose iron supplementation of breast-fed infants at 4-9 months of age had a negative effect on
linear growth and head circumference. This adverse impact was more evident in Sweden than in Honduras. The finding is consistent with the hypothesis that iron supplementation may pose greater risks in iron-replete than in iron-deficient populations, as baseline iron status was considerably higher in Sweden than in Honduras. Only one other study has shown a negative effect of iron supplementation on growth: among 47 iron-sufficient Indonesian children 12-18 months of age, weight gain (but not length gain) was significantly lower in those given iron (3 mg/kg/d) for 4 months than in those given placebo. The authors reported that the growth of the iron-deficient, anemic children in the same study was improved by iron supplementation, whereas growth of children who were iron-deficient but not anemic was unaffected. Other studies have demonstrated either a positive effect of iron treatment (supplementation or use of fortified foods) or no impact on growth of young children. However, in all of these studies, the children were past infancy (2-5 years old) or were not fully breast-fed. Aside from the Indonesian data reported by Idjradinata et al, most investigators have not differentiated between iron-replete and iron-deficient children when examining growth effects.

The mechanism(s) underlying the unfavorable growth effect observed in our study is unclear. The dose given (1 mg/kg/d, averaging 6-9 mg/d) is lower than the amount given in most other iron supplementation trials. Nonetheless, it is possible that the infants absorbed an excessive amount of iron, as we have demonstrated that iron absorption is not fully down-regulated even in iron supplemented infants (see below). Furthermore, the seemingly unregulated increase in Hb seen in infants given iron supplements between 4-6 months, may indicate that "blood growth" is increased in iron supplemented infants during this age interval at the expense of skeletal growth. In mice, hematopoietic growth factors have been shown to increase osteoclast activity, thus modulating bone growth. However, this would not explain the negative effects of iron on longitudinal growth observed at 6-9 months.

Greater morbidity in those given iron could impair growth, but in our analyses the morbidity variables did not account for the growth effects observed. Another possibility is that iron supplements may impair zinc absorption and thus adversely affect zinc status, which is known to be related to child growth. We did not observe any differences in plasma zinc among treatment groups in our study (unpublished data), but plasma zinc may not be an adequate indicator of zinc status. Lastly, iron is a pro-oxidant and thus may have a toxic effect as a producer of free radicals. Infants may be particularly prone to such effects because of their rapid rate of growth.

With regard to morbidity, iron supplementation had a beneficial effect on prevalence of diarrhea among infants with Hb < 110 at 4 months, but an adverse effect on prevalence of diarrhea among those with Hb ≥ 110 g/L. There was no impact on other morbidity outcomes.

**Regulation of Fe absorption**

At 6 months of age, there was no significant difference in iron absorption between iron-supplemented and unsupplemented infants and there was no correlation between iron status variables and iron absorption. This suggests that neither the dietary, nor the stores regulator is fully active at this early age (Paper III). At 9 months of age, mean fractional iron absorption from human milk was significantly higher in unsupplemented infants (37 %) than in iron-supplemented infants (17 %). This was expected, because
unsupplemented infants at 9 months had significantly lower iron stores than iron supplemented infants, as assessed by ferritin levels, and since low iron stores are known to up-regulate intestinal iron absorption in adults.\textsuperscript{11}

We did, however, unexpectedly find that iron absorption was not correlated with indices of iron status including plasma ferritin at 9 months. However, a significant negative correlation was found between dietary iron intake and iron absorption. This suggests that dietary iron intake \textit{per se} is an important regulator of iron absorption at this age, and not merely secondary to influencing body iron stores.

We found no evidence that the size of iron stores would be responsible for the significant increase in iron absorption, which occurred in unsupplemented infants between 6 and 9 months of age. Furthermore, we found evidence that the dietary regulator was active at 9 months but not at 6 months of age. This may suggest that the dietary regulator of iron absorption is immature in the 6-month-old infant, and is subject to developmental changes between 6 and 9 months of age. Several new transporters of iron have been found in enterocytes\textsuperscript{127} but little as yet is known about their expression in human infants. Recent data from animal studies, however, suggest that the expression of one iron transporter, DMT1, is not affected by dietary iron during early infancy, whereas it is in late infancy,\textsuperscript{128} which would support our findings.

