Effects of iron supplementation on iron status, health and neurological development in marginally low birth weight infants.

Staffan Berglund
J.O.H.N.

Järn och Hälsa hos Nyfödda
# Table of Contents

**Table of Contents**
- Abstract vii
- Original papers vii
- Abbreviations viii

**Populärvetenskaplig sammanfattning**
- Bakgrund ix
- Metod ix
- Resultat x
- Slutsatser x

**Background**
- Iron metabolism 1
  - Body compartments of iron 1
  - The flux of iron 2
  - Iron homeostasis 4
  - Diagnostic tools of iron status 6
  - Iron deficiency (ID) 8
  - Iron metabolism in infants 9
- Iron deficiency in infants 11
  - Infants and children at increased risk of ID 11
  - Possible disadvantages of infant ID 12
  - Evidence of impaired neurological development 14
  - Benefits and risks of iron supplementation 16
- Low birth weight infants 18
  - Marginally low birth weight infants (MLBW) 18
  - Morbidity in MLBW children 19
  - Iron requirements in LBW infants 21

**Objectives** 25

**Subjects and methods** 26
- Participants and design 26
  - Study compliance 26
  - Discontinued cases 26
  - Exclusions and inclusions 27
  - Data collection 28
- Methodological considerations 28
  - Definitions and references 28
  - Power and statistical analyses 29

**Results** 30
- Participant characteristics 30
  - Perinatal morbidity 30
  - Proportions of prematurity and SGA 31
Abstract

Background Due to small iron stores and rapid growth during the first months of life, infants with low birth weight (LBW) are at risk of iron deficiency (ID). ID in infancy is associated with irreversible impaired neurodevelopment. Preventive iron supplementation may reduce the risk of ID and benefit neurodevelopment, but there is also a possible risk of adverse effects. More than 50% of all LBW infants are born with marginally LBW (MLBW, 2000-2500g), and it is not known if they benefit from iron supplementation.

Methods We randomized 285 healthy, Swedish, MLBW infants to receive 3 different doses of oral iron supplements; 0 (Placebo), 1, and 2 mg/kg/day from six weeks to six months of age. Iron status, during and after the intervention was assessed and so was the prevalence of ID and ID anemia (IDA), growth, morbidity and the interplay with iron and the erythropoietic hormones hepcidin and erythropoietin (EPO). As a proxy for conduction speed in the developing brain, auditory brainstem response (ABR) was analyzed at six months. In a follow up at 3.5 years of age, the children were assessed with a cognitive test (WPPSI-III) and a validated parental checklist of behavioral problems (CBCL), and compared to a matched reference group of 95 children born with normal birth weight.

Results At six months of age, the prevalence of ID and IDA was significantly higher in the placebo group compared to the iron supplemented infants. 36% had ID in the placebo group, compared to 8% and 4% in the 1 and 2mg/kg/day-groups, respectively. The prevalence of IDA was 10%, 3% and 0%, respectively. ABR-latencies did not correlate with the iron intake and was not increased in infants with ID or IDA. ABR wave V latencies were similar in all three groups. Hepcidin correlated to ferritin and increased in supplemented infants while EPO, which was negatively correlated to iron status indicators, decreased. At follow up there were no differences in cognitive scores between the groups but the prevalence of behavioral problems was significantly higher in the placebo group compared to those supplemented and to controls. The relative risk increase of CBCL-scores above a validated cutoff was 4.5 (1.4 – 14.2) in the placebo-group compared to supplemented children. There was no detected difference in growth or morbidity at any age.

Conclusion MLBW infants are at risk of ID in infancy and behavioral problems at 3 years of age. Iron supplementation at a dose of 1-2 mg/kg/day from six weeks to six months of age reduces the risks with no adverse effects, suggesting both short and long term benefit. MLBW infants should be included in general iron supplementation programs during their first six months of life.
Original papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals (I-IV)


Paper I is reprinted with permission from Pediatrics Copyright © 2010 by the AAP, paper II with permission from Pediatric Research Copyright © 2011 by Wolters Kluwer Health and paper III with permission from American journal of clinical nutrition Copyright © 2010 by the American society for nutrition.
**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>ADHD</td>
<td>Attention deficit/hyperactive disorder</td>
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<tr>
<td>AGA</td>
<td>Appropriate for gestational age</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>CBCL</td>
<td>Child behavioral checklist</td>
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<tr>
<td>EBF6w</td>
<td>Exclusively breastfed at 6 weeks of age</td>
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<tr>
<td>EPO</td>
<td>Erythropoietin</td>
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<tr>
<td>Hb</td>
<td>Hemoglobin</td>
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<tr>
<td>ID</td>
<td>Iron deficiency</td>
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<tr>
<td>IDA</td>
<td>Iron deficiency anemia</td>
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<tr>
<td>LBW</td>
<td>Low birth weight (&lt; 2500 g)</td>
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<tr>
<td>MCV</td>
<td>Mean corpuscular volume</td>
</tr>
<tr>
<td>MLBW</td>
<td>Marginally low birth weight (2000-2500g)</td>
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<tr>
<td>OR</td>
<td>Odds ratio</td>
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<tr>
<td>RR</td>
<td>Relative risk</td>
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<tr>
<td>SD</td>
<td>Standard deviation</td>
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<tr>
<td>SGA</td>
<td>Small for gestational age</td>
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<tr>
<td>TfR</td>
<td>Transferrin receptor concentration</td>
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<tr>
<td>TS</td>
<td>Transferrin saturation</td>
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<tr>
<td>VLBW</td>
<td>Very low birth weight (&lt; 1500g)</td>
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<tr>
<td>WPPSI</td>
<td>Wechsler preschool and primary scale of intelligence</td>
</tr>
<tr>
<td>Z-score</td>
<td>Standard deviation score</td>
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</tbody>
</table>

**Keywords**

Auditory brainstem response, behavior, breast feeding, cognition, erythropoietin, ferritin, growth, hemoglobin, hepcidin, human infant, iron, iron deficiency, iron deficiency anemia, iron status, iron supplementation, low birth weight, morbidity, neurodevelopment, nutritional requirements, randomized controlled trial.
Populärvetenskaplig sammanfattning

Bakgrund


En anledning till att järnbrist har blivit en stor fråga över hela världen är att flera tidigare studier har visat ett samband mellan tidig järnbrist och senare sämre neurologisk utveckling. Man tror att det är bristen på järn i hjärnan som kan vara förklaringen och dessvärre har några av dessa hjärnskador visat sig vara långvariga. Det är därför viktigt att förebygga järnbrist. Men situationen är mer komplicerad då flera ganska nya studier har visat att om man försöker förebygga järnbrist hos de som aldrig hade någon ökad risk att få det, ja då kan man få negativa effekter, till och med försämrad neurologisk utveckling.


Metod

Vi har genomfört en randomiserad studie där vi lottat 285 barn med marginellt låg födelsevikt till tre doser av järntillskott, givet under första levnadshalvåret. En grupp fick inget järn (Placebo) och två grupper fick järn i olika doser (1 respektive 2 mg/kg kroppsvikt och dygn). Vi har sedan undersökt barnen vid olika åldrar, senast vid 3.5 års ålder, för att se vilka
effekter járntillskott hade på blodprover, tillväxt och den neurologiska utvecklingen, mm.

**Resultat**

I det första arbetet visade vi att barnen som inte fick något järn hade ökad risk för järnbrist (36%) och järnbrist med blodbrist (10%) vid sex månaders ålder. Bland dem som fick 2 mg/kg/dygn hade bara 4% järnbrist och ingen hade järnbrist med blodbrist. Risken var extra stor för de som fick sin huvudsakliga näring via bröstmjölk, eftersom bröstmjölk innehåller lite järn.

I det andra arbetet undersökte vi om man kunde uppmäta skillnader i hörselnervens ledningshastighet (hjärnstamsaudiometri). Andra har föreslagit att den kan påverkas av järnbrist. Vi fann inga sådana skillnader utan konstaterade att hörselnervens ledningshastighet inte påverkades.

I det tredje arbetet utvärderade vi de olika mätmetoderna för järnbrist inklusive den nya variabeln hepcidin. Hepcidin har föreslagits vara ett viktigt protein i regleringen av järn i kroppen och vi kunde visa att hepcidin påverkas av järnbrist och järnöverskott, även hos nyfödda. Även det blodbildningsstimulerande hormonet EPO undersökedes och vi fann ett intressant samband med järntillgång i kroppen.

I det fjärde delarbetet utvärderade vi barnen vid 3.5 års ålder avseende IQ och beteendeproblem för att se om den tidiga järnbristen påverkat hjärnan. Vi fann inga skillnader i IQ mellan de olika grupperna men däremot en skillnad i förekomst av beteendeproblem. Barn som inte fått järn under första levnadshalvåret hade 4 gånger ökad risk att ha beteendeproblem, då de skattades av föräldrarna med hjälp av en ofta använd enkät.

**Slutsatser**

Från denna studie drar vi slutsatsen att barn med marginellt låg födelsevikt har ökad risk att få järnbrist under första levnadshalvåret och att járntillskott kan skydda mot det utan att medföra några negativa effekter eller biverkningar. Detta skyddar inte bara mot järnbrist mätt med blodprover utan minskar också risken för beteendeproblem i förskoleåldern. Vår studie är den första randomiserade studie som visat ett samband mellan tidig järnbrist och senare beteendeproblem.
Background

IRON METABOLISM

Iron is the fourth most common element in the earth crust and thought to be the main content of the earth core. Iron is also fundamental for life. Every adult human carries about 3.5-4 g of iron, representing about half a cubic centimeter. Yet this small amount of mineral, relative to the body weight, is essential, being a key building block in several proteins, distributed to three main compartments of the body:

**Body compartments of iron**

**Oxygen transport**

The main compartment of iron in the adult human (~30 mg Fe/kg body weight) is in the circulating blood, where each mature erythrocyte carries about 300 million hemoglobin molecules, containing four heme proteins, with one Fe$_2^+$ atom each. The oxygen carrying heme proteins are also represented by in myoglobin, the protein responsible for oxygen storage and transport in muscles (~4 mg Fe/kg).

**Other tissue proteins**

A smaller but yet as important fraction of the body iron (~2 mg Fe/kg) is present in various tissues as iron containing enzymes and other proteins. The complete list of functional iron-proteins is not understood, but iron is essential in electron transport proteins present in all body cells (i.e. cytochromes), activators of molecular oxygen (i.e. pexoxidases and catalases), and many others.

**Storage**

The third compartment of iron is the storage iron, making the body prepared for unexpected loss or changes in iron absorption. This is a “buffer” compartment, were large changes can occur between a state of iron depletion (~1-2 mg/kg) and repletion (~5-30 mg/kg), without affecting the functional iron compartments. The main storage proteins are ferritin and hemosiderin. Ferritin is found essentially in the liver, spleen, bone marrow, and muscles and each molecule can bind 2500-3000 iron atoms. In a well designed interplay between ferritin-containing cells and the transport protein...
transferrin, a small amount of iron is portioned to circulating plasma, bound to transferrin addressed to the various tasks of iron in the body.¹⁻⁴

**The flux of iron**

*Iron absorption*

Mammals have no mechanism for excreting iron. However, small passive losses (~14µg/kg/day) occur daily from cells lost in the gastrointestinal- and urinary tracts, as well as from the skin. Adding the losses from occasional small bleedings and for women menstrual bleedings, an average loss of 1-2 mg; representing 15-40 µg/kg/day is present in a basal state. Thus, this is also the required mean daily absorption that must take place to maintain iron homeostasis. Furthermore, if sudden losses occur, such as large bleedings from wounds, occult bleedings from tumors, gastric ulcers, and gastrointestinal infections, the absorption must be further up-regulated.

Iron is available in the intestine as heme-bound iron and non-heme iron, depending on type of diet. For heme-iron, representing about 10% of dietary intake, the mechanisms of absorption are poorly understood. However the absorption of heme-iron is 6-7 times more effective compared to non-heme iron, making it an important source. For non-heme iron the absorptive mechanisms are better understood: Non-heme iron is reduced to Fe²⁺ by ferrireductases in the brush border of the enterocyte. When reduced, the iron molecule is transported into the enterocytes via the divalent metal transporter 1 (DMT1). Much of the iron absorbed into the enterocytes is stored there, bound to ferritin and only a fraction is transported through the basolateral membrane by the transport protein ferroportin. This part of the transport is subject to regulation. Since enterocytes are at constant renewal and old cells are lost into the gastrointestinal lumen, ferritin bound iron, not transported further to the plasma will be lost with the desquamated cells.¹⁻⁴

*Erythrocyte-bound iron*

Once transported to the lumen by ferroportin, iron binds to transferrin, which has two binding sites for iron. About 350µg/kg/day of transferrin-bound iron is utilized in the bone marrow and used for erythropoiesis. This represents a daily ten times turnover of the transferrin bound iron, which at any moment is only about 35µg/kg. The transferrin-iron complex has high affinity to transferrin receptor 1 (TfR), located on the surface of the developing erythroid cell. The complex of TfR, transferrin and iron is absorbed into the cell by a well described, essential cycle, ending in incorporation of the iron molecule into the heme protein and secretion of the
empty transferrin protein back to the blood stream. The regulation of erythropoiesis is mainly mediated by erythropoietin (EPO), a hormone produced in the kidney as a response to tissue hypoxia.

