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IRON AND ZINC IN INFANCY: RESULTS FROM EXPERIMENTAL TRIALS IN SWEDEN AND INDONESIA

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“In spite of the spectacular advances in scientific medicine which we have witnessed in the last 20 years, there is still a need for information about a number of fundamental, if quite elementary, matters.”

_E M Widdowson and C M Spray, 1951_
ABSTRACT

Background: Iron and zinc are difficult to provide in sufficient amounts in complementary foods to infants world-wide, resulting in high prevalence of both iron and zinc deficiency. These deficiency states cause anemia, delayed neurodevelopment, impaired growth, and increased susceptibility to infections such as diarrhea and respiratory infections.

Design: Two different intervention strategies; reduction of a possible inhibitor of iron and zinc absorption, i.e. phytate, or supplementation with iron and zinc, were applied to two different populations in order to improve iron and zinc nutrition:

In a high-income population (Umeå, Sweden), the amount of phytate in commonly consumed infant cereals was reduced. Healthy, term infants (n=300) were at 6 mo of age randomized to phytate-reduced infant cereals, conventional infant cereals, or infant formula and porridge.

In a low income population (Purworejo, Indonesia), daily iron and zinc supplementation was given. Healthy, term infants (n=680) were at 6 mo randomized to supplementation with iron, zinc, a combination of iron and zinc, or placebo.

Blood samples, anthropometrical measurements, and data on infant neurodevelopment and morbidity were collected. Also, in the Swedish study, detailed information on the dietary intake was recorded.

Results: In the Swedish study, the reduction of phytate had little effect on iron and zinc status, growth, development or incidence of diarrhea or respiratory infections, possibly due to the presence of high contents of ascorbic acid, which may counteract the negative effects of phytate. In the Indonesian study, significant negative interaction between iron and zinc was evident for several of the outcomes; Hb and serum ferritin improved more in the iron only group compared to placebo or the combined iron and zinc group. Further, supplementation with iron alone improved infant psychomotor development and knee-heel length, whereas supplementation with zinc alone improved weight and knee-heel length compared to placebo. Combined iron and zinc supplementation did decrease the prevalence of iron deficiency anemia and low serum zinc, but had no other positive effects. Vomiting was more common in the combined group.
Analyses of dietary intake from the Swedish study showed that dietary iron intake in the 6-11 mo period was significantly associated with Hb, but not serum ferritin at 9 and 12 mo, whereas the opposite was true in the 12-17 mo period, i.e. dietary iron intake was significantly associated with serum ferritin, but not Hb at 18 mo.

**Conclusions:** The phytate content of commercial infant cereals does not seem to contribute to poor iron and zinc status of Swedish infants as feared. However, the current definitions of iron and zinc deficiency in infancy may overestimate the problem, and a change in the recommended cutoffs is suggested. These studies also indicate that dietary iron is preferably channeled towards erythropoiesis during infancy, but to an increasing amount channeled towards storage in early childhood. This suggests that in evaluating dietary programs, Hb may be superior in monitoring response to dietary iron in infancy, whereas S-Ft may respond better later in childhood. However, as shown in this study, increasing Hb may not necessarily be an indicator of iron deficiency, as more dietary iron increased Hb regardless of iron status.

In the low-income setting combined supplementation with iron and zinc resulted in significant negative interaction. Thus, it is not possible to recommend routine iron-zinc supplementation at the molar concentration and mode used in this study. It is imperative that further research efforts are focused at finding cost-effective strategies to prevent iron and zinc deficiency in low-income populations.
LIST OF ABBREVIATIONS

ANCOVA Analysis of co-variance
ANOVA Analysis of variance
ARI Acute respiratory infection
BRS The behavioral rating scale of the Bayley Scales of Infant Development
BSID The Bayley Scales of Infant Development
CC In SINUS: commercially available MCD and porridge group
CHN-RL The Community Health and Nutrition Research Laboratories, Gadjah Mada University, Yogyakarta, Indonesia
CI Confidence interval
DMT1 Divalent metal transporter 1
EZP Exchangeable zinc pool
HAZ Height-for-age z-score compared to the WHO/NHCS reference population
Hb Hemoglobin
ID Iron deficiency
IDA Iron deficiency anemia
IF In SINUS: cow's milk based infant formula and porridge
MCD Milk-based cereal drinks
MCV Erythrocyte mean cell volume
MDI The mental development index of the Bayley Scales of Infant Development
NHANES The second National Health and Nutrition Examination II
PDI The psychomotor development index of the Bayley Scales of Infant Development
PR In SINUS: phytate-reduced MCD and porridge group
RBC Red blood cells
RR Incidence rate ratio
S-Cu Serum copper
S-Fe Serum iron
S-Ft Serum ferritin
SINUS Study on Infant Nutrition in Umeå, Sweden
S-TfR Serum transferrin receptor
S-Zn Serum zinc
TIBC Total iron binding capacity
WAZ Weight-for-age z-score compared to the WHO/NHCS reference population
WHO The World Health Organization
WHZ Weight-for-height z-score compared to the WHO/NHCS reference population
ZD Zinc deficiency
ZINAK Zinc and Iron Supplementation to Infants in Purworejo, Indonesia
The thesis is based on the following papers:


II. Lind T, Persson LÅ, Lönnerdal B, Stendlund H, Hernell O. Effects of weaning cereals with different phytate content on growth, development and morbidity: a randomized intervention in infants from 6 to 12 mo of age. *Acta Paediatrica* accepted with revisions.


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**PAPERS I-V**
THE CHALLENGE

Exclusive breast-feeding, i.e. giving no additional food or drink but breast milk, is the recommended type of feeding to term infants during the first 6 mo of life (1). However, after 6 mo of age the demand of the growing infants for some nutrients, particularly micronutrients, i.e. vitamins and trace minerals present in low concentrations in the diet, increases beyond the amounts supplied from breast milk only, prompting addition of other foods or supplements to the infant’s diet (2). The composition and provision of these foods, ensuring adequate amounts of energy as well as essential nutrients to sustain growth, development and a general state of health of the growing infant or young child continue to be a challenge to parents, health care providers and the scientific community (3).

INFANT NUTRITION

After 6 mo of age, when breastmilk alone becomes increasingly short in providing the infant with sufficient amounts of micronutrients to sustain growth and development, the need for complementary foods becomes evident. The term complementary foods has in this thesis a broad definition, including both foods that are given in addition to breast milk (2) and all other foods that are used during the transition from the liquid diet typical of small infants to family foods. Complementary foods may consist of transitional foods, i.e. foods specifically designed to meet the nutritional needs of infants and young children, or family foods, meaning the that the infant consumes the same food as the rest of the family.

The composition of complementary foods and how they are used vary with country and culture, but there are some universal aspects. One aspect, common to both low-income and high-income settings, is that they are often plant-based (4-6). Although plants are excellent sources of energy and nutrients, plant constituents can also greatly affect nutrient bioavailability, i.e. the fraction of a particular nutrient or other substance that is actually available for absorption by the body. Polyphenols, for example, found in many vegetables inhibit iron absorption (7). Another aspect of complementary foods is that the content of meat and other sources of animal protein tends to be low, also in the most affluent settings (5). Meats and
other animal protein sources are not only excellent sources of protein and energy, but also contain many essential nutrients, and increase the absorption of others, for example, animal protein enhances zinc absorption. The intestinal absorption of micronutrients thus is affected by the composition of the diet, with factors both enhancing and inhibiting absorption.