Even though we found no correlation between iron absorption and various measures of iron status in this study, it cannot be excluded that such correlations might be possible to demonstrate in a larger cohort of infants with a wider range of iron status.

In an iron-replete adult man, absorption is reduced so that the size of iron stores does not increase further, even if iron supplementation is given for an extended period of time.\textsuperscript{11} We found that mean fractional iron absorption from the reference dose in iron supplemented infants was 7-9\% at 6 and 9 months of age, suggesting that iron absorption cannot be fully down-regulated even in iron-replete infants. If fractional absorption of the supplemental iron drops (about 8 mg/d) is 8\% in iron replete infants at 6-9 months of age, this would result in an absorption of 0.64 mg daily. This is supported by our observation that Swedish infants who received iron supplementation from 4 months, and who must have had generous iron stores at 6 months, yet had a continued positive effect on ferritin of iron supplementation between 6 and 9 months (Paper I).

**Sex differences**

At 4 months of age, we found lower Hb, MCV and plasma ferritin and higher ZPP and TfR in boys than in girls; all of these seemingly suggesting greater ID in boys. Sex differences in various iron status variables have been reported in a few previous studies of older infants, and have generally been attributed to possible sex differences in growth or diet.\textsuperscript{6,129} The observed sex differences at 4 months in our study cannot be attributed simply to growth-related factors, since they remained when controlling for birth weight and postnatal weight gain. Furthermore, dietary factors are not likely to be responsible, since all infants were exclusively breast-fed at this time.

At 9 months, the sex differences for MCV and ZPP were at least as large as at 4 months: boys had a mean of 3.5 fL lower MCV and 20\% higher ZPP compared to girls. The sex differences in MCV and ZPP remained significant when controlling for possible explanatory variables and were not affected by iron supplementation. At 9 months, Swedish boys had approximately 40-70\% lower ferritin values than girls, the
larger difference observed in infants with lower birth weight. This is compatible with our previous finding that Swedish 12 months old boys had 31% lower mean ferritin compared to girls. Since iron supplementation did not affect the sex differences in these variables, we conclude that the lower MCV values and higher ZPP values in boys, as well as the lower ferritin in Swedish boys, were not caused by ID. One possible explanation might be hormone-mediated differences in metabolism. It is known, for example, that serum insulin and leptin concentrations are different in male and female infants during this period, even when correcting for body weight or body mass index. Although the mechanism is not known, differences in lean versus fat body mass synthesis may indirectly affect the internal kinetics of iron metabolism. To our knowledge, such interactions have not been studied in experimental animals or human subjects.

In contrast to Swedish infants, Honduran infants showed no sex difference in ferritin at 9 months of age. This site-dependent sex difference was observed also at 4 months, suggesting that the sex difference in ferritin may be determined by genetic or maternal factors differing between populations. This may explain the contradictory findings in previous studies of cord blood ferritin, some of which have shown significantly lower values in boys compared to girls, and others which have not.

In unsupplemented infants with a birth weight of < 3500 g, boys had 9 g/L lower Hb than girls at 9 months, but there was no sex difference in Hb in infants with a birth weight of ≥ 3500 g. This suggests that the Hb difference in unsupplemented infants at 9 months may be explained by ID in boys, since low birth weight is directly associated with low iron stores at birth. Our findings are supported by the results from a study of healthy, British infants. In that study, no sex difference in Hb was seen at 8 months of age, but in a follow-up of the same infants at 18 months of age, significantly lower Hb was observed in boys, suggesting a greater risk for boys to develop IDA.

In unsupplemented infants at 9 months of age, boys had 1.6 – 3.3 mg/L higher TfR than girls, the larger difference being observed in infants with lower birth weight (<3500g). However, in iron-supplemented, compliant infants, there was no sex difference in TfR at 9 months. This suggests that boys - especially those with a lower than average birth weight - are at higher risk for ID, as measured by TfR, and that this can be prevented by iron supplementation.