Erythrocytes have a life cycle of \(~120\) days and old cells are phagocytosed by macrophages, mainly in the spleen (MPS-cells). The MPS-cells store iron bound to ferritin and release it to the blood by ferroportin, the same transport protein as enterocytes. The macrophage release of iron is the main source of circulating transferrin bound iron \(\sim 300\mu g/kg/day\).1-4

**Other targets of transferrin-bound iron**

The transferrin-bound iron not used for erythropoiesis is transported in similar manner to other cells in need of iron. If the circulating iron exceeds the needs, it is incorporated into storage cells, mainly in the liver hepatocytes. Also hepatocytes use TfR receptors as capturers of iron and ferroportin for excretion. For tissues separated from the blood such as the brain, the transport mechanisms are not yet understood. Transferrin bound iron cannot cross the blood-brain barrier and thus, other mechanisms must be involved. The proportions of iron compartments and the utilization of iron are summarized in Figure 1.

![Diagram of iron compartments and daily utilization of iron per kg of a human adult.](image)

*Figure 1. Iron compartments and daily utilization of iron per kg of a human adult.*
Iron homeostasis

The total body iron is regulated by changing iron absorption in the intestine and it has been shown, in adults, that the DMT1 and the ferroportin-transporters are correlated strongly to iron status. Furthermore, iron absorption is regulated by the degree of erythropoiesis and the rapid utilization between iron compartments indicates a well designed regulation. Yet, the actors of iron homeostasis and particularly their signaling and regulation are not fully understood. However, during the last decade, rapid progress has occurred within this research field, not least due to the discovery of the iron regulatory protein hepcidin.

Hepcidin

Hepcidin is produced in the liver, released into the blood stream and acts as an inhibitor of ferroportin (figure 2). Through this mechanism it can down regulate the release of iron from the enterocytes in the intestinal tract, the iron storage cells in the liver, muscles and spleen, or particularly the large flow of iron from the MPS-cells, trapping iron from phagocytosed erythrocytes.

Regulation of hepcidin

Most research on hepcidin is based on cellular or animal models and the clinical experience is poor. As of today there are five separately described, sometimes opposing regulators of hepcidin.

1. **Transferrin saturation.** Hepcidin synthesis increases as response to increased transferrin-bound iron, thus maintaining a relatively constant level of circulating iron. The mechanism by which this occurs is not fully understood, but includes the transferrin receptor 2 (TfR2), the HFE protein and the co-receptor HJV, all three associated with the disease hemochromatosis, characterized by an inappropriate iron absorption and tissue incorporation.

2. **Iron stores.** In a yet even less well understood pathway, including the protein BMP6, iron stores in the hepatocytes are integrated with the signal of transferrin saturation. Thus, a dual input both from iron stores and circulation iron affects hepcidin expression.

3. **Erythropoietic signals.** Hepcidin is reduced as a response to increased erythropoiesis, suggesting that the largest consumer of iron can affect absorption and cellular release. The association between iron absorption and erythropoiesis was known before the discovery of hepcidin. This association is of particular clinical interest since it has been suggested that
the hepcidin inhibiting effect from erythropoiesis is more powerful than the stimulating effect from iron stores, and might be an explanation why patients with hemolytic diseases, who typically has increased erythropoiesis and replete iron stores (iron loading anemias, e.g. thalassemia), suffer from poor down regulation of iron absorption.  

4. **Inflammation.** Hepcidin is increased in inflammation. The mechanisms have been partly described and include the cytokines Interleukin 1 and 6, which interact with hepatocytes and stimulate hepcidin expression. The physiological effect is that circulation iron is reduced to starve a potential microbe from iron. Clinically, this sometimes causes a problem in cases of prolonged autoimmunity and malignancy, where the reduced iron availability causes anemia of inflammation.

5. **Hypoxia.** In cellular models, hepcidin has also found to be decreased in situations of hypoxia, assumed to be in concordance with the effect of erythropoiesis.

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**Figure 2. Illustration of the iron regulatory effect of hepcidin (Hep) and its regulation.** Hepcidin expression in liver cells is stimulated by circulation transferrin bound iron (Fe-Tf), iron stores and inflammation and down regulated by erythropoietic activity and ID. Hepcidin down-regulates ferroportin (Fpn) in enterocytes, and iron-storing cells.
**Diagnostic tools of iron status**

From a clinical view point the ability to evaluate iron status in an individual is of great importance. There are several diseases associated with disorders of iron metabolism, and there is a need of reliable diagnostic tools. Depending on the question, several of the actors in iron metabolism could contribute with such information.\(^{10-12}\)

**Ferritin**

Functionally being the protein involved in iron storage, ferritin is a common tool for assessing iron stores, and the most accepted.\(^{12}\) Even though the underlying mechanism is not understood it is a fact that the level of serum ferritin is in equilibrium with body stores and thus used as an indirect measure. It is estimated that one µg/L ferritin corresponds to 120µg storage Fe/kg body weight.\(^{11}\) Ferritin concentration declines early in a state of iron deficiency (ID) and serum levels below 12-15µg/L indicates depleted stores and increased levels indicate iron overload.\(^{10}\) A limitation in the use of ferritin is that it is also an acute phase reactant, increasing in states of inflammation, unrelated to iron status. Another limitation is that it does not indicate the degree of functional iron depletion.\(^{10-12}\)

**Iron, transferrin and transferrin saturation**

Transferrin is increased in ID and serum iron levels are reduced. However there is a diurnal variation and the variables should be interpreted with caution, particularly since transferrin-bound iron represents less than one tenths of a percent of iron in the body. Calculating transferrin saturation (TS), i.e. the ratio of serum iron and the total binding capacity of transferrin (TIBC), gives a better but yet not completely reliable measure of available serum iron. TS is used both as an indicator of ID and iron overload.\(^{10-12}\)

**Serum transferrin receptors (TfR)**

Erythropoetic cells and other cells in need of iron signal by increased TfR concentration at the cell surface. Similar to ferritin, a proportional amount is assumed to be circulating in serum and can be measured. Increased TfR is associated with early tissue iron need. Further, it has been suggested that TfR concentration is not affected by inflammation, making it an important complement to ferritin. However international standards are missing and the assays are expensive. Furthermore, reference values in infants and children are scanty.\(^{10-12}\)
Mean corpuscular volume (MCV)

As iron availability in erythropoiesis reaches a critically low level, erythrocyte production is maintained but with decreased hemoglobin content, resulting in decreased erythrocyte cell size, measured as mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), or mean corpuscular hemoglobin concentration (MCHC), all late signs of ID.\textsuperscript{10-12}

Hemoglobin (Hb) and hematocrit

Since most of the body iron is bound to hemoglobin, a state of prolonged ID will result in decreased hemoglobin levels and erythrocyte volume fraction (EVF or hematocrit). Traditionally, hemoglobin measures have become the key measure of ID and international standards, reference- and cutoffs-levels are available, though they are repeatedly questioned.\textsuperscript{12} However, being an indirect measure if ID, anemia (low hemoglobin) should be interpreted with cautions and other causes considered.\textsuperscript{10-12}

Other indicators

\textit{Erythrocyte protoporphyrin (EPP)} is the precursor of heme and becomes elevated when iron availability is low.\textsuperscript{10-13}

\textit{Erythrocyte zinc protoporphyrin (ZPP)} is produced as zinc replaces missing iron in the formation of heme. It has a high sensitivity but poor specificity to identify ID.\textsuperscript{10-12}

\textit{Bone marrow iron staining} has been considered golden standard of measuring iron stores. However the procedure is far too complicated to be used clinically.\textsuperscript{10-12}

\textit{Hemoglobin response to iron supplementation} is a common practice to evaluate if present anemia is associated to ID. An increase of 10g/L in hemoglobin indicates prior deficiency. But the method has been questioned, particularly in infants.\textsuperscript{10-12, 14}

\textit{TfR/ferritin ratio}. The logarithm of this ratio has been suggested to correlate better than any individual measure to total body iron.\textsuperscript{15} However its advantage over ferritin alone has been questioned.\textsuperscript{10-12}
**Iron deficiency (ID)**

ID is considered present when available iron supply for physiological function is inadequate. It is the most common micronutrient deficiency in the world and a public health problem.\textsuperscript{10} The World Health Organization (WHO) estimates that the global prevalence of anemia is 25% (47% in preschool aged children) and advocate that ID is the most significant contributor.\textsuperscript{16-17} However estimating the prevalence of ID in the world or even in subgroups of populations is difficult, since the measures of functional supply are indirect, and difficult to interpret.\textsuperscript{12, 15}

The process of ID-development theoretically progress through three stages:
1. Storage iron depletion
2. Early functional deficiency (or iron deficient erythropoiesis)
3. Established functional deficiency (or iron deficiency anemia)

Separating two stages of functional deficiency underlines that there could be impaired tissue availability, before the Hb level declines, causing a risk of damage in other functional compartments of iron, i.e. the brain.\textsuperscript{12} The challenge lies in identifying this stage.

<table>
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<th>Biomarkers of iron deficiency</th>
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<td>Ferritin</td>
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<td>Normal</td>
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<tr>
<td>Storage iron depletion</td>
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<tr>
<td>Early functional deficiency</td>
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<tr>
<td>Established functional deficiency</td>
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| Table 1. Iron status indicators and their suggested change during stages of ID. |

**Biomarkers of iron deficiency**

There is an ongoing debate among researchers on which biomarkers best identify ID, particularly the second stage. In screening programs anemia alone is commonly used, although most agree that it is not specific, since anemia may result from other causes. Neither is it sensitive since ID in the two earlier stages by definition is overreached.\textsuperscript{12} Since functional deficiencies
are of most clinical concern, also ferritin has been questioned, being a marker of iron stores and not tissue availability. Furthermore, as mentioned, in presence of inflammation, ferritin cannot be trusted.\textsuperscript{10-12, 15} For remaining indicators, there are several pros and cons but a common problem is lack of international standards and established cut offs. Instead, the use of combinations of 3-4 biomarkers has been suggested, defining ID when 2 out of 3 or 2 out of 4 indicators are abnormal.\textsuperscript{12} Table 1 summarizes the available indicators of iron status and their theoretical change during stages of ID.

\textbf{Iron metabolism in infants}

Much of what is known of iron metabolism is based on research of adults and cannot be applied directly on children and particularly not on infants. The reasons for this are several.

In opposite to the stable distribution between iron compartments in adults, the rapidly growing infant experiences both large redistributions and an overall increase of tissue bound iron. Iron requirements are not restricted to the iron needed to compensate for small losses from skin and gastrointestinal tract, but also include the iron needed in the rapid expansion (growth) of erythroid mass and other tissues. Assuming a mean growth rate of 24 g/day during the first six months of life\textsuperscript{18} and 35 mg of functional iron per kg body weight corresponds to an iron requirement of \(~150\ \mu g/kg\) per day, compared to the estimated 15-40 \(\mu g/kg/day\) assumed in adults (see above).

To meet this, the infant with normal birth weight is born with elevated iron stores. The average iron content of a newborn is about 75 mg/kg,\textsuperscript{19} decreasing to about 45 mg/kg at one year of age.\textsuperscript{20} By redistribution of the iron compartments, these iron stores are enough to meet the needs during the first 4-6 months of life, with no exogenous iron needed, explaining why breastfed infants do not develop ID during their first half year of life; even though human milk has low iron content.\textsuperscript{5}

During this redistribution, not only are iron stores decreasing compared to the newborn. Also the hemoglobin concentration is decreasing during the first 6-8 weeks of life, making further iron available to the expanding erythropoietic mass and other growing tissues.\textsuperscript{21-22} Nevertheless, iron stores will be close to depleted at 4-6 months of life, and requirements of exogenous iron rapidly increase during the second half year of life, making term infants, at particular risk of ID from about six months of age (see below).\textsuperscript{20, 23} Figure 3 summarizes the redistribution of iron assumed during the first 12 months of life.
Infant iron regulation

Our understanding of the homeostatic mechanisms in infant iron metabolism is poor. Mostly knowledge are based on animal models and studies on adults. The validity of this approach has not been confirmed. Since there is a rapid, physiological change in iron compartments and erythropoiesis, the infant’s homeostasis is clearly different from that of adults and regulatory mechanism could theoretically be different, or even absent. For instance, it has been suggested that the ability to up regulate iron absorption, develop between six and 9 months of age and that it prior to six months of age is immature. Such immature regulation might be physiological since it would allow iron stores and erythropoetic mass to rapidly change without affecting absorption and cellular release, but it would also put infants at particular risk of iron overload. The role of hepcidin in infant iron metabolism has not been evaluated. Theoretically, absence or poor sensitivity for hepcidin could explain the immaturity suggested.