There are also many important differences among complementary foods, particularly between high-income and low-income settings. Whereas in the former setting it is common to give industrially manufactured, fortified and nutritionally balanced foods, in the latter it is more often home-produced from locally available staples. Although studies of complementary foods in developing countries indicate that the energy and protein content may be adequate, they may be short in micronutrients; in most low-income settings, intakes of iron, zinc and vitamin B₉ are inadequate, and in some populations the same is true for riboflavin, niacin, calcium, thiamin, folate, vitamin C and vitamin A (6, 8, 9).

Although providing adequate amounts of energy and some nutrients, diets may contain low absolute amounts of other nutrients, or nutrients may have low bioavailability, leaving the infant or child at risk of specific nutrient deficiencies. The symptoms of these micronutrient deficiencies may appear subtle, although the impact on health both in the short and long run is substantial. Such vitamin and mineral malnutrition, sometimes called hidden hunger, is amongst the most common nutritional disorders in infancy and childhood world-wide (10). This thesis concerns two of these micronutrients, iron and zinc, how they affect health and well-being of term infants, and how two different nutrient interventions can influence iron and zinc nutriture during the second half of infancy and early childhood.

IRON

Iron is the fourth most abundant element in the Earth’s crust and one of the cheapest (11), still iron deficiency is the most prevalent nutritional disorder in the world (12). UNICEF estimates that 40-50% of children under five years of age in low income countries suffer from iron deficiency (ID), and that overall some 2 billion people world-wide are anemic, with an even greater number suffering from ID (Figure 1) (13).
Iron is essential for a number of important proteins and metalloenzymes in the body, which often use its ability to alternate between two oxidation states – ferrous ($\text{Fe}^{2+}$) and ferric ($\text{Fe}^{3+}$) iron – in their function. Hemoglobin (Hb) and myoglobin are among the more well-known iron-dependent compounds, but others include cytochromes, peroxidases, and non-heme proteins such as ribonucleotide reductase and lactoferrin. Thus, iron is essential in the function of the living organism from oxygen transport to DNA synthesis.
Iron metabolism

The human iron pool is mainly regulated by intestinal uptake from the diet (14), since epithelial losses and losses through urine, stool and sweat under normal circumstances are small (15). The human body has no active way to discharge excess iron.

Iron homeostasis has recently been reviewed (16-18) (Figure 2). Absorption of non-heme iron is concentrated to the gastroduodenal junction. The iron is transported across the apical membrane of mature enterocytes by divalent metal transporter 1 (DMT1, formerly Nramp2 or DCT1).

Figure 2. Absorption of iron and zinc from the small intestine is affected by enhancers and inhibitors in the intestinal fluid. Dietary iron is transported across the apical membrane of the enterocyte by DMT1. The mechanism by which dietary zinc crosses into the enterocyte is less clear, although the transporter ZIP4 has been suggested. At the basolateral side of the enterocyte, iron is exported to the portal circulation by ferroportin1, whereas zinc is transported by Zn-T1. In the circulation iron is bound to transferrin and zinc mainly to albumin. Because of their omnipresence in the human body, deficiency of iron and zinc affect many different organs and tissues.
This protein channel may also transport other metals, e.g. zinc, cadmium, lead, manganese and cobalt (19). At the basolateral side of the enterocyte, iron is transported to the portal circulation, supposedly by ferroportin1 (formerly Ireg1 or MTP1). The export of Fe$^{2+}$ is facilitated by hephaestin, a transmembrane protein similar to ceruloplasmin, which also oxidizes Fe$^{2+}$ to Fe$^{3+}$.

Ferric iron is then bound to transferrin and transported to tissues in need of iron, most notably erythropoietic cells in the bone marrow for the production of hemoglobin. Transferrin receptors on the cell surface bind the transferrin-Fe$^{3+}$ complex, and the complex is taken into the cell using endocytosis. The ferric iron is again oxidized to Fe$^{2+}$, which DMT1 transports from the endosomes to the cytosol. The intracellular iron is then transported across the mitochondrial membrane, where it is used for incorporation into heme, used for other purposes in the cell or stored in cytosolic ferritin. Circulating erythrocytes are destroyed by macrophages in the reticuloendothelial system. Bound to ceruloplasmin, iron is moved out of the reticuloendothelial macrophages and delivered to transferrin for recycling.

Surplus iron may be stored in ferritin, a large multifunctional and multunit protein, capable of reversibly binding as many as 4500 iron atoms as iron oxide (20). Ferritin is found in the cytosol of most cell types, most notably in the liver and various macrophages found in the liver, spleen and bone marrow, but it is also the storage form of iron in normal erythroblasts undergoing maturation. It plays a key role in iron homeostasis because of its capability to regulate the intracellular labile iron pool.

Intracellular Fe$^{2+}$ can participate in the generation of oxygen radicals, which can severely damage cell organelles including the DNA. Through its enzymatic properties, ferritin converts Fe$^{2+}$ to Fe$^{3+}$, which is then stored in the ferritin mineral core as iron oxide (21). Ferritin may thus detoxify not only highly reactive Fe$^{2+}$, but also oxygen radicals harmful to the organism. Ferritin is found in small quantities in human serum, although the origin of this ferritin is unknown.
Measures of iron status

Hematological indices: Hemoglobin and MCV

In infants as well as adults, about two-thirds of the body iron is bound to Hb in the red blood cells. In the absence of available iron, Hb begins to decline, thus Hb distinguishes between anemic and non-anemic iron deficiency (ID) states. However, anemia, i.e. low Hb, may be caused by deficiencies of other nutrients, e.g. vitamin B₁₂ and folic acid, as well as a number of other conditions including hemolytic states, inflammation, and defective hemoglobin such as thalassemia.

Thus, anemia alone does not necessarily imply iron deficiency. Mean cell volume (MCV), a measure of the size of red blood cells, can give guidance in discriminating between microcytic anemia, caused by for example, ID; normoblastic anemia such as in hemolytic states; and megaloblastic anemia, caused by for example vitamin B₁₂ deficiency. In ID, zinc is incorporated into protoporphyrin instead of iron, thus zinc protoporphyrin (ZPP) to heme ratio can be measured as an index of iron-deficient erythropoiesis (22, 23).

Biochemical indicators: Serum ferritin, serum iron and serum transferrin receptors

Although the exact origin and role of ferritin in serum is still shrouded in uncertainty, serial phlebotomy in adults has indicated a strong correlation between serum ferritin (S-Ft) and the magnitude of iron stores, decreasing in iron deficiency and increasing in iron overload states, such as hemochromatosis. Not surprisingly, it is widely used as an indicator of iron stores, not only in adults, but also in infants and children (24, 25). Serum ferritin is iron-poor (<5% saturation) and carries carbohydrates on its surface indicating active secretion of the protein to the serum. However, S-Ft acts as an acute phase reactant and in the presence of inflammation it may increase considerably, which may mask depleted iron stores (26-28). This limits the value of serum ferritin as a marker of iron stores, especially in the face of inflammation and infection, the latter being common in childhood.