Thus, the sex differences in Hb and TfR suggested that boys might be at higher risk for "true" ID. We did not find any evidence that this increased risk would be caused by sex differences in weight gain, food intake, erythropoietic activity, iron absorption or infections (Paper IV). Remaining possibilities include that girls may have higher iron accretion in utero and/or that girls may have lower basal iron losses than boys. It is not unlikely that such mechanisms to preserve body iron have developed especially in girls to protect them later in life from iron deficiency related to menstrual bleedings.

Cutoffs

Reference values

This is the first study reporting reference values for iron status variables in exclusively breast-fed infants at 4 and 6 months of age. The results have widespread implications, since exclusive breast-feeding is generally recommended during the first 6 months of life. The sample size in this study is smaller than in most other studies on reference values for other age groups, resulting in limited statistical power. However, the advantage
of our dataset is the strict definition of breast-feeding, the presence of an iron-supplemented as well as an unsupplemented group, and the wide range in iron status resulting from the two-country design, all of which are essential for evaluating iron status cutoffs during infancy.

As expected, reference values at 6 and 9 months were different depending on the approach used. We suggest that "iron replete" infants best represent healthy infants, since this approach theoretically would neither include any individuals with ID, nor would it be biased by excessive iron intake (Paper V). Suggested 2 SD cutoffs for iron replete infants at 4, 6 and 9 months are presented in Table 3. These were close to the 2 SD cutoffs of the iron supplemented infants for all variables except ferritin, for which iron supplemented infants would yield considerably higher cutoffs. This does not necessarily mean that iron supplemented infants have more "normal" ferritin concentrations in serum than iron replete infants. Since iron absorption in infants cannot be down-regulated effectively (see above) and since plasma ferritin is not homeostatically regulated, it is not surprising that iron supplemented infants had higher ferritin values.

For Hb, our suggested -2 SD cutoffs of 105-100 g/L (Table 3) are lower than the commonly used cutoff of 110 g/L at 6-12 months of age. Our results are supported by a study of 1175 unselected British 8-month-olds in which the -2 SD cutoff for Hb was 95 g/L.74 This is close to our suggested level of 100 g/L at 9 months, but our level may be more appropriate since we excluded infants with possible ID. The number of infants in the current study is too small to allow for accurate calculation of separate reference values for boys and girls, but there was no significant sex difference in mean Hb in iron replete infants (p=0.907).

For MCV, our suggested -2 SD cutoffs of 73-71 fL (Table 3), based on Swedish infants, are similar to those reported in a study of Finnish infants fed iron-fortified formula.75 The difference in MCV between Honduran and Swedish infants may be due to genetic or environmental factors which we have not identified. A methodological problem cannot be completely excluded, since MCV was the only variable that was analyzed with different methods at the two sites. We are not aware of any previous reports on geographical variations in MCV, but regardless of the reason, this may be important to consider in future studies.

For ZPP, our suggested +2 SD cutoffs of 75-90 μmol/mol heme (Table 3) are similar to the cutoff of 80 μmol/mol heme suggested for children of various ages.18

For ferritin, our suggested --2 SD cutoff of 5 μg/L at 9 months (Table 3) is considerably lower than the commonly used cutoff of 10-12 μg/L.

For TfR, our suggested +2 SD cutoff of 11 mg/L (Table 3) is similar to the +2 SD cutoff of 11.1 mg/L previously reported for 1-year-old infants, using the same assay.134

**Hb response**

Our results suggest that Hb response to iron given from 4 to 6 months of age is not a relevant criterion for ID (Paper V). One possible explanation for these results may be the persistence of fetal Hb (HbF) production. After birth, the production of HbF is gradually switched to adult Hb (HbA), but at 4 months of age, more than 5% of the newly synthesized Hb is still HbF.67 HbF synthesis may not be regulated in the same way as HbA. We have also suggested that the regulation of intestinal iron absorption is not yet mature at 4-6 months of age (Paper III).

In contrast to the results found for the younger age group, Hb response (> 5 or > 10 g/L) to iron given from 6 to 9 months of age may be a relevant criterion for IDA,
since low Hb, low MCV and high ZPP at 6 months as well as low birth weight and large postnatal weight gain were all predictive of Hb response.