Assessing infant iron status

Defining what is “normal” iron status in the newborn infant is challenging due to rapid physiological changes. The levels of hemoglobin and indicators of iron status at one month can change rapidly until the next and
reference ranges must take age into account. Most commonly, iron status reference ranges have been constructed by examining the range in healthy infants, some exclusively breastfed and others given iron fortified formula or supplements, making the results diverging. However, for infants aged less than six months, such descriptive trials are few and the suggested ranges wide. The correlation with functional outcomes is not known.\textsuperscript{14, 23} Still, hemoglobin and ferritin are considered most efficient at least in monitoring effect of iron supplementation, but their cutoff values have been questioned.\textsuperscript{5, 27}

**IRON DEFICIENCY IN INFANTS**

Due to the rapid growth rate and high requirements, ID is the most common single nutrient deficiency in infants.\textsuperscript{10, 16} Since ID without anemia is difficult to assess (see above), the exact prevalence is unknown. In developed countries, with well nourished children, the prevalence of IDA is < 3-4% \textsuperscript{28-30} However the prevalence of ID among US children aged 12-35 months has been estimated to 9%, and several risk groups are identified, with even higher prevalences.\textsuperscript{31}

*Infants and children at increased risk of ID*

*Exclusively breastfed infants*

As discussed above, exclusively breastfed infants are at risk of having depleted their iron stores at about 4-6 months of age.\textsuperscript{23} If iron-enriched complementary food, infant formula or follow on formula is not added to the diet at that age; there is a substantial risk of established functional ID. At what age exogenous iron, either as iron fortified complementary food or supplementation, should be introduced into the infant’s diet is under debate.\textsuperscript{23, 32-33} Several studies have suggested that this risk is not present before six months of age, in healthy normal birth weight infants, and WHO does recommend exclusive breast feeding until six months of age, based on the numerous benefits that is associated with breastfeeding.\textsuperscript{28, 30} However, recently the American Academy of Pediatrics suggested that breastfed infants are at risk already from four months of age and recommended supplemental iron of 1 mg/kg/day to all breastfed infants receiving more than one half of their daily feeding as human milk, a recommendation that has been questioned.\textsuperscript{31, 33}
Preterm and LBW infants

Iron stores accumulate rapidly in the fetus during the third trimester to reach about 75mg/kg at term birth. Since this process is not complete when born before term, preterm infants are born with lower iron stores proportional to the body weight. Additionally, the postnatal proportional growth rate is mostly more rapid in preterm compared to term infants and preterm infants thus empty their stores of iron more rapidly. Similarly, SGA infants are considered at increased risk due to the rapid catch up growth. There are also trials suggesting lower iron stores in SGA, term infants. Recommendations for preterm/LBW infants are reviewed below.

Prenatal ID

Several factors are associated with fetal/neonatal ID.

**Severe maternal ID** is the most common cause of prenatal ID. Cord blood ferritin as a proxy of iron stores are correlated to the degree of maternal iron status. Infants of anemic mothers, even if not ID at birth, are at increased risk of postnatal ID.

**Maternal diabetes** increases fetal iron demand due to rapid fetal growth and increased erythropoiesis due to hypoxia.

**Maternal smoking and maternal hypertension** are other known risk factors.

Possible disadvantages of infant ID

As mentioned, anemia is the most common marker of ID. The symptoms of anemia are mostly none, but fatigue, palpitations, headache, dizziness, pale skin and insomnia are typical symptoms of severe anemia in adults. If present in infants, they constitute possible disadvantages. Anemia is also associated with impaired growth and lower work capacity in children and adults. But anemia is not the only manifestation of ID. Also immune dysfunction, pica, breath holding spells and restless leg syndrome are associated with ID. However of most concern is that ID in infants and children is associated with impaired neurodevelopment.

ID and brain development

Iron is essential for neurogenesis and in differentiation of brain cells. Simultaneously, brain growth is rapid during the last trimester of pregnancy and during the first two years of life, even more rapid than the rest of the
body. Not surprisingly, research in animal models, mostly rodents, has shown an association between ID in infancy and impaired brain development and function.38-39, 41-43

The effects on brain development are depending on timing, duration and severity of ID.38, 41-42 Several of the alterations in the brain, observed in animal models of ID are long lasting and do not reverse if iron therapy is added.39 The existence of a critical window of irreversible damage has been suggested.43 Furthermore, it has been suggested that iron is prioritized for erythropoiesis over other functions, and consequently, functional deficits in the brain may appear before IDA occurs.17, 38-39 There are three domains of brain function, that are most studied in ID animal models.

**Neurotransmitter metabolism.** ID alter the synthesis and catabolism of neurotransmitters such as dopamine, serotonin and norepinephrine.39, 42 Dopamine is important in regulation of higher functions such as cognition, emotion, reward and motivated behavior particularly active in striatum and the basal ganglia.41-42 In rodent models of moderate ID, dopamine and serotonin metabolism are altered, proportionally to the degree of ID. Some of these alterations persists into adulthood.39, 41 There is also evidence of behavioral alterations in these rats such as delayed sensory-motor reflexes, less exploration and more hesitancy, all associated with dopamine functions.39, 41 Recent studies in monkeys with infant ID showed similar effects of prolonged lower dopamine levels.39

**Morphological alterations and dendritic structure.** ID causes decreased dendritic growth and reduces the interneuronal connections in hippocampus, a structure central to recognition and memory.38-39, 42 The findings are accompanied by behavioral alterations (learning and trace conditioning), and persists into adulthood despite iron therapy.38, 41

**Myelination.** Oligodendrocytes, the cells that produce myelin, are particularly sensitive to ID. In rodent models, ID causes altered composition and amount of myelin in large regions of the brain and these changes persists.39, 42

**Human studies**

When applying the knowledge of ID and brain development from animal models on human infants, the situation becomes more complicated. Behavioral development is multi-factorial. An association between ID and development might be explained both by a direct damage to brain tissues and by an indirect effect on the child’s exploratory behavior or parental interactions.44 An association can also be confounded by disadvantages in
family structures, causing both increased risk of ID and behavioral impairment, without a direct causality. Figure 4 summarizes the possible associations between ID and child behavior as summarized by Osendarp.\textsuperscript{43} Below follow a summary of available research in human infants and toddlers with or at risk of ID.

![Figure 4. Possible explaining causalities of the association between iron deficiency and child behavior. Reproduced from Osendarp\textsuperscript{43} with permission.](image)

**Evidence of impaired neurological development**

There are a substantial number of trials and reports on the effect of IDA in infants and children. Fewer have examined infants with ID without anemia, mostly due to difficulties in identifying them. Most are cross sectional or longitudinal case-controlled trials and few are randomized interventions of iron supplementation. Furthermore, most studies investigated the neurological functions at time of, or short after ID/IDA and compared to controls but few have performed follow up studies, to assess long term consequences. There are several reviews of published trials.\textsuperscript{44-46}

**Case-controlled trials**

An association between IDA and neurological performance has been suggested in several of these trials. Most commonly, studies have shown impaired mental (cognitive) or motor achievements in infants and young children with IDA compared to controls.\textsuperscript{39, 45-46} In trials evaluating cognitive
scores, a difference of 6-15 points was seen in infants with IDA compared to iron replete infants. Differences in behavioral and social-emotional development has also been reported. IDA infants are found less pleased, more hesitant, less playful and with functional isolation. In trials performed on infants with prenatal ID (infants of diabetic mothers), infant were found to have poorer recognition memory and behavioral tests. From these case-controlled trials, a few include follow-up after treatment of the IDA. Most but not all reported persisting deficits in some domains of neurodevelopment, but the follow up period was mostly short.

More important from a public health perspective are the assessments of long-lasting effect by early ID and IDA. In follow ups of pre-school and school aged children with IDA in infancy, a few case-controlled trials suggest evidence of long-lasting impairment in cognition, behavior, motor development and increased educational problems. In a trial on Costa Rican infants, impaired cognitive and behavioral achievement was found at follow-up at five, 11-14 and 19 years of age in those who were diagnosed with IDA in infancy, compared to a iron replete control group.

A limitation of these case-control trials is that that infants with IDA are more likely to have socio-demographic risk factors and these can confound the results (figure 4). Although researchers have tried to statistically control for these confounders, uncertainty still remain of causality between ID and poor development, and the association has been questioned.

Neurophysiologic outcomes

To improve research of the association between ID/IDA and neurodevelopment, a few trials have analyzed neurophysiologic functions. Auditory brainstem response (ABR) is a measure of conduction speed in the auditory nerve, and a proxy of myelination of neurons. Case controlled-trials of infants with IDA compared to controls have shown prolonged ABR-latencies, suggesting an impaired myelination of the neurons. In one trial, the impaired latencies persisted, and even magnified at follow-ups until four years of age, suggesting an irreversible damage. Other case-controlled trials have shown alterations in EEG, eye-blinking patterns, and visual evoked potentials, all attributed to IDA.

Randomized controlled trials

Some randomized placebo-controlled trials (RCT) have analyzed the effect of iron supplementation of infants with already existing IDA. A few of those have shown a positive effect on mental, motor or language development. But
again, the results are diverging, possibly depending on the time, duration and severity of ID/IDA or the time and duration of supplementation.\textsuperscript{44} In a recent meta-analysis, Sachdev et al concluded that there is evidence of a small but significant benefit on mental development, but not in motor development.\textsuperscript{53} However they included all ages and found that the effect was lesser in young children and infants. As for mild/moderate ID without anemia there is insufficient data.\textsuperscript{44}

RCTs of preventive iron supplementation are few. In a recent meta-analysis, only five randomized trials assessing neurological development following preventive iron supplementation to infants below 3 years of age was identified.\textsuperscript{54} The authors concluded that none showed benefits in mental development while three trials showed positive effect on psychomotor development.\textsuperscript{55-59} None presented follow up data above 18 months of age. Similarly, Grantham-McGregor recently concluded that there is evidence of improved psychomotor development but not enough evidence to state causality between iron supplementation and improved cognitive development in infants and small children. With respect to long term effects, there is no available data.\textsuperscript{44}

**Benefits and risks of iron supplementation**

Given the suggested association between ID and impaired neurological development, iron supplementation is recommended to infants diagnosed with, or at risk of ID.\textsuperscript{10, 60} However humans have no ability to excrete iron and iron, being a pro-oxidant, is a potentially toxic drug. Thus, possible negative effects or overdosing must be considered, especially if supplementation or fortification is recommended in populations or parts of populations, where individual follow up is impossible.\textsuperscript{61} Notably, such negative (adverse) effects have recently been reported, causing concerns in the field.\textsuperscript{61-62}

**Anemia**

Most trials of iron supplementation to infants at risk of or with diagnosed IDA show increased hemoglobin levels. As discussed above, this has been suggested as a reliable measure of present IDA. However, an increase in hemoglobin concentration is in itself of no benefit to a child, unless it results in short or long term functional benefits.
**Growth**

A few studies on ID infants have shown a positive effect of iron supplementation on growth\(^6\) but a recent meta-analysis of 27 iron supplementation trials to children below five years of age showed no overall effect on growth.\(^6\) What is of more concern is that four trials have suggested a negative effect on growth when analyzing iron replete infants separately, underscoring the importance of identifying the correct risk groups for supplementation.\(^6\)-\(^9\)

![Figure 5. The balance of possible benefits and risks of iron supplementation to infants.](image)

**Infections**

Iron is also an essential nutrient to pathogens i.e. bacteria, fungi and viruses. As part of the immune response in humans, circulating iron is reduced, to reach less iron available to pathogens. Theoretically, this mechanism can be broken during iron supplementation and it has been suggested that iron supplementation can increase the risk of infections, supported by results from some clinical trials.\(^6\) Most recent evidence relate to the risk of Malaria. A large iron supplementation trial in Zanzibar had to be terminated due to increased risk of severe Malaria infection in supplemented infants.\(^7\) Also in this study, the adverse effect was most pronounced in those initially iron replete.\(^6\)
Neurological development

The possible positive neurological effects of iron supplementation to infants at risk of ID were reviewed above. However a very recent trial, have suggested that iron supplementation might also have adverse effects on neurodevelopment. Healthy Chilean infants were randomized to infant formula with different levels of iron fortification from six to 12 months of age (12.7 mg/L vs. 2.3 mg/L). At 10 years, infants fed the high iron formula had significantly lower scores on visual motor integration and memory, and a non-significant trend of lower IQ and arithmetic achievement. The negative effects were not present in those with lowest initial hemoglobin levels, further supporting the hypothesis that iron replete infants are at risk of adverse effects when given excess iron.71

Summary

Iron supplementation can benefit millions of infants and possible improve neurodevelopment if given to those at risk of ID. But due to possible adverse effects, identifying the correct risk groups for general iron supplementation requires large randomized trials and correct interpretations of the outcomes. The balance of pros and cons is summarized in figure 5.

LOW BIRTH WEIGHT INFANTS

Low birth weight (LBW) is defined as birth weight less than 2 500g. Unicef estimated in 2009 the global prevalence to 14% and 7% in industrialized countries.72 The prevalence in Sweden is 4.7% (Socialstyrelsen, unpublished).

A substantial amount of research and medical resources in industrialized countries, focus on the more severe forms of LBW, i.e. infants born very LBW (VLBW, < 1 500g) or extremely LBW (ELBW < 1 000g). As illustrated in figure 6, a majority of LBW infants are only moderately LBW (1500-2500g). Actually > 50 % of LBW infants have a birth weight of 2000-2500g, a group of infants rarely studies. Below we refer this group to marginally low birth weight (MLBW). The situation is similar when studying age categories of preterm infants. Of 12.3% preterm infants in US 2008, 8.8% were born late preterm (34-36 weeks of gestation).73

Marginally low birth weight infants (MLBW)

MLBW infants are a heterogeneous group, including infants born moderately preterm (31-33 weeks), late preterm (34-36 weeks) and term (> 37 weeks),
each category to varying degree including SGA infants. The MLBW SGA infants can further be categorized into intrauterine growth restricted (IUGR), where the small size is caused by poor nutrition in late gestation, but also constitutionally small infants with genetic predisposition for small size.

Due to this heterogeneity, the knowledge of MLBW infants is poor since they are mostly excluded from clinical trials of both term and preterm infants. However during recent years, MLBW and late preterm infants have been more prioritized. One reason being that it has been suggested that also this group is at risk for increased mortality and morbidity, both perinatally and later in life.\textsuperscript{74-75}

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{figure6.png}
\caption{Distribution of weight categories in US and Swedish LBW infants.}
\end{figure}

\textbf{Morbidity in MLBW children}

LBW is associated with increased disadvantages in life such as death during infancy and childhood, congenital anomalies, respiratory infections, neurodevelopmental impairment, school failure and growth problems.\textsuperscript{76} Even though the risk is lower in MLBW infants compared to those VLBW, it is still increased compared to normal birth weight infants.\textsuperscript{77}

\textbf{Neonatal morbidity}

Late preterm and MLBW infants compared to full-term infants are at increased risk of feeding disabilities, temperature instability, hypoglycemia,
respiratory distress, jaundice and septicemia. The mortality rate is 3-fold higher than for term infants.