Serum iron (S-Fe) measures the transferrin-bound iron in serum and together with the transferrin concentration, which determines how much iron can be bound in plasma, i.e. the total iron binding
capacity (TIBC); it is used as another functional assessment of iron status. These measures are however subject to considerable variation; for S-Fe there is a diurnal variation with lower concentrations in the afternoon and also during acute phase reactions, and similar to S-Ft, TIBC increases during acute phase reactions, which limits their usefulness, and its discontinuation in the diagnosis of ID has been suggested (23, 29).

In the search for more specific tools to measure iron stores serum transferrin receptor (S-TfR) has been identified as an indicator of intracellular iron demand and hence a measure of iron status (30). These receptors are found most abundantly on cells involved in erythropoiesis (31), and they are progressively found in serum as iron stores are depleted. Unlike S-Ft, S-TfR is not affected by inflammation (32). However, the measurement of S-TfR has not yet been standardized and various commercial kits have different cut-offs. This together with insufficient data on S-TfR as an indicator of ID in infants and young children has so far limited its use (23). A novel indicator of iron status, especially in the diagnosis of IDA in children, is the reticulocyte hemoglobin content (33), although its exact role remains to be elucidated.

Iron deficiency

Iron deficiency is considered to develop in discreet steps, which are reflected by the biochemical markers of iron status in blood. First, with iron needs surpassing demands, iron stores are depleted, which is seen in the blood as diminishing S-Ft values. Several cut-off values for S-Ft have been proposed to determine when depleted iron stores are present; ranging from 10-16 µg/l, but so far no consensus has been reached, in part because of lacking information on the functional consequences of different levels of iron stores. As iron status deteriorates, there is also evidence of increasing hematological and biochemical changes due to iron-deficient erythropoiesis (changing blood indices, e.g. decreasing MCV, decreasing transferrin saturation and increasing erythrocyte zinc protoporphyrin) as well as signals of increasing cellular iron demand (increasing S-TfR). The last stage is frank anemia, reflected in a low Hb (34). This is the indicator most commonly used when evaluating ID, although a low Hb is, as already stated, frequently caused by other factors, i.e. infections, other non-nutritional causes or non-iron nutritional causes (26, 35).
Definition of iron deficiency

With the plethora of iron status measures available, multiple definitions of ID have been proposed (36-41) (Table 1). In this thesis the definitions suggested by WHO (40) are used, i.e. from 6 mo of age; Hb <110 g/L, defining anemia; and S-Ft <12 µg/L, defining depleted iron stores. In conjunction to these, when available, the following are used: MCV <73 fl, defining iron-deficient erythropoiesis (41), transferrin saturation <16%, indicating low levels transferrin-bound iron, or as recently has been suggested S-TfR >11 mg/L, indicating increased cellular need for iron (36). However, all these definitions and cutoffs have been challenged in infants and young children as they are to a large extent extrapolated from values in older children and adults (23, 36). Hence, there is a need for age appropriate cut-offs for the various iron-status indicators (23, 36, 37), particularly in infants.

Table 1. Suggested cut-offs for various iron status indicators in infancy according to various authors.

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Hb (g/L)</th>
<th>S-ferritin (µg/L)</th>
<th>MCV (fl)</th>
<th>Transferrin saturation (%)</th>
<th>S-TFR (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WHO (40)</td>
<td>&lt;110</td>
<td>&lt;12</td>
<td>73</td>
<td>16</td>
<td>NA</td>
</tr>
<tr>
<td>Domellöf et al 2002 (36)</td>
<td>6 mo: &lt;105 9 mo: &lt;110</td>
<td>6 mo: &lt;9 9 mo: &lt;5</td>
<td>&lt;71</td>
<td>NA</td>
<td>&gt;11</td>
</tr>
<tr>
<td>Dallman et al 1980 (59)</td>
<td>&lt;110</td>
<td>&lt;10-12</td>
<td>70</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Emond et al 1996 (37)</td>
<td>&lt;97</td>
<td>&lt;17</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Looker et al 1997 (281)</td>
<td>&lt;110</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Virtanen et al 1999 (259)</td>
<td>&lt;106</td>
<td>&lt;9</td>
<td>&lt;72</td>
<td>&lt;5</td>
<td>&gt;11</td>
</tr>
</tbody>
</table>

NA – not available
Consequences of iron deficiency

There are a number of adverse functional consequences of ID alone or in conjunction with anemia, including decreased work capacity (42-44), impaired regulation of body temperature and heat production in cold environments (45), and increased uptake of lead (46). ID reportedly also affects growth (47) and the immune system (48), but it is the suggested, and possibly irreversible effect of ID on cognitive and psychomotor development of infants and young children that brought it onto the public health agenda (49-52). Studies on auditory brainstem responses and visual evoked potentials suggest that ID alters neuron myelination in infants (53, 54). Effects of ID on cognitive functioning may also be evident in school-aged children (55).

Causes of iron deficiency

Iron deficiency is caused by inadequate intake or low bioavailability of dietary iron to meet the organism’s metabolic needs, or by non-nutritional factors such as parasite infestation, particularly intestinal parasites such as hook worm, or blood loss, including obstetric practices (56-58). The metabolic needs for iron are affected by physiological demands such as rapid growth, pregnancy, lactation, and increased losses (e.g., menstruation). Thus, infants, children and women during their fertile years are particularly prone to develop ID. In children, the highest prevalence of ID is found between 4 mo and 3 years of age (59). There are estimates that more than half of the children worldwide have ID, mainly due to poor iron stores to begin with due to low birth weight and other fetal factors, low absolute iron intake in the diet, poor bioavailability of the dietary iron, and, in endemic areas infestations with hookworm, or a combination of these factors (40).

In infants and young children, dietary factors are strongly contributing to the problem of ID. These factors include short breastfeeding periods, early introduction of foods that interfere with the uptake of iron from breast milk, and use of complementary foods with low iron content or with low bioavailability of iron (60, 61). In some high-income settings, the prevalence of ID among infants and children has decreased due to increased prevalence of exclusive breastfeeding, increased use of iron-fortified foods and decreased use of cow’s milk in infancy and early childhood (62, 63), although low absolute iron intake has also been reported from
affluent societies (64). Early introduction of and high intake of unmodified, i.e. non-iron fortified cow’s milk is associated with increased risk of ID also in high-income settings (65). In other parts of the world, complementary foods continue to be low in iron, or contain large amounts of inhibitors or inadequate quantities of enhancers, leading to an inadequate intake and/or absorption of iron and eventually to high prevalence of ID (66, 67).

Iron in the diet

Two types of iron are available in the diet: heme and non-heme iron. Heme iron is mainly found in animal products, while non-heme iron is largely found in plant foods. The bioavailability of heme iron is high and not influenced much by the composition of the food (68), although calcium has been shown to decrease its absorption (69). In contrast, the bioavailability of non-heme iron is greatly influenced by various dietary enhancers (e.g. meat and vitamin C) and inhibitors (e.g. polyphenols, phytate, calcium, tannins) (70). Therefore, absorption of non-heme iron can vary between <1% to >50% (7). Despite this, non-heme iron is the quantitatively most important source of iron in diets world wide, especially among infants and children in low-income countries (66).