The fact that the -2 SD cutoff for Hb (<105 g/L), had a high specificity (95%) but low sensitivity (44%) to predict Hb response to iron, suggests that there is a considerable overlap between the distribution of Hb in anemic (as defined by Hb response) and non-anemic (as defined by lack of Hb response) individuals. This overlap, combined with the absence of any known risk indicator capable of identifying individuals at risk for neurological or other disturbances, makes it difficult to choose an appropriate cutoff for clinical or epidemiological use. However, we suggest using the -2 SD cutoff (<105 g/L) at least until the validity of Hb response as a definition of IDA has been proved in infants of 6 months of age. Further studies, using neurodevelopmental or other functional outcome, are necessary to determine if mild "anemia" defined as a Hb response to iron of 5-10 g/L has any pathological significance.

We found that the erythrocyte variables (Hb, MCV and ZPP) were significant predictors of Hb response at 6-9 months, whereas the two plasma variables (ferritin and TfR) were not. Assuming that Hb response is a valid definition of IDA, this suggests either that ferritin and TfR at 6 months may be poor measures of iron stores or, alternatively, that the size of iron stores in the growing 6-month-old is poorly related to subsequent Hb synthesis. In the unselected, unsupplemented population, TfR did not increase until 9 months, which may suggest that TfR (and maybe ferritin) are not useful for diagnosis of ID until that age.

It was not surprising to find that growth variables were useful predictors of IDA at 6 months, as defined by Hb response to iron (Paper V). Our data suggest that breast-fed infants may be at risk for IDA when their weight has increased to >2.2 times the birth weight. Our study did not include low birth weight infants (<2500 g), but we found an increased risk for IDA in infants with a birth weight of less than approximately 3100 g. Since weight measurements are much less expensive than laboratory analyses, this information may be useful for targeting interventions.

**New definition of IDA**

Using a conventional definition of IDA, 29% of unsupplemented Honduran infants were classified as having IDA at 9 months (Fig 8). Of the Swedish unsupplemented infants, 20% had Hb <110 g/L and 29% had ferritin <12 µg/L at 9 months (Paper I), which is similar to previous results. However, using a strict, multiple criteria definition, the prevalence of IDA was only 2.9% (Fig 8). The discrepancy between the proportion of infants with low Hb or ferritin and the proportion with IDA motivates re-evaluation of the commonly used cutoff levels for infants.

Using the alternative definition based on 2 SD cutoffs (Table 3), 14% of unsupplemented Honduran infants and 3% (a single infant) of unsupplemented Swedish infants were classified as having IDA at 9 months (Fig 15). Of the Swedish unsupplemented infants at 9 months, only a single infant had Hb <100 g/L and this infant also had ferritin <5 µg/L at 9 months and was classified as having IDA. Thus, using our suggested cutoffs, the false positive rate of the Hb cutoff (proportion of anemic infants without IDA) was reduced from 86% to 0%.
CONCLUSIONS AND PERSPECTIVES

Iron supplementation of breast-fed infants?

Iron supplementation of term, breast-fed infants from 4-6 months to at least 9 months of age can effectively prevent IDA in a socio-economically disadvantaged population with a high prevalence of IDA. However, for several reasons, a cautious approach is warranted with regard to routine iron supplementation during infancy: 1) We found that iron-replete infants were unable to effectively down-regulate iron absorption. 2) We found a seemingly unregulated Hb response to iron in iron replete infants at 4 months. 3) We found a retardation of longitudinal growth and head growth in iron-supplemented Swedish infants.

Thus, in more affluent countries, and in those with a wide range in economic and nutritional status, universal iron supplementation of breast-fed infants may cause unwanted effects in a significant proportion of the population. Although targeting is costly and sometimes impractical, it may be a safer option in such situations.

National screening programs are not feasible because of high costs and a lack of sensitive and specific biochemical markers for ID, but smaller programs should perhaps be set up in areas containing a high number of at-risk children (e.g. inner cities with large numbers of socio-economically disadvantaged families).