**Neurodevelopmental impairment**

SGA and preterm LBW infants are associated with cognitive disabilities, ADHD and other behavioral problems. It has been suggested that LBW contributes to 14% of all cases of attention-deficit/hyperactivity disorder (ADHD). The risks increase with decreasing gestational age and birth weight. However several recent trials have suggested that compared to term normal birth weight infants, also moderately LBW infants have increased risk if impaired neurodevelopment. The increased risks have been attributed to perinatal complications such as intra-ventricular hemorrhage, sepsis, metabolic complications, painful procedures, and maternal separation. Another common explanation is that there are confounding socio-demographic factors causing both LBW and neurological problems. However the association between LBW and neurodevelopment persists in several trials even when adjusting for such confounders.

**Metabolic and cardiovascular risk**

Barker and colleagues showed in early 1990s that LBW was associated to cardiovascular disease and diabetes. The “Barker hypothesis” suggests that early fetal or perinatal starvation causes a disturbance in metabolic programming, resulting in increased morbidity later in life. Several trials have confirmed an association between LBW and obesity, cardiovascular disease, diabetes, hypertension, and other markers of metabolic syndrome and LBW is now considered a risk factor of later cardiovascular disease. However a recent meta-analysis concluded that there is no overall risk of obesity in LBW children suggesting that the association only relates to subgroups. Others have suggested that the growth acceleration, following prenatal or perinatal starvation might be the reason to inadequate metabolic imprinting, suggesting that the risk could be reduced by feeding interventions.

**Nutritional disadvantages**

Except for the practical feeding problems present due to immaturity or during neonatal morbidity, LBW infants require special feeding interventions in several domains. Multi-component fortifier is recommended to infants born < 32 weeks of gestation and special pre-term formula to non-breastfed VLBW infants. However for MLBW and late preterm infants, WHO
concluded in a report from 2006 that there is insufficient data to make recommendations on preterm infant formula content, Vitamin D, Phosphorus, calcium, Vitamin A, Zinc or multi-component fortifiers. For iron, WHO recommends supplementation from 6-8 weeks of age but conclude that there is a paucity of data on its effect on mortality, morbidity, and neurodevelopment particularly in MLBW infants.98

**Iron requirements in LBW infants**

As discussed above, preterm and SGA LBW infants theoretically deplete their iron stores earlier than term, normal birth weight infants due to rapid growth and lower iron stores at birth. This is supported by the clinical experience all around the world, where VLBW infants receive iron supplements to adjust iron stores and hemoglobin levels. Early research on bone marrow aspirates in preterm infants suggested that age of depletion is around 1-3 months of age followed by a fall in Hb-levels, referred to as late anemia (figure 7).34, 99 However, evidence for beneficial effect of iron supplementation to LBW infants is limited to a few trials mostly from the 1960s - 1980s. The knowledge of dose, age to start supplementation, duration, as well as the long term consequences is unsatisfactory.98, 100 This is of particular concern since it has been suggested that preterm infants exert no control over iron absorption and are at particular risk of iron overload.34

![Iron stores diagram](image)

*Figure 7. Development of anemia of prematurity has been suggested to appear in two steps, first the physiological drop in Hb-levels at birth and second a late phase of anemia suggested to be caused by depleted iron stores. Modified from Shaw et al.34 with permission.*
Identified trials assessing iron supplementation to LBW infants are summarized in table 2. In brief, there is evidence from developed countries that LBW infants < 2000g are at increased risk of developing IDA during the first six months of life, and that iron supplementation starting at about 4-8 weeks of life, has a preventive effect.

**MLBW and late preterm**

MLBW infants are included in several trials but we found only one trial analyzing MLBW infants separately. In that, the prevalence of ID in MLBW breastfed infants was 75% at six months compared to 22 % in the group of infants fed iron-fortified formula (8mg/L). However, only 26 MLBW infants were analyzed.\(^{101}\) In opposite, Halliday et al analyzed moderately and late preterm infants of 28-36 weeks of gestation and mean birth weight 2020g. They found no effects of iron supplementation from 7 days of age, or 8 weeks of age compared to placebo solution including only ascorbic acid.\(^{102}\) In secondary analyses they compared late preterm infants (33-36 wk) with moderately preterm infants (28-32 wk), and found iron status changes during the first half of infancy to be very similar, suggesting that iron stores deplete similarly in the two subgroups.\(^{103}\) In contrast, Lundström showed that risk of anemia increase with decreasing birth weight.\(^{104}\)

**Diverging recommendations**

The paucity of conclusive data from MLBW infants is underscored by the diverging recommendations to this group. A recent questionnaire sent to all Swedish neonatal centers revealed that MLBW infants are prescribed iron in doses from 0 – 2.2 mg/kg/day during the first six months of life. None of the centers recommended prolonged supplementation beyond six months of age (unpublished). Similar disparity of policies were reported from a British survey of 57 neonatal units,\(^{105}\) and in a retrospective case-control trial of LBW infants in New Zeeland.\(^{106}\) In 2003, the British recommendations were to restrict iron supplementation to LBW infants below 1800g,\(^{107}\) while the American Academy of Pediatrics (AAP) recommend 2 mg/kg/day from 1-12 months of age to all LBW and preterm infants who are breastfed and 1 mg/kg/day to those formula fed.\(^{108}\) The ESPGHAN Committee on nutrition recommended an intake of 2-3 mg/kg/day to preterm infants with no separate recommendations to late preterm or MLBW infants and without specifying how this could be interpreted in clinical interventions.\(^{109}\)
<table>
<thead>
<tr>
<th>Study</th>
<th>Included infants</th>
<th>Objective</th>
<th>Outcome</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>James 1960&lt;sup&gt;110&lt;/sup&gt;</td>
<td>205 LBW &lt;2000g</td>
<td>Intramuscular iron (250mg x 5) when body weight was 2000g vs. no treatment (Randomized)</td>
<td>Hb from 1-6 mo and incidence anemia → 6 mo</td>
<td>Sign diff. in Hb from 2-6 mo** Incidence of anemia until 6 mo: 0 vs. 90%**</td>
</tr>
<tr>
<td>Hammond 1960&lt;sup&gt;111&lt;/sup&gt;</td>
<td>66 preterm 1300-2500g</td>
<td>Intramuscular iron (200-400mg) vs. no treatment (Randomized)</td>
<td>Iron status and incidence of Anemia → 12 mo</td>
<td>Sign diff. in Hb from 3-12 mo* Incidence of anemia until 6 mo: 9 vs. 23%*</td>
</tr>
<tr>
<td>Gorten 1964&lt;sup&gt;112&lt;/sup&gt;</td>
<td>145 preterm mean 1.9kg</td>
<td>Fortified formula (12mg/L) vs. non fortified formula from birth (Randomized)</td>
<td>Hb from 1-18 mo and incidence of Anemia → 18 mo</td>
<td>Sign. difference in Hb from 3*, 4 – 18 mo** Incidence of anemia until 6 mo: 2 vs. 35% * (Large drop-out)</td>
</tr>
<tr>
<td>Lundström 1977&lt;sup&gt;114&lt;/sup&gt;</td>
<td>177 LBW breastfed, 1050-2000g</td>
<td>2mg/kg/d oral suppl. from 2 wk to 6 mo vs. no iron. (Randomized)</td>
<td>Hb, iron status and incidence of Anemia → 6 mo</td>
<td>Sign. difference in Hb, MCV, TS, ferritin from 3 mo. Anemia in 0 % vs. 77% **</td>
</tr>
<tr>
<td>Halliday 1983&lt;sup&gt;112&lt;/sup&gt;</td>
<td>49 preterm mean 2020g, 33.1 weeks</td>
<td>12mg/day from 7 days of age vs. 8 weeks vs. ascorbic acid (Randomized)</td>
<td>Iron status → 12 mo</td>
<td>No sign. effect on Hb, iron, TS, transferrin or ferritin</td>
</tr>
<tr>
<td>Iway 1986&lt;sup&gt;111&lt;/sup&gt;</td>
<td>45 LBW 1000-2499g (26 MLBW)</td>
<td>15 breastfed vs. 30 formula fed (8mg/L). (Case-control)</td>
<td>Iron status at 6 mo and incidence of ID → 6 mo</td>
<td>Sign. difference of Hb**, MCV**, Ferritin** and ID (86 vs. 33%)**. No difference in RBC, TIBC and TS. MLBW: 75% vs. 22% ID*</td>
</tr>
<tr>
<td>Olivares 1992&lt;sup&gt;113&lt;/sup&gt;</td>
<td>84 LBW mean 2194g</td>
<td>3 mg/kg/day oral from 2-4 mo vs. no suppl. Stratified in 3 groups; Preterm/SGA, P/AGA, or Term/SGA (Randomized)</td>
<td>Hb and ferritin at 4 mo</td>
<td>Sign. difference in Hb and ferritin for Preterm/SGA and Preterm/AGA*, but no difference in Term/AGA</td>
</tr>
<tr>
<td>Hall 1993&lt;sup&gt;114&lt;/sup&gt;</td>
<td>79 LBW formula fed, &lt; 1800g</td>
<td>Formula of 15 vs. 3 mg/L from 21 days until discharge. (Randomized)</td>
<td>Iron status at discharge and 8 weeks post discharge</td>
<td>Ferritin, MCV sign. higher at discharge* and 8 wks post** Prevalence low ferritin 8 weeks post discharge lower**</td>
</tr>
<tr>
<td>Borigato 1998&lt;sup&gt;115&lt;/sup&gt;</td>
<td>45 preterm mean 1935g</td>
<td>Potatoes cooked in iron pots vs. aluminum ports from 4-12 mo of age, added to suppl. of 2mg/kg/day (Randomized)</td>
<td>Iron status and prevalence anemia at 12 mo</td>
<td>Sign. effect on Hb*, hematocrit*, MCV*, EPP* and ferritin** Anemia in 36 vs. 74%*</td>
</tr>
<tr>
<td>Griffin 1999&lt;sup&gt;116&lt;/sup&gt;</td>
<td>81 preterm &lt;1750g</td>
<td>Infant formula of 5 vs. 9 mg/L (Randomized)</td>
<td>Iron status at 6 mo</td>
<td>No sign. differences</td>
</tr>
<tr>
<td>Friel 2001&lt;sup&gt;117&lt;/sup&gt;</td>
<td>58 LBW formula fed, &lt; 2500g</td>
<td>Formula of 20.7 vs. 13.4 mg/L from discharge (Randomized)</td>
<td>Iron status and developmental index at 12 mo</td>
<td>No sign. differences</td>
</tr>
<tr>
<td>Thom 2003&lt;sup&gt;106&lt;/sup&gt;</td>
<td>81 LBW &lt; 2500g</td>
<td>Low vs. high iron intake past month (Case control)</td>
<td>Prevalence ID at 10 mo</td>
<td>Sign. increased risk of ID if low iron intake. OR 13.4 **</td>
</tr>
<tr>
<td>Aggarwal 2005&lt;sup&gt;118&lt;/sup&gt;</td>
<td>73 SGA, term Breastfed, &lt;2500g</td>
<td>3mg/kg/day vs. no supplementation (Randomized)</td>
<td>Hemoglobin at 4 wk, 8 wk, and after 2 mo</td>
<td>Sign. difference at 4 wk and 8 wk* but not on follow up</td>
</tr>
<tr>
<td>Franz 2000&lt;sup&gt;119&lt;/sup&gt; Steinmacher 2007&lt;sup&gt;120&lt;/sup&gt;</td>
<td>133 LBW &lt; 1301 g</td>
<td>2-4 mg/kg/d as soon as enteral feeding tolerated vs. start at 61 d of age (Randomized)</td>
<td>Ferritin and incidence ID at d 61. Neurodevelopment at 5.3y</td>
<td>No difference in ferritin and 15 vs. 40% ID** at day 61. Non-sign. trend of improved cognitive and psychomotor development at 5.3y. No effect on behavior.</td>
</tr>
</tbody>
</table>

Table 2. Identified randomized or case-controlled trials assessing iron supplementation to LBW and preterm infants. *p < 0.05. **p < 0.001
SUMMARY AND UNSOLVED QUESTIONS

Iron is essential to humans, distributed between three main compartments of the body; oxygen transport proteins, other tissue proteins and storage. The hormone hepcidin regulates the release from stores and enterocytes. Present diagnostic tools of iron status are difficult to interpret since they are collected from plasma or serum, which is only a proxy of what happens in the tissues in need of iron. This is unsatisfactory, since ID is the most common micronutrient deficiency, in need of trustable diagnostic tools.

**Novel diagnostic tools of iron availability, particularly to detect early functional ID needs improvement and those present needs to be evaluated based on functional outcomes.**

The iron homeostasis in infants is different from adults in needs of exogenous iron and in the proportions of compartments.

**The mechanisms of iron regulation, the role of hepcidin and the validity of iron status indicators in infants are poorly known.**

The need of iron for tissue growth in infants is \(~10\) times greater per kilogram than for adults and infants are at increased risk of ID. ID in infancy is associated with poor neurological development, supported primarily by research on animal models, but also from case control trials, showing an association of ID in infancy and impaired neurodevelopment. But since a disadvantaged environment might be a confounder of this association there is no undisputable causality proven. A few randomized trials have suggested causality between ID and impaired short term psychomotor development but none on cognition or behavior. Other trials have shown possible adverse effects on growth, infections, and even cognitive development if iron is given to those already replete.