Ascorbic acid (vitamin C) is an important enhancer of iron absorption (71), acting in the stomach and duodenum primarily by reducing Fe³⁺ to Fe²⁺, thus improving iron solubility, which is higher in the Fe²⁺-state. At an ascorbic acid:Fe molar ratio of 4:1, ascorbic acid prevents the negative effects of inhibitors of iron absorption inhibitors, such as calcium, polyphenols, phytate and soy (72-74). However, the effects of ascorbic acid on non-heme iron from the complete diet has been more modest than from single meal studies (75). A high intake of ascorbic acid, either as fruit or vegetables is associated with higher Hb in children up to 18 mo (76), and increased uptake of ascorbic acid is one of the suggested strategies to improve iron bioavailability, also in low-income settings (77, 78).
ZINC

Zinc is like iron a divalent metal, but considerably less abundant (79). Although zinc has been know to be essential for living organisms since the latter part of the 1900\textsuperscript{th} century (80), it is only within the last 40 years that it has been established as an essential nutrient for humans (81). Reasons for this, though zinc is ubiquitously present in the cells of living organisms, including humans, are that actual zinc status is difficult to measure and symptoms of deficiency are subtle. Thus, an estimate of the global prevalence of zinc deficiency (ZD) has been difficult to achieve. Estimates using indirect measures, such as the zinc content in the global food supply, shows that 49% of the world’s population is at risk of inadequate zinc intake (79), of which the majority is found in low-income countries. An estimate based on the disability adjusted life years lost (DALY) places ZD behind vitamin A deficiency, but before ID in importance (82).

Zinc is essential to the function of a number of enzymes, structural components and regulatory proteins vital to the human organism. It has unique properties, although in contrast to iron it does not display redox chemistry. Thus, zinc functions in milieus where generation of free radicals would be deleterious. Zinc is involved in several of the basic functions of the cell and acts catalytically as well as structurally and regulatory, affecting cell growth and differentiation. Examples of zinc-dependent compounds are RNA nucleotide transferases, carbonic anhydrases, and zinc fingers in various proteins. Zinc may also act as a factor regulating gene expression, e.g. for metallothionein, and may regulate apoptotic cell death (83). About 60% of total body zinc is found in muscle tissue and 30% in bone, whereas plasma zinc only accounts for 0.1% (84).

Zinc metabolism

Zinc homeostasis is primarily regulated through zinc absorption and excretion in the gastrointestinal tract, although some zinc is excreted in the urine. Epithelial losses and losses in sweat are small, but in adult men losses in semen can be substantial (85). The jejunum has the highest rate of absorption (Figure 2), but zinc is also absorbed from the duodenum and ileum (83). Both absorption and excretion are actively, and maybe independently, regulated. Decreasing zinc intake increases fractional absorption of zinc and
increasing intake increase fecal excretion. Excretion responds faster to changes in dietary zinc intake, but absorption of zinc has the capacity to adjust for larger fluctuations in zinc intake (86). In infants, fractional absorption varied from 29% to 54% as zinc intakes decreased from 111 to 15 µmol/d (87).

Several classes of zinc transporters have been identified (88). The first transporter identified, ZnT-1 (89), is found in virtually all organs. In the duodenum and jejunum it is located to the basolateral membrane, exporting zinc from the enterocyte to the circulation. Increased dietary zinc intake increases ZnT-1 levels in the intestine indicating a function of the transporter in zinc retention (90). A recently discovered transporter, ZIP4, mutated in patients with acrodermatitis enteropathica, a sometimes lethal disease of zinc metabolism, is located at the apical membrane of enterocytes and may function as the main transporter of zinc into the enterocyte, and has been suggested to have a key regulatory function in dietary zinc absorption and cellular zinc homeostasis (91, 92).

Experimental studies indicate that humans can regulate their whole body zinc over a 10-fold change in intake. In adult men fed a zinc-restricted diet homeostatic mechanisms to control body zinc included reduced urinary and fecal excretion and reduced plasma zinc concentration, with urinary and plasma zinc concentrations responding faster than fecal zinc (93).

Through stable isotope studies in adults an exchangeable zinc pool (EZP) has been identified (94). This pool mixes with plasma and is thought to ascertain zinc supply to zinc-dependent biological processes. In infants, the EZP also seems to relate to crucial variables of zinc homeostasis (87). Hence, EZP is positively correlated to the total amount of absorbed zinc and endogenous fecal zinc excretion, but is not correlated to fractional absorption of zinc. This would indicate that the regulation of total absorbed zinc is limited, that endogenous zinc excretion is both dependent on and responsive to the individual’s zinc status, and that intraluminal zinc and, possibly, presence of factors affecting absorption such as phytate, affect fractional absorption more than the infant’s zinc status (87).
Measures of zinc status

There is still no single, sensitive and specific measure of zinc status. Plasma or serum zinc (S-Zn) has been most commonly used. A zinc-restricted diet significantly decreases S-Zn (85), but the assay is affected by a number of other factors, e.g. infections, environmental contamination, and time since last meal, which limits its value (95, 96). However, in a study from Peru, children with clinical signs of infection (fever, respiratory infections, diarrhea or anorexia) did not differ significantly in S-Zn from those without infection, whereas those with biochemical evidence of infection, i.e. elevated C-reactive protein or leukocytosis had significantly lower S-Zn compared to those with normal laboratory tests (26). S-Zn decreases after meals (97), although this effect may be blunted in infants; Michaelsen et al found no correlation between duration of fasting and S-Zn in Danish infants 2-9 mo of age (98). Technical predicaments such as hemolysis of the blood sample or zinc contamination from syringes, needles, test tubes, etc., may falsely elevate S-Zn (99). Other measures used in the assessment of zinc status have been hair zinc, urine zinc, zinc content of leukocytes, and the activity of zinc-containing enzymes such as alkaline phosphatase, or the protein metallothionein and serum thymulin. In most cases sensitivity and specificity of these tests have been low, the analyses laborious to perform, or the results difficult to interpret (95). Assessment of dietary zinc intake has also been used (66). Although this method provides a feasible way to quantify zinc intake, which may be an indirect proxy for zinc status, it requires information on the zinc content of local foods as well as presence of dietary factors affecting zinc bioavailability and the likely absorption of zinc from these diets. Thus, in spite of these short-comings, from a pragmatic point of view, determination of serum or plasma zinc concentration remains the best reasonable method to assess zinc status, especially at the group level (100).

Zinc deficiency

Aspergillus niger, a fungus causing black mold on foodstuffs, was the first biological system where zinc was shown to be essential [J Raulin 1869, cited in (80)], but it was not until the early 1960s that ZD was described in humans (81). Dietary factors, i.e. low absolute intake of zinc, the presence of phytate or other inhibitors of zinc absorption, absence of enhancers of zinc absorption such as animal
protein, and increased losses as in diarrheal states, all contribute to the development of significant ZD (101, 102).

Definition of zinc deficiency

With the problems of assessing zinc status, defining ZD has been equally difficult. Based on data from the second National Health and Nutrition Examination Survey (NHANES II), a much used definition of low S-Zn has been <10.7 µmol/L (<70 µg/dL) in persons sampled in the morning after an overnight fast and <9.2 µmol/L (<60 µg/dL) in non-fasting persons sampled in the afternoon (103). A recent reanalysis of the NHANES II data (104) has suggested a lower 2.5 percentile cutoff of 9.9 µmol/L (65 µg/dL) in children <10 years. However, the children examined in NHANES II were all 3 years or older. Population-based data in infants are restricted to two surveys; one from Canada (n=62, age 1-6 years), which observed the 2.5 percentile at 10 µmol/L (105), and a more recent from Australia (n=467, age 9-62 mo.), which suggested the lower 2.5 percentile at 9 µmol/L (106).