Dietary recommendations

Exclusive breast-feeding is recommended until about 6 months of age. At this age, complementary foods should be introduced, but breast-feeding should be continued until at least 1 year of age.

We did not find any effects of complementary food iron intake on iron status, suggesting that the bioavailability of complementary food iron is lower than the bioavailability of iron drops given between meals. Furthermore, this suggests that, iron fortification of complementary foods is not likely to give any negative effects on growth, and can therefore be recommended also to iron replete populations. The optimal level of iron fortification of infant foods, however, remains controversial and a more detailed discussion on this topic is beyond the scope of this thesis.

Our results are compatible with the following general recommendations concerning appropriate complementary foods for the prevention of IDA at 6-9 months of age: Offer a wide variety of foods, ensure adequate vitamin C in meals, include iron-rich foods e.g. meat and iron-fortified foods, exclude foods that inhibit iron absorption (high fiber cereals, tea, coffee) and avoid cow's milk and unfortified formulas.

Definition of IDA

There is need for a complete re-evaluation of the laboratory criteria for IDA in infants. New cutoffs based on iron replete, breast-fed, unsupplemented infants are presented in Table 3. Notably, we suggest that the cutoff for Hb at 9 months is 100 g/L rather than 110 g/L and that the cutoff for ferritin at 9 months is 5 µg/L rather than 10-12 µg/L. We suggest an alternative multiple criteria definition for IDA: Low Hb in combination with one of the following: high ZPP, low ferritin or high TfR, based on the 2 SD cutoffs from Table 3. Further studies are needed to clarify the relation between laboratory criteria for ID and IDA and clinical outcomes such as impaired neurological
development in infants, on which the disease definitions should be based.

Considerations for future studies

Infant age
The reason why pediatrics has evolved as a separate specialty is that children are not "small adults". Likewise, infants are not small children, and even infants aged 4, 6 and 9 months are very different from one another in certain respects of iron metabolism.

We observed different patterns of regulation of iron absorption at 6 and 9 months of age. Furthermore, we have shown that at 4-6 months of age, Hb increases as a response to iron supplementation regardless of initial iron status, suggesting that Hb response to iron is not useful as a criterion of IDA in infants below 6 months of age, at least not in infants without severe anemia. This latter observation is important and should lead to a more cautious interpretation of Hb outcome in any study where iron supplementation is given to infants below 6 months of age.

Also, ferritin and TfR may have limited value for the diagnosis of IDA before the age of 9 months. This latter result was unexpected and needs to be confirmed in other studies.

Infant sex
We found significant sex differences in Hb, MCV, ZPP, ferritin and TfR at 4, 6 and 9 months, seemingly suggesting a higher prevalence of ID in boys. At 9 months, boys had an approximately 10-fold increased risk for being diagnosed with IDA. These differences remained when controlling for growth and diet. The sex differences in some variables (MCV, ZPP and ferritin) may be due to genetic or hormonal factors whereas the differences in some other variables (Hb and TfR) most likely reflect a truly increased risk for ID in boys.

Sex differences have to be taken into account in future studies on iron status in infants and there may be a need to develop sex-specific cutoff levels for some iron status variables.

Dietary iron intake
We have for the first time demonstrated the importance of the dietary regulator of iron absorption in infants. The impact of recent dietary iron intake needs to be taken into consideration in the design of future studies of iron absorption in infants, since dietary differences may at least partly explain the variable absorption rates found in previous studies. Moreover, the dietary regulator might prove to be a valuable compensatory mechanism in partly breast-fed infants with a low-iron diet. However, an up-regulation of iron absorption in infants with a low-iron diet may lead to a reduced effect of dietary interventions and may explain why we found no correlation between complementary food iron intake and iron status at 9 months in Swedish or Honduran infants.

Iron requirements of term, breast-fed infants
In this study, the observed prevalence of IDA in unsupplemented Swedish infants at 9 months of age was lower than theoretically expected, suggesting that iron requirements of healthy, term, breast-fed infants are lower than previously assumed.