**There is no proven causality between ID in infancy and impaired long term neurodevelopment, particularly cognitive or behavioral.**

LBW infants are the largest risk group of ID in developed countries. But this is confirmed only in those with moderately or very LBW (\(<2000g\)). The most common LBW infants are those born with marginally low birth weight (MLBW).

**It is not known if iron supplementation benefit or harm MLBW infants.**
Objectives

The general objective of this trial was to test the hypothesis that marginally low birth weight infants (MLBW, 2000-2500g) benefit from iron supplementation given during their first six months of life.

The specific objectives were to assess possible benefits and adverse effects of iron supplementation from six weeks to six months of age, given to healthy, Swedish infants, with regard to the following outcomes (respectively paper in parenthesis):

- Iron status, growth and morbidity until six months of age (I).
- Auditory brainstem response at six months of age (II).
- Hepcidin, erythropoietin and its interplay with iron status from six weeks to six months of age (III).
- Cognitive and behavioral effects at 3.5 years of age (IV).
Subjects and methods

PARTICIPANTS AND DESIGN

This was a prospective, randomized, placebo-controlled, double blinded trial including 285 healthy MLBW infants and 95 term, normal birth weight controls born in Umeå (n=44+15) and Stockholm (n=241+80) between March 2004 and November 2007. In brief, the 285 MLBW infants were stratified for sex and study center and randomized to three different doses of iron supplements, given as oral drops between six weeks and six months of age (Figure 8).

![Study design diagram](image)

Figure 8. Study design.

Study compliance

Of the included infants, 24 (8%) dropped out during intervention and another 18 (6%) until the follow up at 3.5 years. Compliance to the intervention was measured by a daily checklist, filled in by the parents and by weighing the bottles. In 62 cases (22%), the compliance to the intervention was <70%, of which 43 infants belonged to the iron supplemented groups. Poor compliance was equally distributed among the groups.

Discontinued cases

Infant iron status was assessed at six weeks and 12 weeks of age. Due to ethical considerations, infants diagnosed with anemia by then, were further evaluated by a pediatrician. Those with suspected IDA were prescribed iron supplementation and thereby discontinued the intervention. However, they continued as unblinded participants and were included in some analyses (see
below). Thirteen infants were prescribed iron at six weeks and 9 infants at 12 weeks of age.

Figure 9. Trial profile of the participants when analyzing according to the intention to treat principle in non anemic MLBW infants (Paper I, II and IV).

**Exclusions and inclusions**

The primary outcomes, presented in paper I, II and IV, assessed the group effect in infants non anemic at baseline (preventive trial). Thus the 16 infants with anemia at six weeks were excluded. The analyses were preformed according to the intention to treat principle, including also discontinued cases. During the intervention, another two infants were diagnosed with blood disorders (beta-thalassemia and ABO-immunization) and were excluded from all analyses. Another child was diagnosed with an autism-disorder (22q11-deletion syndrome) preceding the follow up and was excluded in analyses at 3.5 years. The trial profile in figure 9, illustrates the participant flow in these main analyses. In paper III and in some secondary analyses of the ‘per protocol’ group-effect, other inclusion and exclusion criteria were used as summarized in table 3.
### METHODOLOGICAL CONSIDERATIONS

#### Definitions and references

ID was defined using a combination of criteria. Ferritin, MCV, TS and TfR was analyzed at 12 weeks and six months and ID was defined when two out of these four biomarkers were outside the reference range (Paper III, table 1). In concordance, infants with none of the biomarkers outside these cutoffs were considered iron replete (IR). The cutoffs were chosen based on term, normal birth weight infants since the “null-hypothesis” of the present trial
was that MLBW infants similar to term, normal birth weight infants are not at risk of ID during the first six months of life.

SD-scores (z-scores) for anthropometric data were calculated, corrected for gestational age at birth, using a recently published Swedish growth reference with continuous standards from 24th week of gestation to 24 months of age. SGA was defined as birth weight 2 SD below normal (z-score < -2).

**Power and statistical analyses**

A prestudy power analysis showed that a group size of 64 would detect a difference of 0.5 SD between 2 groups, corresponding to a difference at six months of 4 g/L in mean Hb (expected mean ± SD 119 ± 7 g/L²⁸), 1 cm in length (expected mean ± SD 67.5 ± 2.4 cm⁶⁶), 0.1 ms in CCT (expected mean ± SD 4.55 ± 0.2 ms⁵¹) and 5% in IDA prevalence and a difference at 3.5 years of 7.5 points in IQ-scores. An actual group size of 95 was decided to allow for a dropout rate of 20% and a poor compliance rate of 15%.

Two factor ANOVA and Bonferroni post hoc test were used when comparing mean and Fisher’s exact test was used when comparing proportions. To explore possible confounders, baseline and background variables were tested in univariate and multivariate models. Continuous outcomes with a non-normal distribution (ferritin, hepcidin and EPO) were log-transformed in all calculations and transformed back for presentation as geometric means and SD. The distributions of morbidity outcomes and CBCL-scores were as expected severely skewed and analyses were performed using Kruskal Wallis rank sum test.

<table>
<thead>
<tr>
<th></th>
<th>Intervention</th>
<th>Follow up</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6 wk</td>
<td>12 wk</td>
</tr>
<tr>
<td>Blood sample</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Anthropometry</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Food diary</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Morbidity checklist</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Auditory brainstem response</td>
<td>← Continuous</td>
<td>→</td>
</tr>
<tr>
<td>Morbidity questionnaire</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>WPPSI-III (Cognitive score)</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Child behavioral checklist</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>

*Table 4. Summary of data collection during and after intervention, for details, see each paper (I-IV).*
Results

PARTICIPANT CHARACTERISTICS

Baseline and background characteristics of participants are presented in each paper separately. The effectiveness of randomization was successful, i.e. we found no baseline or background variables with significant differences between the randomization groups, neither when analyzing all included infants (n=283), those included in the intention to treat analyses (n=267) or those remaining at six months and 3.5 years respectively (I-IV).

![Bar chart showing neonatal morbidity in analyzed MLBW infants (n=267). There was a significant difference between preterm and term infants in all prevalence's but hypoglycemia (p < 0.001).]

**Perinatal morbidity**

Only infants healthy at six weeks of age were assigned. However, minor perinatal morbidity was common and 45% of infants had been admitted to neonatal ward. The perinatal diagnoses found in the delivery records are summarized in figure 10. There was a significant difference between term and preterm infants in proportions of perinatal diagnoses (p<0.001), suggesting stratified secondary analyses for the main outcomes.
**Proportions of prematurity and SGA**

As assumed, the MLBW infants represented a heterogeneous group. The degree of prematurity and growth restriction was widely varying as illustrated in figure 11. However notably, there are no clusters of typical subgroups but rather a continuum of different degree of prematurity and growth restriction, suggesting several possible subgroups and cutoffs in stratified analyses.

![Figure 11. Gestational age and SD-score of birth weight for all 285 assigned MLBW infants.](image)

**Socio-demographic background**

There was an overall high educational level of the parents. Only 1.3% of MLBW mothers and 4.5% of fathers had an education ≤ 9 years (Swedish elementary school) while 61% and 59%, respectively had ongoing or finished studies at university level at the time of inclusion. This could be interpreted as an inclusion bias; however we find it more likely to reflect the high educational level in these university cities, since the general acceptance to participate was high and the proportions were similar in the reference group. There was a minor proportion of non-Scandinavian parents (20%), equally distributed between the groups (p=0.853), most commonly from Iraq (2.6%), Thailand (1.1%) and Bosnia (1.1%).
Dietary habits and sources of iron

There are no specific recommendations on feeding practices to MLBW infants in Sweden and the parents were encouraged to follow standard Swedish recommendations. Figure 12 illustrates the feeding practices at different ages. There was a high proportion of breastfed infants at six and 12 weeks, whereof > 50% exclusively breastfed. At six months, 67% were breastfed but only 6.8% exclusively.

From the 3-days food diaries we calculated the mean iron contribution from the three dietary sources at each age (Paper I, table 3). The mean dietary iron intake during the intervention was 0.32 mg/kg/day and ranged from 0.04 - 1.9 mg/kg/day depending mostly of the proportion of iron enriched diet.

![Figure 12. Proportions of feeding regimes at different ages. B=Breast milk, F=Infant formula and C=Complementary foods, including follow on formulas.](image)

MAIN RESULTS

Effects on iron status (I, III)

At six months of age, there was a significant, dose-dependent, positive effect on Hb, MCV, Ferritin, iron and TS and a corresponding negative effect on transferrin and TfR, all suggesting increased iron stores and/or reduced signs of ID (Paper I, table 2). Compared to placebo the mean (95% CI) increase in Hb was 3.8 g/L (1.2 to 6.4) and 8.0 g/L (5.2 to 10.8) in the 1 and 2 mg group respectively, suggestive of established functional iron deficiency in the placebo group.
Further evidence of improved iron status was found using the multiple criteria of ID. There was a high prevalence of ID and IDA at six months in the placebo group, dose dependently and effectively reduced in supplemented infants (Figure 13). The relative risk of ID for placebo vs. the 2mg-group was 9.4 (95%CI, 3.0 to 29.7). Adding the nine infants discontinuing the intervention at 12 weeks of age increased the cumulative incidence of IDA to 12.5% in the placebo group, compared to 6.5% and 2.2% in the 1mg and 2 mg/kg/day-group respectively. In Table 5, the prevalence’s of ID when using single indicators are presented. The results are similar. These robust and concurrent results suggest that unsupplemented MLBW infants are at risk of ID, even though the exact prevalence is dependent of the definitions used.

<table>
<thead>
<tr>
<th>ID Indicator</th>
<th>Placebo</th>
<th>1 mg/kg/d</th>
<th>2 mg/kg/d</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferritin &lt; 12µg/L</td>
<td>23.8%</td>
<td>5.5%</td>
<td>3.6%</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>MCV &lt; 71 fl</td>
<td>21.7%</td>
<td>3.9%</td>
<td>3.7%</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>TS &lt; 10%</td>
<td>35.4%</td>
<td>10.7%</td>
<td>9.0%</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Tfr &gt; 11µg/L</td>
<td>43.2%</td>
<td>20.3%</td>
<td>20.8%</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Table 5. Prevalence of ID at 6 months of age in MLBW infants supplemented with different doses of iron supplements from 6 weeks to 6 months, using four different single indicators of ID (n=231-241). * Fisher exact test.
**Effects on Growth (I)**

There were no significant differences between the groups in absolute anthropometric values or z-scores at six months (Paper I). Figure 14 illustrates the growth during intervention, including unpublished data from 12 weeks and 19 weeks.

![Graph showing growth data](image)

*Figure 14. Length and weight growth until 6 months of age in 267 MLBW infants randomized to different doses of iron supplementation from 6 weeks to 6 months of age. Data are mean with 95% CI.*

There was a rapid catch up growth from birth to 12 weeks of age with a mean change in z-score of +1.2 in weight and 1.4 in length. This catch up growth was more pronounced in SGA infants but evident also in preterm and AGA infants. Only 10% of infants had a weight decrease in z-scores and 7% had a length decrease. At 12 weeks the mean weight was 5.0 kg which was more than double the mean birth weight (2.3 kg) and 79% had doubled their birth weight. Altogether, this suggests a rapid catch-up growth from birth to 3 months of age in most MLBW infants.

**Effects on morbidity (I)**

From the morbidity checklists, filled in by the parents during intervention, longitudinal prevalence of the following symptoms was calculated: Hard stools, loose stools, diarrhea, fever, abdominal pain, crying, vomiting, cough, breathing difficulties, rhinitis, nasal obstruction, rash and tiredness. The distribution of these variables was skewed and zero days with each symptom was the most common answer. Figure 15 illustrates two of the outcomes,
hard stools and abdominal pain. There were no significant difference
between the groups in any of these ($p=0.375$ and $0.919$ respectively), or in
any other symptom, suggesting that oral iron supplementation have no short
term side effects.

![Figure 15. Longitudinal prevalence of abdominal pain and hard stools reported by the parents during supplementation of different doses of iron supplements between 6 weeks of 6 months of age in MLBW infants (n=207).](image)

**Effects on auditory brainstem response (II)**

Three latencies of ABR were assessed at six months of age; absolute wave I, and V latencies and the central conduction time (CCT), whereof wave V latencies and CCT have been associated with impaired myelination and ID.\textsuperscript{51} Unfortunately, there was only a complete measure available in 126 cases. However for wave V latencies, 218 measures were available for analysis. In univariate analyses the results were confounded by sex, study centre and head circumference and the group effects were calculated adjusting for these and for postmenstrual age. There were no significant differences between the groups in absolute wave I and V latencies but CCT was significantly higher in the 2mg/kg/day-group, suggesting a possible adverse effect (Figure 16). The mean difference in CCT between the placebo and the 2 mg-group was 0.15 ms (95% CI, 0.05 to 0.25) representing, an effect size of 0.6 SD. However, we found no association with iron status indicators or mean iron intake, and the difference was less pronounced when excluding infants with poor compliance (per protocol). All together, this suggests that the effect on CCT was not caused by iron supplements.
Hepcidin (III)

Hepcidin was significantly different between the intervention groups at 12 weeks (p=0.005) and six months (p=0.007) of age. The effect was similar in the 1, and 2 mg-group (paper III, figure 2) and the effect of iron supplements on hepcidin during intervention, analyzed when adding the two iron groups is presented in figure 17. While the levels in the placebo-group stayed relatively stable, hepcidin was increased in supplemented infants. Furthermore, the infants with ID at six months had significantly lower levels of hepcidin at 12 weeks and six months compared to the levels at baseline (paper III, table 2), and highly significantly lower than those iron replete (figure 17). All together the results suggest that the expression of hepcidin is changed as a response to both iron loading and ID, likely signaling for reduced and increased iron absorption and iron release respectively. In concordance, hepcidin was correlated to iron intake. 80% of those belonging to the 10th percentile at six months had a low iron intake (<0.4 mg/kg/day) and 75% were diagnosed with ID (paper III, figure 3).