Consequences of zinc deficiency

Due to the ubiquitous presence of zinc in the cell, ZD affects the basic mechanisms of the organism including growth, appetite, skeletal maturation, sexual development, and the immune system (81, 83, 107). Acridermatitis enteropathica, a potentially lethal inborn error of metabolism causing malabsorption of zinc, instigates desquamating dermatitis, diarrhea, mood changes, anorexia, growth retardation, alopecia, and recurrent infections. The condition is readily treatable with oral or parenteral zinc (108, 109). However, the vast majority of cases with ZD has much more obscure symptoms including growth failure, increased susceptibility, severity and duration of infectious disease episodes, and possibly altered behavior (110-112).
IRON AND ZINC REQUIREMENTS

Iron requirements

The healthy, full-term infant is born with iron stores which, in breast-fed infants, last up to 6 mo of age (113, 114). The growing infant will increase its total body iron from about 250 mg at 6 mo of age to about 420 mg at 12 mo. The need for iron is estimated to 45 mg per kg weight gain (115). Infants with low birth weight have smaller stores and require supplemental iron from an earlier date (116). The total requirements of absorbed iron in infants (6-12 mo; 80 µg/kg body weight/d) and young children (1-3 years; 35 µg/kg/d) can be divided into those needed for growth (infants 6-12 mo; 60 µg/kg/d, and children 1-3 years; 20 µg/kg/d) and those to cover iron losses (infants 6-12 mo; 20 µg/kg/d, and children 1-3 years; 15 µg/kg/d). Based on these assumptions and an assumed bioavailability of 5-15% depending on the presence or absence of inhibitors or enhancers of absorption in the food, the recommended daily iron intake of infants 6-12 mo is 0.7-2.1 mg/kg/d and of children 1-3 years 0.3-0.9 mg/kg/d (117) (Table 2).

Table 2. Iron and zinc requirements (mg/d) in infants and young children 6-36 mo of age according to bioavailability. From (117).

<table>
<thead>
<tr>
<th>Bioavailability</th>
<th>Fe</th>
<th>Age (mo)</th>
<th>High 15%</th>
<th>Intermediate 12%</th>
<th>Low 10%</th>
<th>Very low 5%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>6-11</td>
<td>6.2</td>
<td>7.7</td>
<td>9.3</td>
<td>18.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12-36</td>
<td>3.9</td>
<td>4.8</td>
<td>5.8</td>
<td>11.6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Zn</th>
<th>Age (mo)</th>
<th>High 50%</th>
<th>Moderate 30%</th>
<th>Low 15%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6-11</td>
<td>2.5</td>
<td>4.1</td>
<td>8.4</td>
</tr>
<tr>
<td></td>
<td>12-36</td>
<td>2.4</td>
<td>4.1</td>
<td>8.3</td>
</tr>
</tbody>
</table>

Zinc requirements

Although breast milk contains relatively low concentrations of zinc, it is highly available (118). The combination of high fractional absorption of available zinc in breast milk, low endogenous fecal zinc excretion and, possibly, stores from birth, ensure adequate zinc status to sustain growth through the first 5 mo of infancy (118,
119). Even in mothers with marginal zinc intake, adequate breast milk zinc output is sustained during these first months of lactation (120). However, in mothers from both high-income and low income settings, zinc concentrations in breast milk fall dramatically during the infant’s first half year (121-125) and zinc supplied from complementary foods is deemed necessary after 6 mo of age (2).

Zinc requirements of infants and children have been extrapolated from adults, as data on zinc metabolism of younger age-groups are lacking (84). In the age-group 6-12 mo some 35 µg zinc/kg body weight/d are needed to cover basal losses and another 35 µg/kg/d are needed for growth, adding up to a total basal need of around 70 µg absorbed zinc/kg/d. In young children, 1-3 years old, the corresponding figure for maintenance of basal losses is 35 µg/kg/d and for growth 15 µg/kg/d, which gives a total of 50 µg absorbed zinc/kg/d. However, prolonged provision at that rate will leave the individual with no reserves in case of further reductions in zinc intake or increased losses as is seen in diarrheal states (102). Hence, a normative physiologic requirement, which is 40% above the basal requirement, has been calculated. In 6-12 mo old infants, this corresponds to 95 µg zinc/kg/d, and in children 1-3 years of age, 70 µg/kg/d.

In the last trimester of pregnancy, the fetus accumulates zinc. Thus, infants born prematurely (<38 weeks of gestation) presumably have smaller zinc stores. Studies show that preterm infants need to retain (i.e. absorption minus endogenous excretion and other losses) at least 326 µg zinc/kg/d to grow at the expected rate (126), and that a higher zinc intake is beneficial to very-low-birth-weight infants (127). Other infants requiring catch-up growth, e.g. infants recovering from malnutrition or infection also have increased zinc needs. A modest estimate has been that an underweight (5th percentile of the WHO reference population) infant at one year requires 114 µg absorbed zinc/kg/d to reach the 40th weight-percentile within a reasonable amount of time (84). Zinc supplementation has been efficient in the rehabilitation of malnourished infants (128), though high doses may have detrimental effects (129).

As for iron, zinc absorption is influenced by a number of dietary factors. Animal and fish protein are both rich sources of zinc and important stimulators of zinc uptake (130). On the other hand, phytate is one of the most important inhibitors, and is likely to contribute significantly to the world-wide problem of inadequate
zinc status [reviewed in (101)]. With diets differing in zinc bioavailability, different recommendations regarding the lower limits of population mean zinc intake per day are given for diets with high (i.e. 50%), moderate (30%) or low bioavailability (15%) (117). Table 2 gives the recommended intakes of infants and young children. A high zinc bioavailability diet is low in cereal fiber, has a low phytate:zinc molar ratio (i.e. <5), and has adequate protein from mainly animal sources. A diet with moderate bioavailability has a phytate:zinc molar ratio of 5-15, though not exceeding 10 if a considerable part of the energy intake comes from unrefined cereals, and does contain animal protein sources. A low bioavailability diet is high in unrefined cereals, low in animal protein and has a phytate:zinc molar ratio >15. Especially phytate:zinc molar ratios >15 are known to compromise zinc status (131, 132).

Phytic acid

Phytic acid (myo-inositol hexaphosphate; Figure 3) is a constituent of plants such as seeds, roots, tubers and vegetables, where it functions as the storage form of phosphorus. The focus on phytic acid in human nutrition has largely been due to its ability to chelate metals. In the intestinal lumen it is negatively charged over a wide pH-range and can thus chelate cations such as Ca\(^{2+}\), Zn\(^{2+}\), Fe\(^{2+}\) and Mg\(^{2+}\). These chelates are either insoluble or difficult to hydrolyze during digestion, and thus difficult to absorb by humans and other monogastric animals. A chelate of phytic acid and a mineral is called phytate. In grains such as barley, wheat, rye and rice, most of the phytate (90%) is found in the outer layers of the seed. Thus, whole meals of cereals have higher contents of phytic acid than do flours with lower extraction rates, roots, tubers and vegetables. Western diets typically include low extraction flour and roots, tubers and vegetables with low phytate content, whereas the typical diet in many low-income countries include whole meal cereals and other plant-based foods with high phytate content. The difference in daily phytate intake between a Western country, e.g. Sweden, and a less affluent country may be >10-fold (133, 134). Table 3 shows the phytate content in common staple foods. Phytate, in the absence of ascorbic acid, inhibits iron absorption in a dose-dependent manner above a phytate:Fe molar ratio of 1:7 (135), whereas phytate:zinc molar ratios >15:1 have been shown to inhibit zinc absorption (131, 132). Large intakes of phytate have also been associated with large fecal zinc losses (136).
Table 3. The phytate content in common staple foods.