As iron losses are small, iron requirements are determined by 1) baseline total body iron and 2) growth. Estimations of iron requirements of term infants are based on old studies of total body iron in newborns. The most referred study included only five term infants, of which
two had significantly lower total body iron than the others. If these two outliers (of which one probably had suffered a severe hemorrhage) were excluded, the results from that study would suggest a total body iron content of 90-100 mg/kg in a term infant, instead of 75 mg/kg, reducing estimated iron requirements during infancy by 25%.

Honduran infants, however, were at higher risk for IDA, suggesting higher iron requirements of term, breast-fed infants in a socio-economically disadvantaged population, possibly at least partly because of poor nutritional status of their mothers during pregnancy.

Further studies are needed to more accurately assess total body iron in newborns and infants at different risk for IDA. Novel techniques have to be developed to accurately and non-invasively measure total body iron using e.g. improved magnetic resonance imaging or magnetic susceptibility measurement. This would also be useful to validate ferritin and other variables as measures of iron status in infants.

Ultimately, determination of iron requirements should not be based on laboratory values or theoretical calculations. Instead, recommendations should be based on the minimal iron intake that prevents clinical symptoms of ID. More research is therefore needed to clarify the relation between dietary iron intake in infancy and clinical symptoms such as impaired neurological development. Since ID is a global public health problem and since the first year of life is a crucial period for growth and development of the central nervous system, this issue deserves high priority.

Risken för svenska barn att få järnbruistanemi vid 9 månaders ålder var liten: mindre än 3%. Studien ger alltså inte stöd för att svenska barn skulle behöva järndroppar. I Honduras, däremot, hade 29% av barnen järnbruistanemi vid 9 månaders ålder och denna risk kunde minskas till 9% genom att ge förebyggande järndroppar från 4 eller 6 månader ålder. Vi såg en något långsamare tillväxt hos barnen som fått järndroppar och denna skillnad var mer uttalad hos de svenska barnen (7 mm skillnad i långdtillväxt). Tendensen var dock så svag att vid 9 månaders ålder fanns ingen skillnad i långd, vikt eller huvudomfång mellan barnen som fått järn och barnen som inte fått järn. Blotta möjligheten för en negativ effekt på tillväxten, är dock ytterligare ett argument för att man inte bör utföra någon generell rekommendation att ge järndroppar till alla svenska barn. Vissa barn med hög risk för järnbrist, t.ex. för tidigt födda barn, behöver dock järndroppar.

Vi studerade även hur mycket av järnet i kosten som togs upp (absorberades) i tarmen. Vid 6 månaders ålder fann vi att endast 16% av järnet i bröstmjölkken togs upp. Vid 9 månaders ålder fann vi att absorptionen fortfarande var lika låg hos barnen som hade fått järndroppar. Barnen som inte hade fått järn, däremot, tog upp 37% av järnet från bröstmjölen. Vi fann att barn som fick mycket järn i kosten uppsvisade ett lågt jämnupptag och vice versa. Denna ”självreglering” kan delvis förklara att vi inte såg någon skillnad i risk för järnbrist mellan barn som fick olika mängder järn i kosten.

Ett oväntat fynd var att risken att utveckla järnbrist var betydligt större för pojkar än för flickor (10 gånger större), alltså det motsatta förhållandet mot vad som gäller hos tonåringar och vuxna. Detta kunde inte förklaras med skillnader i födelsevikt, tillväxt eller födointag. Ytterligare studier får avgöra om detta skall föranleda olika rekommendationer för pojkar och flickor under spädbarnsåret.

Slutligen har vi med utgångspunkt från vår studie gjort en genomgång av de laboratorie-kriterier som vanligtvis används för diagnostik av järnbruistanemi. Vi fann bl.a. att nedre normalgränsen för hemoglobin i blodet (”blodvärdet”) kan sänkas från 110 g/L till 100 g/L vid 9 månaders ålder. Samtidigt kunde vi konstatera att kriterierna för järnbruistanemi är osäkra och att ytterligare studier behövs för att koppla laboratoriefynden till kliniska symptomer, framför allt när det gäller hjärnans och nervsystemets funktion under det viktiga första levnadsåret.
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