In univariate models, hepcidin was correlated to EPO at six months and to iron status indicators at all ages, mostly with ferritin where $R^2=0.13-0.28$, p<
0.001, (paper III, table 3). To explore the predictors of hepcidin, stepwise multivariate linear regression models were performed. At each age, hepcidin was always mostly correlated to ferritin but the models also included other indicators (table 6). Interestingly, the univariate correlation between hepcidin and EPO at six months of age did not remain significant when adjusting for other iron status indicators, suggesting that hepcidin is primary not regulated by EPO (Paper III). In summary, the results suggest that hepcidin is closely correlated to iron availability, particularly to iron stores. It is affected both by iron loading and ID. However, iron status and Hb only predicted 15-33% of the variance of hepcidin suggesting additional predictors, not included in the present trial.

Table 6. Multivariate linear regression models, assessing the relation of hepcidin with iron status, Hb and EPO at 3 different ages. $\beta$ is the adjusted correlation coefficient. NS = non-significant.
**EPO (III)**

Similar to hepcidin, EPO was significantly different between placebo- and iron supplemented groups at six months (p< 0.001, paper III, figure 2). EPO decreased in those supplemented, while the levels in the placebo-group were similar between ages. Furthermore, infants developing ID had increased levels of EPO, both at 12 weeks and six months (figure 18). Interestingly, these significant differences remained after adjusting for Hb-levels (p=0.037 for Placebo vs. 1 or 2 mg/kg/day and p< 0.001 for ID vs. IR).

![Figure 18. Effects of iron supplementation to MLBW infants between 6 weeks and 6 months of age on EPO (left) and retrospective analysis of EPO change in those diagnosed with ID at 6 mo compared to those iron replete both at 12 weeks and 6 months of age (right). Data are presented as geometric means with 95% CI for geometric mean. For details see paper III (n=168 and n=108 respectively).](image)

Univariate and multivariate models were performed to further explore the correlation between EPO and iron status (paper III, table 4). At all ages, EPO correlated negatively to iron status indicators, also when adjusting for Hb-levels. Altogether this suggests a down-regulation of EPO expression in iron replete infants and up-regulation in cases with ID, independent of the effect from hemoglobin. We speculate on a possible negative feedback mechanism from iron availability in erythropoietic cells on EPO-producing cells in the kidney.

**Effects on cognitive scores and behavior (IV)**

In the follow up at 3.5 years of age, neurodevelopment was assessed using the cognitive Wechsler preschool and primary scale of intelligence – third edition (WPPSI – III) and the validated questionnaire Achenbach Child behavior checklist (CBCL) assessing behavior. There were neither significant differences nor insignificant trends in cognitive scores between the intervention groups or compared to controls (figure 19). However, the
The prevalence of behavioral problems, defined as CBCL-scores above validated cutoffs, was increased in the placebo group compared to the iron supplemented infants and compared to controls (figure 20). The absolute risk reduction of CBCL-scores above the Swedish cutoff from iron supplements was 11.3% and the relative risk (RR) decrease was of 0.33 (0.14-0.77) compared to placebo.

The CBCL outcome was significantly confounded by maternal age but the effect of iron supplements remained significant also in a multivariate model, adjusting for this (Paper IV, table 4). We found no confounding perinatal background factors e.g., prevalence of behavioral problems (Swedish cutoff) was not significantly increased in those with perinatal hypoglycemia [RR 1.6 (0.6 – 4.8), p=0.364], those treated for jaundice [RR 0.9 (0.2 – 3.2), p=1.000], or those with any perinatal morbidity [RR 1.0 (0.4 – 2.6), p=1.000]. Furthermore, full scale IQ was similar in those with perinatal morbidity (104.9) compared to those without (103.2), (p = 0.158).

The CBCL outcome was further analyzed based on the seven syndrome-based subscales, included in the checklist (Paper IV, figure 2). Most pronounced difference between iron supplemented infants and placebo was the subscales emotionally reactive and attention but the trend was similar in most subscales. Examples of symptoms from the CBCL suggesting emotionally reactive children are; ‘disturbed by change’, ‘moody’, ‘worries’, and ‘panics’. Symptoms associated with attention behavior are; ‘can’t concentrate’, ‘can’t sit still’, ‘clumsy’, and ‘wanders away’. In summary, the
results from paper IV suggests that unsupplemented MLBW infants are at risk of behavioral problems and that the risk is reduced by iron supplementation.

Figure 20. Prevalence of behavioral problems defined as total CBCL score above suggested cutoffs. Three different cutoffs were used; two based on the CBCL manual (US pediatric reference) and one based on a Swedish reference. P-values for placebo-group vs. controls are presented, analyzed with fisher’s exact test.

In table 7, the iron status at 12 weeks and six months are presented for the 12 cases in the placebo group with CBCL-score above Swedish 90th percentile. There was no significant correlation between those indicators and the outcome on CBCL.
POSSIBLE INTERACTIONS

Due to the heterogeneity of MLBW infants and the possible impact on future recommendations to a large group of infants, particular focus was put on assessing interactions. Theoretically, the outcomes of the intervention might be different or even reverse in subgroups.

Infant feeding

The group effect on ID at six months interacted with feeding regime at six weeks (p=0.002) and 12 weeks (p=0.004), but not significantly at 19 weeks or six months. The dichotomous dietary variable, with the most significant interaction was presence or not of exclusive breastfeeding at six weeks of age (EBF6w, p<0.001). Stratified analyses showed that the risk of ID, and IDA, and the effect on iron status indicators at six months, was increased in those EBF6w (Paper I, table 4). We interpret this as if EBF6w is a proxy for low dietary iron intake, since all non-EBF6w was partially or exclusively fed iron fortified infants formula (figure 12). This is supported by the calculations of mean (95%CI) dietary iron intake (intervention excluded) from six weeks to six months, which was 0.13 mg/kg/d (0.11 to 0.15) in EBF6w-infants vs. 0.59 mg/kg/d (0.52-0.66) in those fed any formula at baseline.

<table>
<thead>
<tr>
<th>Iron status</th>
<th>Exclusively breastfed at 6 wk</th>
<th>Formula fed at 6 wk</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Placebo</td>
<td>1 mg</td>
<td>2 mg</td>
</tr>
<tr>
<td>ID, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IDA</td>
<td>25 (56)</td>
<td>6 (14)</td>
<td>2 (5.0)</td>
</tr>
<tr>
<td>Growth</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight SD-score</td>
<td>-0.93</td>
<td>-0.67</td>
<td>-0.83</td>
</tr>
<tr>
<td>Length SD-score</td>
<td>-0.37</td>
<td>-0.38</td>
<td>-0.50</td>
</tr>
<tr>
<td>Headcirc. SD-score</td>
<td>-0.31</td>
<td>-0.28</td>
<td>-0.33</td>
</tr>
<tr>
<td>ABR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wave V (ms)</td>
<td>6.27</td>
<td>6.23</td>
<td>6.25</td>
</tr>
<tr>
<td>CCT</td>
<td>4.47</td>
<td>4.51</td>
<td>4.63</td>
</tr>
<tr>
<td>WPPSI-III</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Full-scale IQ</td>
<td>107.1</td>
<td>107.3</td>
<td>104.4</td>
</tr>
<tr>
<td>Verbal IQ</td>
<td>108.8</td>
<td>109.2</td>
<td>106.2</td>
</tr>
<tr>
<td>Performance IQ</td>
<td>103.3</td>
<td>103.8</td>
<td>101.4</td>
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</table>

<table>
<thead>
<tr>
<th>CBCL broadband scores</th>
<th>Total CBCL T-score</th>
<th>US clinical, n (%)</th>
<th>US subclinical</th>
<th>Swedish 90th</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>43.9</td>
<td>3 (7.5)</td>
<td>5 (12.5)</td>
<td>7 (17.5)</td>
</tr>
<tr>
<td></td>
<td>43.3</td>
<td>2 (6.5)</td>
<td>4 (12.9)</td>
<td>5 (16.1)</td>
</tr>
<tr>
<td></td>
<td>42.4</td>
<td>1 (3.4)</td>
<td>1 (3.4)</td>
<td>2 (6.9)</td>
</tr>
<tr>
<td></td>
<td>42.8</td>
<td>2 (5.6)</td>
<td>2 (5.6)</td>
<td>2 (5.6)</td>
</tr>
<tr>
<td></td>
<td>0.739</td>
<td>0.269</td>
<td>0.038</td>
<td>0.140</td>
</tr>
<tr>
<td></td>
<td>0.870</td>
<td>1.000</td>
<td>0.376</td>
<td>0.376</td>
</tr>
<tr>
<td></td>
<td>0.831</td>
<td>0.598</td>
<td>0.768</td>
<td>0.959</td>
</tr>
</tbody>
</table>

Table 8. Analyses of the main outcomes, stratified based on infant feeding practice at 6 weeks of age. aP-values are ANOVA for growth, ABR and IQ, Kruskal Wallis for T-scores, and Fischer’s Exact for proportions. bEBF6w X Group interaction calculated by GLM. Data are mean or n(%).
There was no similar significant interaction with EBF6w and the outcomes of growth, ABR, cognitive scores or behavioral problems but a suggestive trend of improved verbal-IQ and length was found in formula fed infants, not seen in the overall analyses (Table 8). No adverse effects were found in the subgroups, neither in those belonging to the 4th quartile of dietary iron intake (> 0.55 mg/kg/day, data not shown).

**Gestational age and size for age**

As presented in paper I, there was an interaction between the group effect and prematurity (< 37 wk) on Hb and MCV at six months. However, for remaining iron status indicators, there were no interactions and for all outcomes, there was a similar trend between the subgroups, suggesting benefits in both, even though the group effect was stronger for preterm infants (Paper I, table 5). All main outcomes in term and preterm infants separately are summarized in table 9. An alternative stratification was performed based on presence or not of SGA at birth. The results were similar to the preterm/term-stratifications (data not shown) with the exception that SGA/AGA interacted with the group effect on ID-prevalence (p=0.035). In AGA-infants the difference between placebo and 2mg/kg/day-group was more pronounced (44 vs. 0%) compared to SGA infants where the difference was 24 vs. 9%. However, the difference was significant in both subgroups.

<table>
<thead>
<tr>
<th>Iron status</th>
<th>Placebo</th>
<th>1 mg</th>
<th>2 mg</th>
<th>p*</th>
<th>Preterm</th>
<th>1 mg</th>
<th>2 mg</th>
<th>p*</th>
<th>Inter*</th>
</tr>
</thead>
<tbody>
<tr>
<td>ID, n (%)</td>
<td>1 (30)</td>
<td>3 (9.4)</td>
<td>2 (5.9)</td>
<td>0.015</td>
<td>18 (41)</td>
<td>4 (9.5)</td>
<td>1 (2.2)</td>
<td>&lt;0.001</td>
<td>0.380</td>
</tr>
<tr>
<td>IDA</td>
<td>2 (5.4)</td>
<td>1 (3.1)</td>
<td>0 (0)</td>
<td>0.644</td>
<td>6 (14)</td>
<td>1 (2.4)</td>
<td>0 (0)</td>
<td>0.008</td>
<td>0.295</td>
</tr>
<tr>
<td>Growth</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight SD-score</td>
<td>-1.36</td>
<td>-1.11</td>
<td>-1.26</td>
<td>0.478</td>
<td>-0.39</td>
<td>-0.42</td>
<td>-0.25</td>
<td>0.670</td>
<td>0.499</td>
</tr>
<tr>
<td>Length SD-score</td>
<td>-0.76</td>
<td>-0.68</td>
<td>-0.75</td>
<td>0.928</td>
<td>0.02</td>
<td>-0.05</td>
<td>0.30</td>
<td>0.256</td>
<td>0.445</td>
</tr>
<tr>
<td>Headcerc. SD-score</td>
<td>-0.69</td>
<td>-0.62</td>
<td>-0.77</td>
<td>0.808</td>
<td>-0.17</td>
<td>-0.22</td>
<td>0.20</td>
<td>0.068</td>
<td>0.135</td>
</tr>
<tr>
<td>ABR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wave V, mean (ms)</td>
<td>6.17</td>
<td>6.18</td>
<td>6.25</td>
<td>0.253</td>
<td>6.28</td>
<td>6.20</td>
<td>6.28</td>
<td>0.262</td>
<td>0.459</td>
</tr>
<tr>
<td>CCT</td>
<td>4.40</td>
<td>4.43</td>
<td>4.60</td>
<td>0.008</td>
<td>4.53</td>
<td>4.55</td>
<td>4.66</td>
<td>0.242</td>
<td>0.741</td>
</tr>
<tr>
<td>WPPSI-III</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Full-scale IQ, mean</td>
<td>103.5</td>
<td>100.2</td>
<td>104.7</td>
<td>0.584</td>
<td>106.7</td>
<td>107.1</td>
<td>104.4</td>
<td>0.544</td>
<td>0.352</td>
</tr>
<tr>
<td>Verbal IQ</td>
<td>104.9</td>
<td>104.0</td>
<td>108.0</td>
<td>0.614</td>
<td>107.3</td>
<td>108.9</td>
<td>107.2</td>
<td>0.746</td>
<td>0.473</td>
</tr>
<tr>
<td>Performance IQ</td>
<td>102.5</td>
<td>96.1</td>
<td>100.4</td>
<td>0.388</td>
<td>104.2</td>
<td>103.2</td>
<td>100.0</td>
<td>0.400</td>
<td>0.404</td>
</tr>
<tr>
<td>CBCL broadband scores</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total CBCL T-score</td>
<td>45.4</td>
<td>41.5</td>
<td>42.1</td>
<td>0.458</td>
<td>42.1</td>
<td>42.8</td>
<td>42.0</td>
<td>0.684</td>
<td>0.373</td>
</tr>
<tr>
<td>&gt; US clinical, n (%)</td>
<td>2 (6.3)</td>
<td>0 (0)</td>
<td>1 (3.1)</td>
<td>0.772</td>
<td>3 (7.7)</td>
<td>2 (5.0)</td>
<td>1 (2.3)</td>
<td>0.442</td>
<td>0.697</td>
</tr>
<tr>
<td>&gt; US subclinical</td>
<td>5 (15.6)</td>
<td>0 (0)</td>
<td>1 (3.1)</td>
<td>0.046</td>
<td>4 (10.3)</td>
<td>2 (5.0)</td>
<td>1 (2.3)</td>
<td>0.256</td>
<td>0.443</td>
</tr>
<tr>
<td>&gt; Swedish 90th</td>
<td>7 (21.9)</td>
<td>1 (3.6)</td>
<td>2 (6.3)</td>
<td>0.074</td>
<td>5 (12.8)</td>
<td>3 (7.5)</td>
<td>2 (4.7)</td>
<td>0.380</td>
<td>0.421</td>
</tr>
</tbody>
</table>