<table>
<thead>
<tr>
<th>Staple foods</th>
<th>Phytate (µmol/100 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rye</td>
<td>1280</td>
</tr>
<tr>
<td>Wheat</td>
<td>1450</td>
</tr>
<tr>
<td>Oat</td>
<td>1430</td>
</tr>
<tr>
<td>Barley</td>
<td>940</td>
</tr>
<tr>
<td>Wheat bran</td>
<td>4330</td>
</tr>
<tr>
<td>Rice, brown</td>
<td>1120</td>
</tr>
<tr>
<td>Rice, polished</td>
<td>1000</td>
</tr>
<tr>
<td>Maize</td>
<td>1300</td>
</tr>
<tr>
<td>Soy bean</td>
<td>2000</td>
</tr>
<tr>
<td>Lentils</td>
<td>1400</td>
</tr>
<tr>
<td>Roots and tubers</td>
<td>180-530</td>
</tr>
</tbody>
</table>

Calculated from (133, 262).
Figure 3. Phytic acid is negatively charged in the intestinal lumen, giving it an ability to chelate various metal cations. These complexes, called phytates, are difficult or impossible to hydrolyse during digestion, rendering the metals unavailable for absorption.

IRON AND ZINC INTERACTION

Sharing several chemical characteristics, it has been suspected that iron and zinc may interfere with one another with regard to absorption and metabolism. It has been shown that high concentrations of inorganic iron given in water inhibit zinc absorption (137-139) and zinc given in water inhibits iron absorption (140, 141). Solomons and Jacob (137) found that 25 mg Fe added to a water solution with 25 mg Zn decreased plasma zinc, whereas lower total amounts of minerals (10 mg Fe and 5 mg Zn) and an iron:zinc ratio of 2:1 had no effect on plasma zinc levels (142). However, Sandström et al (139) found no effect on zinc absorption when the iron:zinc ratio was 1:1 or 2.5:1 in a water solution, but decreased zinc absorption when the iron:zinc ratio was 25:1. Crofton et al showed that iron and zinc given in a 1:1 ratio decreased iron absorption (141), but that a 2:1 iron:zinc ratio had no effect on iron absorption. Rossander-Hultén et al found that an iron:zinc ratio of 1:4 significantly reduced iron absorption when given as a water solution but with no effect on iron absorption.
when given as a meal (140). No effect was seen of a high iron:zinc molar ratio on zinc absorption when given as infant foods (143-145).

Exactly by which mechanism iron and zinc interact is not known. One may be through a common transport mechanism. Previous studies indicated that iron and zinc shared a common transporter, i.e. DMT1 (19). However, later research has shown that though DMT1 does not transport zinc *per se*, zinc does affect the DMT1 level but there may be other, more important, common iron-zinc pathways (146). It is also not known why iron and zinc given together in foods seem not to interact. The two minerals are affected by some of the same inhibitors, e.g. phytate, though iron to a greater extent than zinc. In addition, the effects of iron and zinc may counteract each other in some situations, i.e. zinc has been shown to improve growth in nutritionally deprived infants (110), whereas iron may negatively affect growth in iron replete children (147-149). However, with both iron and zinc deficiency being highly prevalent and possibly present in the same groups of infants, the interaction between the two minerals is an important research issue.

**Strategies to prevent iron and zinc deficiencies**

Interventions to improve iron and zinc nutriture in infancy and childhood include optimizing complementary food nutrient density and enhancing the bioavailability of the minerals present, as well as avoiding foods that inhibit or displace iron and zinc in the diet. Complementary foods are cereal- or vegetable-based in most settings. If these foods are centrally produced, fortification is an attractive alternative, especially in the combination with ascorbic acid (78, 150). Adding other enhancers such as meat or other animal protein sources may also improve iron and zinc bioavailability (130, 151). Unmodified cow’s milk has a low content of iron, and consumption of large amounts of such milk has been associated with poor iron status in infancy and childhood (65), whereas iron-fortified cow’s milk formula is associated with improved iron status (152, 153). Food processing techniques such as fermentation, germination and milling may affect iron and zinc bioavailability through phytate degradation (154, 155).
If the complementary foods are home produced, supplementation with iron and zinc, or mixing it with foods as sprinkles or fat-based spreads may be feasible alternatives (9, 156, 157). However, which strategy to ensure adequate iron and zinc nutriture is most effective depends on the setting; fortification, addition of meat to complementary foods, and avoidance of large amounts of unmodified cow’s milk seem to work well in more affluent settings. Although iron and zinc supplementation has been suggested in low income settings (9), no consensus exists on how to effectively accomplish this, with remaining questions concerning dosage, interactions, avoidance of accidental poisonings, costs, compliance and distribution. Further, knowledge about which indicators are most accurate when evaluating such interventions is also missing. In the present thesis, two strategies, phytate-reduction of infant cereals in a high-income setting, and giving supplemental iron and zinc in a water solution to infants in a low income setting, are evaluated.
THE SCENE

THE SITUATION IN SWEDEN AND UMEÅ

The kingdom of Sweden is a high income country with a population of about 9 million. Infant and child health has improved greatly during the past century with an infant and under-five mortality rate at 3/1000 live born being among the lowest in the world (158). The nutritional situation among infants and children is considered good (159-163).

Umeå is a university town in the northern part of Sweden with a population of 105 000 (Figure 4). The town hosts a university hospital and 7 primary health care centers. In 1997, the infant mortality rate in the municipality of Umeå was 2.5/1000 live born and the rate of low birth weight (<2500 g) was 31.6/1000 live born. At one week of age 94% of infants were exclusively breast-fed, and at 6 mo 46% were exclusively and 32% partially breast-fed (164). The average annual income for people employed in the public sector was USD 29 457 (165).
Surprisingly, a cross-sectional study, performed in the 1990s (166) under the umbrella of the Euro-Growth multi-center study (65), showed that 26% of 12 months-old term infants had low S-Ft (<12 µg/l) and 36% low S-Zn (<10.7 µmol/l). Interpreting low S-Ft as depleted iron stores and low S-Zn as indicative of sub-optimal zinc status, these were unexpectedly high prevalence figures in seemingly well-nourished infants fed according to current recommendations. It was concluded that the most likely explanation to the findings was that the high intake of infant cereals, rich in phytate, impaired the bioavailability of iron and zinc leading to iron and zinc deficiency.

In Sweden, the intake of commercially manufactured, cereal-based products is high from 6 mo of age, as both milk-based cereal drinks (MCDs, essentially a liquid cereal eaten from a bottle) and porridges are consumed, but the intakes of infant formula, conventional follow-on formula and unmodified cow’s milk are low (161). The MCDs and porridges are composed of cooked cereals, skim milk powder, and vegetable fat. They are fortified with minerals (iron and calcium) and vitamins (vitamin A, C, D, E, B₆, B₁₂, thiamine, niacin, folic acid and pantothenic acid). With this composition they comply with European Union legislation (167). These products contribute substantially to the intakes of micronutrients during the second half of infancy (161, 168, 169).

THE SITUATION IN INDONESIA AND PURWOREJO

The republic of Indonesia is the forth most populous country in the world with a population of over 210 million. About 6 000 of the nation’s 13 500 islands are inhabited, Java being the most populated, harboring almost 2/3 of the population. Being a low income country, malnutrition among infants, children and women are important public health problems in Indonesia.

In 1997, the infant mortality rate was 52.2 and 45.2 per 1 000 live births and the under-five mortality rate was 70.6 and 59.9 per 1 000 live births at the national level and in Central Java, respectively (170). Malnutrition reportedly contributed to 40% of the infant mortality and 60% of the child mortality. The differences between regions and between social groups are substantial in Indonesia; children in eastern Nusa Tenggara and Kalimantan, in the rural areas and among the poorest socio-economic groups have the lowest
weight-for-age, while the situation is somewhat better on Java and Bali, in the urban areas and among the more affluent.

Data on anthropometrical status from 1995 show that at the national level, 11% and 13% were wasted [weight-for-height z-score (WHZ) <-2 SD compared to the WHO/NHCS reference population], 25% and 42% were stunted [height-for-age z-score (HAZ) <-2 SD], and 18% and 34% were underweight [weight-for-age z-score (WAZ) <-2 SD] in infants 6-12 mo and children <5 years, respectively (171). In Central Java Province, 29% of children <5 years were reported underweight (172) and that 38% of the children were stunted (173).

Purworejo is a predominantly agricultural district in the southern part of Central Java (Figure 4). The district covers 1 034 km² and the population was some 730 000 in 1994. The district is the location of a health surveillance project run by the Community Health and Nutrition Research Laboratories (CHN-RL), Gadjah Mada University, Yogyakarta, Indonesia. For this surveillance 14 868 households have been selected. The infant mortality rate in the surveillance area is reported at 49.2 (174) and the prevalence of low birth weight (< 2 500 g) was 60/1000 live born in 1995-96 (175). The prevalence of malnutrition in children <24 mo (Figure 5), based on two longitudinal cohort studies within the surveillance system (176, 177), indicates a pattern of increasing malnutrition in the population during the first 2 years of life typical of many low-income Asian countries (178). The median breastfeeding duration was 22 mo, but only 7% received colostrum, and at 1 mo of age a quarter of the infants were receiving complementary foods, a figure that increased to 70% at 4 mo (179). These figures are similar to those reported at the national level (170).
Figure 5. Prevalence (%) of stunting, underweight and wasting among infants and young children in the CHN-RL surveillance area at 6, 9, 12, 18 and 24 mo of age.

The frequency of bottlefeeding at 12 mo was reported at 16% in the surveillance area (179). The complementary foods used in the area are plant-based and contain little animal protein and low amounts of iron and zinc (180). This, together with large amounts of phytate renders the diet inadequate in terms of iron and zinc, and together with early introduction of complementary foods, reducing the bioavailability of breastmilk iron (60), places the infants at high risk of developing micronutrient deficiencies.

A report from Semarang on the north coast of Central Java (181), shows that even though breastfeeding initiation was close to 100%, 61% received pre-lacteal feeds. More than one third of breastfed infants were given supplementary food within the first two weeks, and this number increased steadily during the first year of life. Exclusive breastfeeding was uncommon, as was the use of colostrum. The principal foods given were grains, fruits and sugar, with little or no meat, fish or other animal source of nutrients. Close to half of the mothers gave tea with or without sugar to their infants.
Nationwide household surveys in 1992 and 1995 report anemia prevalence in under-fives at 56% and 40%, respectively [referred in (182)]. Dijkhuizen et al reported from a cross-sectional study in West Java (183) in infants 2.4-10.5 mo of age (n=155), who were assessed together with their mothers, that 57% of the infants had Hb <110 g/L, 54% had plasma retinol <0.70 µmol/L, but only 20% had S-Ft <15 µg/L and 17% S-zinc <10.7 µmol/L. In Central Java, Dibley and co-workers found 38% with Hb <110 g/L among children 6-48 mo of age (n=666) (Dibley 1995, unpublished data). From the same cross-sectional study in Central Java, Kjolhede et al reported that 58% had S-retinol <0.70 µmol/L, with the lowest S-retinol levels found in infants 6-11 mo of age (184). Since the late 1970s, Indonesia has a national program for vitamin A supplementation of children after the first year of life (185). Goiter due to iodine deficiency is endemic on all larger Indonesian islands, and some 20% of the population lives in areas with the goiter rate being greater than 10% (186). Among elementary school children in Purworejo, 11.5% of boys and 7.6% of girls were found to have mild to moderate goiter (D. Ismail 1996, personal comm.).
THE OBJECTIVE

The overall objective of the two randomized trials reported in this thesis was to prospectively investigate the effect of dietary or supplemental iron and zinc on iron and zinc status, infant linear growth, cognitive development, and incidence of childhood infectious diseases during the first 6-12 mo of age in a high-income (Umeå, Sweden; the SINUS study) and a low-income (Purworejo, Indonesia; the ZINAK study) setting.

The more specific aims were to:

- Assess whether phytate-reduced MCD and phytate-reduced porridge or infant formula and conventional porridge fed to Swedish infants from 6 to 12 mo of age would improve iron (Hb, MCV, S-Ft, S-TfR) and zinc (S-Zn) status, affect growth (weight, length, knee-heel length), mental and psychomotor development or the incidence of diarrheal and acute respiratory infections, measured during the second half of infancy compared to infants fed conventional MCD and porridge.

- Record the dietary intake of Swedish infants to explore the effects of dietary iron intake on iron status until 18 mo of age.

- Examine if daily supplementation with iron and zinc given alone or in combination from given to Indonesian infants 6 to 12 mo of age would improve iron (Hb, S-Ft, S-TfR) and zinc (S-Zn) status, growth (weight, length, knee-heel length), mental and psychomotor development or the incidence of diarrhea and acute respiratory infections compared with placebo, with emphasis on possible interactions between the two minerals for the biochemical and functional outcomes.
THE TRIALS

Two trials compose the basis of this thesis. The SINUS (Study on Infant Nutrition in Umeå, Sweden) study is described in detail in papers I – III and the ZINAK\(^\text{¶}\) (Zinc and Iron Supplementation to Infants in Purworejo, Indonesia) study is described in papers IV – V. The papers are summarized in Table 4. The two trials originate from a common problem of presumed or actual iron and zinc deficiencies due to inadequacies in the complementary diet during the latter half of infancy and their functional consequences and possible prevention.

The studies focus on iron and zinc nutritional status and deficiency with two different intervention strategies; reduction of a possible inhibitor of iron and zinc absorption, i.e. phytate, or supplementation with iron and zinc, appropriate for the different circumstances in which the trials took place. The studies share several common features; both use a randomized, controlled trial design; both follow infants from 6 mo of age, the age at which complementary feeding is recommended to commence; and both assess several of the same biochemical as well as functional outcomes.

There are also several differences in the design and execution of the trials due to the conditions of the populations studied, the actual problems prevalent in these settings, and the presumed ways to alleviate these problems. Thus, this thesis is not a presentation of an intervention trial in two different settings, but an effort to address a common problem in two different populations by different intervention strategies. Results and consequences are discussed separately as well as across the studies and inferences are drawn to the local populations as well as to the global scene, when appropriate.