Table 9. Analyses of the main outcomes, stratified based on gestational age at birth.

aP-values are ANOVA for growth, ABR and IQ, Kruskal Wallis for T-scores, and Fischer’s Exact for proportions. bPreterm/term X Group interaction calculated by GLM. Data are mean or n(%).
Baseline iron status

We hypothesized two possible interactions with baseline iron status. First, the positive effects of iron supplements might be restricted to the subgroup with low iron stores. Second, as suggested by others, possible adverse effects might be present in those with elevated iron stores at baseline. Since the present trial assessed iron supplements, starting at six weeks of age, when Hb levels are generally low despite elevated iron stores, we found it more relevant to assess baseline iron status based on ferritin levels. We used different methods for stratification. In paper I, a median split of baseline ferritin showed no adverse but rather an improved effect in those with higher baseline ferritin (Paper I, table 7). In paper II, only those below the 25th and above the 75th percentile were compared. There was no interaction with ABR-latencies. In table 10, the same principle for stratification was used with regard to all other main outcomes. There was a significant interaction with cognitive scores, suggesting benefits of 2 mg/kg/day among infants with high baseline ferritin. However, this effect was not dose-dependent and the number of infants was low, suggesting that interpretations would be done carefully. More important was that no adverse effects were seen in any of the subgroups.

<table>
<thead>
<tr>
<th>Ferritin 6 wk &lt; 81µg/L</th>
<th>Ferritin 6 wk &gt;180µg/L</th>
<th>Inter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ID, n (%)</td>
<td>Placebo 1 mg 2 mg 2 mg p\textsuperscript{a}</td>
<td>Placebo 1 mg 2 mg 2 mg p\textsuperscript{a}</td>
</tr>
<tr>
<td>IDA</td>
<td>7 (33.3) 3 (16.7) 3 (16.7) 0.453</td>
<td>6 (31.6) 1 (5.9) 0 (0) 0.013</td>
</tr>
<tr>
<td></td>
<td>1 (4.8) 1 (5.6) 0 (0) 1.000</td>
<td>1 (5.3) 0 (0) 0 (0) 1.000</td>
</tr>
<tr>
<td>Growth</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight SD-score</td>
<td>-0.46 -0.61 -0.42 0.791</td>
<td>-1.26 -1.02 -0.64 0.242</td>
</tr>
<tr>
<td>Length SD-score</td>
<td>-0.12 -0.55 0.41 0.014</td>
<td>-0.66 -0.62 -0.20 0.380</td>
</tr>
<tr>
<td>Headcirc. SD-score</td>
<td>-0.28 -0.18 -0.11 0.872</td>
<td>-0.81 -0.38 -0.37 0.391</td>
</tr>
<tr>
<td>WPPSI-III</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Full-scale IQ, mean</td>
<td>105.0 107.1 98.3 0.356</td>
<td>102.2 93.9 110.2 0.012</td>
</tr>
<tr>
<td>Verbal IQ</td>
<td>107.8 108.4 100.9 0.400</td>
<td>103.0 100.7 113.1 0.015</td>
</tr>
<tr>
<td>Performance IQ</td>
<td>100.4 104.2 96.0 0.511</td>
<td>100.8 87.7 104.7 0.015</td>
</tr>
<tr>
<td>CBCL broadband scores</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total CBCL T-score</td>
<td>43.1 41.3 43.5 0.806</td>
<td>43.6 44.9 44.9 0.919</td>
</tr>
<tr>
<td>&gt; US clinical, n (%)</td>
<td>3 (17) 0 (0) 0 (0) 0.100</td>
<td>0 (0) 0 (0) 1 (6) 1.000</td>
</tr>
<tr>
<td>&gt; US subclinical</td>
<td>3 (17) 0 (0) 0 (0) 0.100</td>
<td>2 (12) 0 (0) 1 (6) 0.765</td>
</tr>
<tr>
<td>&gt; Swedish 90\textsuperscript{th}</td>
<td>3 (17) 0 (0) 1 (7) 0.367</td>
<td>3 (18) 2 (13) 2 (12) 1.000</td>
</tr>
</tbody>
</table>

Table 10. Analyses of the main outcomes in the subgroups representing the first and fourth quartile of ferritin levels at 6 weeks as a proxy for iron stores at baseline. \textsuperscript{a}P- values are ANOVA for growth and IQ, Kruskal Wallis for T-scores, and Fischer’s Exact for proportions. \textsuperscript{b}Ferritin at 6 wk X Group interaction calculated by GLM. Data are mean or n(%).
**Sex differences**

Previous trials have suggested that boys have lower Hb-levels and iron stores in infancy, possibly putting them at increased risk of ID.\textsuperscript{5, 121} In the present trial we found few differences in the main outcomes between boys and girls. The increased risk of ID and behavioral problems was present in both sexes. E.g. 21.2\% of boys and 13.2\% of girls in the placebo group had a CBCL-score above the Swedish 90\textsuperscript{th} percentile and the overall prevalence was 8.7\% in boys and 8.2\% in girls (controls included).
Discussion

This was the first randomized trial assessing the effects of iron supplementation to MLBW infants. We found a high risk of ID and IDA in unsupplemented infants, particularly those exclusively breastfed at six weeks of age. All examined indicators of iron status, Hb, the newly discovered enzyme hepcidin and EPO, significantly changed as a response to the supplements. We found no overall effects on the functional outcomes; growth, morbidity, cognitive scores or on the neurophysiologic variable ABR V-latencies. However at 3.5 years of age, behavioral problems were increased in non-supplemented children, suggesting late neuro-developmental negative effects, caused by ID in infancy.

IRON DEFICIENCY IN MLBW INFANTS

As reviewed in table 2, there are at least four previous trials, suggesting that unsupplemented LBW infants are at increased risk of IDA during their first six months of life.104, 110-112 In those, the prevalence of anemia in unsupplemented infants was 23-90%, which was higher than the prevalence found in unsupplemented infants in the present trial (10%). This is not surprising, since only one of these111 included MLBW infants, and none presented the IDA-prevalence in MLBW infants separately. Most infants of the present trial (86%) were weaned to complementary foods before six months of age, and Swedish complementary foods are mostly iron fortified. Furthermore, ~50% of the present infants were exclusively or partially fed iron fortified formula. Nevertheless, the prevalence of ID and IDA found, is high compared to previous trials of term, normal birth weight infants in developed countries,28, 30 suggesting that MLBW infants similar to those born with VLBW, and opposite to normal birth weight Swedish infants, benefit from iron supplementation. This is in similar with one previous case control trial by Iway.101

It has been suggested that the stores of iron are depleted as the infant has doubled its body weight, and the 75mg per kilogram at birth is “diluted” to 35-40mg/kg, corresponding to the minimum level before functional iron deficiency occurs.5, 20 Since preterm infants have lower stores at birth35 the time for this depletion might occur even before. This is in concordance with the present trial, in which most infants had doubled their size at 12 weeks of age, a time point when also mean ferritin and TS significantly decreased in unsupplemented infants and in those developing ID (Paper III).
ASSOCIATION WITH NEURODEVELOPMENT

The public health benefit of protecting infants from ID is dependent of its possible association with functional deficits. Previous recommendations of iron fortification and supplementation are based on negative neurodevelopmental effects, suggested from case-control trials of term infants. For LBW infants, there is only one previous trial reporting on the effects of iron supplementation on long term neurodevelopment. This showed a suggestive trend of improved neuro-cognitive and psychomotor development in VLBW infants given iron supplementation from about two weeks of age compared to those supplemented from two months of age, suggesting that VLBW infants deplete their iron stores before two months, and that early iron supplementation benefits neurodevelopment. They found no effect on behavior.119-120 In the present randomized trial, we assessed three outcomes correlated to neurodevelopment.

ABR-latencies

Roncagliolo et al suggested that ABR-latencies are irreversibly prolonged in infants with IDA during infancy. If this association is true, ABR would constitute a promising indicator of functional ID and a proxy for impaired neurodevelopment.51 In the present trial there was no such association which is in similar to the findings in ABR wave V latencies.119-120 Several possible interpretations are discussed in paper II and in summary, we suggest that ABR-latencies is not a sensitive measure of impaired myelination caused by ID, or that there is no such impairment in infants with the degree of ID we found in our study population. Interpretations of the ABR-outcomes were limited by the large number of incomplete measures, the unexplained difference between study centers, and the unexpected prolonged CCT-latencies in the 2mg-group. As discussed in paper II, we consider the latter a type I-error, unrelated to iron supplements. This is further supported by the cognitive scores at 3.5 years, where there was no negative effect in the 2 mg-group. Furthermore, there was no association between CCT at six months and full scale IQ at 3.5 years (r=0.024, p=0.801 when analyzed with linear regression).

Cognitive scores

In concordance with the findings in ABR wave V latencies, there was no significant correlation between iron supplements and cognitive scores at 3.5 years, even though stratified analyses of those with high baseline ferritin indicate possible cognitive benefits in this subgroup. The results might suggest that causality with infant ID does not exist, at least not in MLBW
infants with mild or moderate ID. The previous case-control trials suggesting an association with cognitive scores, usually compared infants with IDA and controls, while the placebo infants of the present trial mostly had mild ID and only a small proportion had IDA. This might suggest that cognition is affected only in cases of established functional deficiency, and that Swedish MLBW infants are not at increased risk. Other possible explanations are that the trial was underpowered, or that the measures might have been made at too early age. The predictive value of WPPSI-tests on later cognitive scores at 3 years is limited, underscoring the importance of school age follow ups.

**Behavior**

The odds ratio (OR) for a CBCL-score above the US subclinical cutoff in supplemented infants, adjusted for maternal age was 0.22 (0.06-0.78) compared to non-supplemented infants. This suggests a causal relationship between iron supplements in infancy and later reduced behavioral problems, likely explained by reduced functional ID. This interpretation is further supported by the very similar baseline and background factors, and the fact that we found no confounding effect from perinatal factors. The association between ID and behavior is supported by animal research and a few previous case-control trials, but the present is the first randomized trial to suggest causality. Interestingly, other recent trials have suggested a close correlation between LBW and behavioral problems, without assessing the impact of iron status. E.g., Lindström et al recently suggested that late preterm birth (33-34 weeks) increased the risk of ADHD –medication [OR 1.3 (1.1-1.4)] and similar, being born SGA significantly increased the risk [OR 1.4 (1.2-1.6)]. In Swedish extremely LBW infants (ELBW, < 1000g) the parents reported more problems with internalizing behaviors and attention, which is similar to the CBCL-profile of the present trial. However, since ELBW infants are mostly iron supplemented and the perinatal complications are much more severe, it is likely that the etiology is different and the interpretations from the present trial should be limited to moderately and marginally LBW or late preterm infants.

Interestingly, recent research in school-aged children with ADHD have suggested an association between behavioral problems and ID, supporting such association. However in contrast to the present trial, that association was reversible and the mechanisms might be different.

**ADVERSE EFFECTS**

As noted above, previous trials on term infants have shown adverse effects of iron supplements on growth, infections, and neurodevelopment, when given
to iron replete infants. Iron repletion in those trials was mostly identified by normal Hb-levels. Most notable, Lozoff et al recently suggested that iron fortified formula given between six to 12 months of age to iron replete infants, could have a negative effect on IQ later in life. In that case iron repletion was indicated by high or normal Hb at baseline. The adverse affects have been suggested to be caused by iron overload, possible due to an immature iron metabolism. In the present trial, we found no adverse effects on growth, morbidity, or neurodevelopment, neither in subgroups of high baseline ferritin or in those with high amount of iron intake from diet (table 10 and 8). However, the stratified analyses should be interpreted with cautious due to low numbers and we cannot completely exclude the possibility of adverse effects in individuals. The results from the present trial nevertheless suggest that the intervention is safe with regard to functional outcomes regardless of initial iron stores and feeding.

Surprisingly, those with highest baseline ferritin at inclusion had instead more benefits from iron supplementation of 2 mg/kg/day on cognition and ID-prevalence, with an opposite trend in those with low iron stores. However, this effect was not dose-dependent, might be confounded by gestational age or might result from type I-error.

**OPTIMAL IRON INTAKE IN MLBW INFANTS**

It is not surprising that the prevalence of ID was further increased in infants mostly breastfed, since the intake of iron in this group was lower and the imbalance between iron needs and stores reasonably occurred more rapidly. However, also formula fed infants of the present trial had an increased risk of ID (table 8), suggesting that partial feeding of iron fortified formula is not enough to cover the needs of MLBW infants. This is further supported by two other studies, reporting a reduced but still high risk of ID or IDA in LBW infants fed iron fortified formula. In contrary, two other trials have suggested that fortified formula is enough to protect LBW infants from ID. In a small randomized trial, Helliday et al, concluded that moderately and late preterm formula fed infants do not benefit from iron supplementation if fed iron enriched formula. They did not present the level of fortification. Griffin et al assessed two levels of iron in post discharge infant formula administered to preterm infants (<32wk) and found no benefits of 8mg/L compared to 5 mg/L. However, the latter trial included infants having been blood transfused. In conclusion, it is likely that feeding with fortified formula reduces the risk of ID but depending on the level of fortification, the overall iron intake, and the degree of prematurity, it is only partially protecting LBW infants from ID.
The mean total iron intake calculated in the present trial contributed to important information to further explore optimal iron intake. Interestingly, cases of both ID and behavioral problems were associated with an iron intake of < 1 mg/kg/day (Paper I and IV). Assuming an absorption coefficient of 10-20%\(^1\) this would correspond to the ~150 µg/kg theoretically assumed to be needed for tissue growth during the first six months of life (above). However it should be noted that iron utilization and absorption can be different depending of its mode of administration.\(^5\), \(^12\) Since there were no adverse effects in the 2 mg-group of this trial, it is likely that a general supplementation of 2 mg/kg/day should be recommended to reach this minimum intake, taking compliance into account. Lower intake could be considered in those preferably fed iron enriched formula, however giving such a recommendation is beyond the scope of the present trial and requires further randomized trials.

**Risks of iron overload**

The risk of iron overload cannot be underestimated when general iron supplementation is considered to large groups of infants. The recently suggested possibility of impaired IQ at school-age in healthy, iron replete infants fed iron enriched formula from 6-12 months of age is alarming and underscores the importance of selecting the correct populations to target and the possible risks of iron overload.\(^7\)

Two later trials have compared different levels of fortification in post discharge formula, given to LBW infants and concluded that this did not change the iron status at six and 12 months respectively, suggesting no benefits from increased iron intake.\(^116\)-\(^117\) Friel et al also assessed cognitive development at 12 months and found no positive or adverse effects of increased level of iron fortification (20.7 vs. 13.4mg/L), corresponding to 2.8 vs. 2.1 mg/kg/day of total iron intake at 3 months of age.\(^117\) In opposite, but in a different setting, Borigato et al showed that preterm Brazilian infants with mean birth weight of 1935g did benefit from additional iron added to a standard supplementation of 2 mg/kg/day, suggesting that the interpretations above, might be restricted to developed countries with well nourishes populations.\(^115\) As for the present trial we have shown that supplementation at a dose of 1-2 mg/kg/day in combination with standard infant formulas and complementary foods is safe. Nevertheless, infant formulas on the Swedish market are mostly enriched with 4-8 mg/L and regarding iron overload, the results from the present trial must be interpreted with caution in countries with higher levels of iron content in infant formulas.
TERM VS. PRETERM

The positive effect of iron supplementation on ID- and IDA-prevalence was present in the subgroups term and preterm as well as in the subgroups SGA and AGA, even though it did not reach significance for all variables. The results on MCV, Hb and ID-prevalence suggest that preterm and AGA MLBW infants are at further increased risk, compared to those born at term and/or SGA. This could be interpreted as if the lower iron stores in preterm infants contribute to a higher risk of iron depletion than does the catch up growth in SGA, term infants. However, even though decreased compared to preterm infants, the benefits in term/SGA LBW infants of iron supplementation are significant or suggestive, and as discussed further in paper I, it is reasonable that the overall finding of the present trial is applicable to all otherwise healthy MLBW infants. Few previous intervention trials have separated LBW into preterm and term infants but two trials reported increased risk in preterm LBW infants. In contrast to us, Olivares et al concluded that full-term LBW infants do not develop ID before 4 months of age; however they supplemented all infants from 4 months of age.

IRON STATUS INDICATORS

The main outcomes of the present trial suggest that MLBW infants are at risk of ID before six months of age, with possible functional deficits in neurodevelopment. Thereby, the cohort constitutes an excellent human model of ID, with the possibility of retrospective assessment of early iron status indicators.

Traditional indicators of ID

In paper III, figure 2, the different indicators of iron status are compared between the groups. Ferritin, Hb, MCV, TS and TfR changed over time in all groups. This illustrates the rapid physiological changes in iron status seen during the first six months of life, and the difficulties of determining normal ranges. Nevertheless, the change over time interacted with intervention and all variables differed significantly at six months between the groups, suggesting that all are useful indicators of iron status in MLBW infants. At 12 weeks of age, only ferritin and TS differed significantly between the groups, suggesting them to be better indicators of early ID, compared to MCV and TfR. This is in concordance with the theoretical stages of ID (table 1).

To illustrate the predictive value of the indicators for ID, infants with ID at six months were compared with those iron replete (paper III, table 2). All of
the four indicators were significantly different between these groups. But there was a large variance and none had separated ±2 SD-ranges, suggesting that there is a wide overlap making cutoffs of early ID difficult to establish.

**Hepcidin as ID-indicator**

Unsupplemented infants and particularly infants developing ID had lower mean levels of hepcidin (Paper III). The decrease was significant at 12 weeks suggesting it to be an early indicator of ID, similar to ferritin and TS. In contrast to ferritin, the levels of hepcidin were more similar between ages, at least in unsupplemented infants, but at each age, they correlated with ferritin. Taken together, we interpreted this as if hepcidin correlates to iron stores and might indicate early storage iron depletion. Possibly, this opens the possibility that less age dependent cutoffs can be established, making hepcidin a clinically useful marker if iron status. However, further trials are needed since several unanswered questions remain in how to interpret hepcidin in infants such as diurnal variation, relation to time of feeding and sensitivity to ID. But most urgent is the need of established reference ranges in healthy, term, normal birth weight infants, likely compared to current gold standards.

The hepcidin-association to iron availability was stronger than the association to EPO, suggesting that the stimulating effect from erythropoiesis on hepcidin expression is not as contributing as the iron-sensing pathway. This is contrary to recent results from animal models.129

**Iron status and later behavioral problems**

The group-effect of iron supplementation on behavior, prompted further retrospective correlation analyzes with iron status in infancy. If the cases of behavioral problems were explained by ID, their iron status in infancy would contribute with important clues on how to identify functional ID. However, there were no significant correlations between CBCL-scores, or cases of behavioral problems and iron status indicators (Paper IV), e.g. only 3 of the 19 cases of CBCL-score above the Swedish cutoff at 3.5 years were diagnosed with ID at six months, which was similar to the overall prevalence. However, since behavioral problems are multi-factorial, such a correlation would not have been expected to be strong. If the absolute risk-increase was explained completely by ID, only ~11% or 7 cases in the placebo group would be attributed to ID, and the trial was not powered to detect differences in such small groups. Actually, five of the cases had ID at 12 weeks or six months and interestingly, another two infants had a hepcidin level below the 10th percentile (6.2ng/mL) at 12 weeks, theoretically representing most of the
group difference (7-12%). Nevertheless, only one of those cases had IDA at six months, none had all four (-five) ID-indicators outside the cutoff, and the iron status was similar in placebo infants not developing behavioral problems.

The results suggest two important interpretations. First, the iron status indicators have poor sensitivity and/or specificity to functional ID. Individual assessment cannot separate those at risk from those not at risk. Second, MLBW infants with mild ID, indicated only by one or two iron status variables and without IDA, are at risk of behavioral problems. Probably in combination with other risk factors such as genetic or environmental impact, some unsupplemented MLBW infants developed behavioral problems. Interestingly, recent animal models of moderate ID have shown an association to behavioral problems, supporting the interpretation.130

STRENGTH AND LIMITATIONS

Randomized controlled trials are considered to contribute the highest level of evidence in clinical practice. The randomization of the present trial was successful with respect to background factors, perinatal complications and baseline iron status, suggesting that the results are reliable. Furthermore, the trial was powered to detect differences of 0.5 SD, suggesting that differences of public health benefits or harm would be detected. Finally, the low dropout rate further contributed to trustable results and a possibility to perform the follow up at 3.5 years. However, some limitations should be considered:

Study compliance

Compared to the low dropout rate the present trial had relatively low compliance to the intervention. 23% of infants received less than 70% of iron drops and 16% less than 50%. This likely contributed to reduced power and possible effects of iron supplementation could have been overseen. Nevertheless, by monitoring compliance, we were able to perform secondary analyses per protocol and thereby confirm or reject some findings.

Data collection

There are some concerns in data collections. First, several iron status indicators have a diurnal variation and are also changed as a response to recent feeding. We did not synchronize the time of venipuncture or its relation to feeding, and this might have biased the results. Furthermore,
complete set of data was not collected in all infants and the numbers analyzed varied.

**Lack of power**

The group sizes were calculated based on overall group effects. Since only 36% of the infants in the placebo group had ID, the functional impairments hypothesized, would theoretically only be seen in this subgroup, thus reducing power in group analyses. We cannot exclude the possibility of small, undetected group effects. Furthermore, since the MLBW infants, by definition is heterogeneous with regard to SGA, prematurity, feeding etc, several possible interactions are possible and the trial was not primary powered to detect all of them.

**Possible confounders**

We monitored several important background and baseline factors to exclude biased results from confounders. However, there are theoretically still possible confounders affecting the results. E.g., it has been suggested that prenatal ID should be considered in studies of postnatal ID. Prenatal ID was only indirectly monitored by the baseline iron status at six weeks, but cord blood iron status would have improved the interpretations. However, maternal smoking was low, most mothers were given iron supplements during pregnancy and none of the infants was born large for gestational age, suggesting that the risk of prenatal ID is limited. The study design by definition reduces the risks of biased results and the main results are unlikely biased by an unknown confounder.
Conclusions and recommendations

From this randomized, placebo-controlled, double-blinded trial, assessing the effect of preventive iron supplementation to MLBW infants during their first six months of life, the following is concluded:

- Otherwise healthy, unsupplemented, Swedish MLBW infants are, similar to VLBW infants, and in contrast to term, normal birth weight infants, at increased risk of ID and IDA at six months of age. The increased risk is present in both term and preterm MLBW infants, even though more pronounced in preterm infants. Infants exclusively breastfed at six weeks of age are at particular risk.

- Iron supplementation from six weeks to six months of age in a dose of 2 mg/kg/day significantly reduces the risk to levels similar to healthy term, normal birth weight infants with no positive or adverse effects on growth or morbidity.

- Latencies of auditory brainstem response at six months of age are not shortened in iron supplemented MLBW infants at risk of ID and not increased in MLBW infants with mild or moderate ID. The use of ABR as a proxy for impaired myelination caused by ID requires further assessment.

- Hepcidin is closely correlated to iron status in infants. It increases as a response to iron loading and decreases in ID. It might be a less age dependent indicator of iron availability but requires further descriptive trials to assess normal ranges.

- Erythropoietin is negatively correlated to iron status, independent of hemoglobin levels. It decreases in iron loading and increases in ID, suggesting a not yet described interplay with iron availability.

- In MLBW infants, hepcidin is more strongly regulated by iron availability than from EPO-levels.

- MLBW children, supplemented with iron from six weeks to six months of age in a dose of 1-2 mg/kg/day have reduced risk of behavioral problems compared to those unsupplemented, even when adjusting for sociodemographic and perinatal problems.
• There is a causal relationship between low iron intake in infancy and long term negative effects on behavior in LBW children.

• At least part of the increased risk of behavioral problems in late preterm and moderately LBW infants suggested by several recent trials might be associated to ID in infancy and could be prevented.

• Hb, ferritin, MCV, TS, TfR and hepcidin during the first six months of life have poor specificity and sensitivity to predict long term behavioral problems associated with ID and should be used cautiously in individual iron status assessments.

• In MLBW infants, a mean total daily iron intake of > 1mg/kg/day from six weeks to six months of age reduces the risk of ID and behavioral problems to levels similar to term, normal birth weight infants. A safe maximum intake is not known but there is no evidence of further improvement from higher intake.

**Recommendations**

Infants born with a birth weight between 2000 and 2500g should be included in general iron supplementation programs during their first six months of life, independent of gestational age at birth, sex, infant iron status and feeding practices. A dose of 2 mg/kg/day from six weeks to six months of age is safe with regard to adverse effects, improves iron status and reduces the risk of behavioral problems at 3 years of age.

**Future studies**

The present trial underscores the importance of long term follow up trials when studying interventions in infancy and we plan to follow the present cohort until seven years of age. Similar long term follow up trials assessing dose, time and duration of iron supplementation of term as well as VLBW infants would improve future recommendations, particularly its safety. With regard to iron status in infants there is an urgent need of improved tools for evaluating infant iron availability. Trials assessing normal ranges as well as changes during functional ID are needed. Both new indicators such as hepcidin, but also the traditional markers of ID, require further evaluation.
Acknowledgements

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