\(^\text{¶}\) The acronym ZINAK stands for Zinc, Iron and aNaK, Indonesian for “child”.

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<table>
<thead>
<tr>
<th>Paper</th>
<th>Title</th>
<th>Objective</th>
<th>Sample</th>
<th>Outcomes assessed</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Effects of weaning cereals with different phytate contents on hemoglobin, iron stores, and serum zinc: a randomized intervention in infants from 6 to 12 mo of age</td>
<td>Examine if extensively phytate-reduced infant cereals, or infant formula and regular porridge reduce the prevalence of depleted iron stores and low serum zinc compared to regular infant cereal</td>
<td>267 infants completing a dietary intervention trial from 6 to 12 mo of age</td>
<td>Hb, MCV, S-Fe, S-Zn, S-Cu, and prevalence of anemia, low S-Fe and low S-Zn</td>
</tr>
<tr>
<td>II</td>
<td>Effects of weaning cereals with different phytate content on growth, development and morbidity: a randomized intervention trial in infants from 6 to 12 months of age</td>
<td>Examine if extensively phytate-reduced infant cereals, or infant formula and regular porridge improve growth, development and health compared to regular infant cereal</td>
<td>263 infants completing a 6 mo dietary intervention trial and follow-up until 18 mo of age</td>
<td>WAZ, HAZ, knee-heel length, BSID MDI and PDI, incidence of diarrhea and respiratory infections</td>
</tr>
<tr>
<td>III</td>
<td>Dietary iron intake affects hemoglobin during infancy but not during the second year of life</td>
<td>A secondary data analysis, assessing possible associations between dietary iron intake during infancy and early childhood and subsequent hematological and iron status.</td>
<td>245 children completing a 6 mo dietary intervention and follow-up until 18 mo</td>
<td>Hb, MCV, S-Fe, S-Fi, S-TiR in relation to dietary iron intake</td>
</tr>
<tr>
<td>IV</td>
<td>A community-based randomized controlled trial of iron and zinc supplementation in Indonesian infants: interactions between iron and zinc</td>
<td>Examine if daily supplementation with iron and zinc improve iron and zinc status compared to iron or zinc treatment alone or placebo</td>
<td>549 infants completing a supplementation trial from 6 to 12 mo of age</td>
<td>Hb, S-Fi, S-TiR, S-Zn, S-Cu, prevalence of low Hb, low S-Fi and low S-Zn, interaction between iron and zinc</td>
</tr>
<tr>
<td>V</td>
<td>A community-based randomized controlled trial of iron and zinc supplementation in Indonesian infants: effects on growth and development</td>
<td>Examine if daily supplementation with iron and zinc improve growth, development or reduces the incidence of diarrhea or respiratory infections compared to iron or zinc treatment alone or placebo</td>
<td>650 infants completing a supplementation trial from 6 to 12 mo of age</td>
<td>WAZ, HAZ, WHZ, knee-heel length, BSID MDI and PDI, incidence of diarrhea and respiratory infections</td>
</tr>
</tbody>
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METHODS

Design and interventions

Table 5 summarizes the main characteristics of SINUS and ZINAK. Table 6 summarizes the data collection and Table 7 shows methods used for analysis of blood samples and collection of anthropometrical and developmental data in the studies.

SINUS

In the Swedish study, infants were assigned to one of 3 study groups; regular infant cereals (commercially available MCD and porridge; CC group), low-phytate infant cereals (phytate-reduced MCD and porridge; PR group) or cow’s milk based infant formula and porridge (IF group). The rationale for this design was to allow for comparisons between both the regular Swedish complementary diet, i.e. phytate-containing infant cereals and a diet with phytate-reduced infant cereals, but also with the internationally more common practice of using a combination of cow’s milk formula and infant cereal, evaluating benefits as well as detriments of these different feeding regimes.

Both breast-fed and non-breast-fed infants were recruited. If the participating infants were breast-fed when entering the study, the mothers were recommended to breast-feed as long as they wished. At the mothers’ own discretion, the study products were introduced into the infants’ diets from 6 mo of age and continued until 12 mo of age, with no other interventions being done. Research nurses supplied the families with the predetermined MCD/formula and porridge ad libitum and free of charge. Semper AB, Stockholm, Sweden, manufactured all study products.

Table 8 summarizes the study products used in SINUS. Figure 6 shows the processes used to reduce the phytate content in the study products. The phytate content of the MCD and porridge was analyzed as individual inositol phosphates by HPLC according to Sandberg and Ahderinne (187).
Figure 6. The process of phytate reduction used in the SINUS study. Oat, whole meal wheat and rye flours were scalded and soaked at pH 4.5, allowing endogenous phytases to act. The slurry was then drum-dried and mixed with low-extraction wheat flour.
Table 5. Main characteristics of SINUS and ZINAK.

<table>
<thead>
<tr>
<th>Study</th>
<th>Study of infant nutrition in Umeå, Sweden</th>
<th>Zinc and iron supplementation to infants in Purworejo, Indonesia</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Acronym</strong></td>
<td>SINUS</td>
<td>ZINAK</td>
</tr>
<tr>
<td><strong>Study area</strong></td>
<td>Umeå, Sweden</td>
<td>Purworejo, Indonesia</td>
</tr>
<tr>
<td><strong>Problem</strong></td>
<td>Swedish infants show a 26% prevalence of low S-Ft and a 35% prevalence of low S-Zn, indicating suboptimal micronutrient status at one year of age. A high intake of phytate-containing cereals may contribute to this situation.</td>
<td>The infants of Purworejo, consuming a diet a high vegetable diet with little animal protein, are at a high risk of developing micronutrient deficiencies during their first year of life. Supplementation with iron and zinc may be a feasible way to provide these micronutrients.</td>
</tr>
<tr>
<td><strong>Design</strong></td>
<td>Randomized dietary intervention trial with 3 study groups; regular infant cereals (commercially available MCD and porridge; CC group) low-phytate infant cereals (phytate-reduced MCD and porridge; PR group) cow's milk based infant formula and porridge (IF group).</td>
<td>Randomized supplementation study with 4 supplementation groups in a factorial design; 10 mg of iron as ferrous sulfate (Fe group) 10 mg of zinc as zinc sulfate (Zn group) 10 mg of both iron and zinc (Fe+Zn group) placebo All in sweet-tasting syrup.</td>
</tr>
<tr>
<td><strong>Study population</strong></td>
<td>Healthy, term infants &lt;6 mo of age from 6 well-baby clinics in Umeå (gestational age at birth 38 – 42 weeks) with birth weights &gt;2 500 g. Infants who at 6 mo had IDA were not recruited. Exclusion criteria were severe and protracted illness and allergy or intolerance to the study products.</td>
<td>Healthy, singleton infants from the CHN-RL surveillance system in Purworejo district who had not passed 6 mo of age and whose mothers had been monitored during pregnancy and birth were considered eligible. Exclusion criteria were Hb &lt;90 g/L and severe and protracted illness.</td>
</tr>
<tr>
<td>Randomization</td>
<td>Block randomization; 12 infants per block</td>
<td>Block randomization; 20 infants per block</td>
</tr>
<tr>
<td>Sample size calculation</td>
<td>Prevalence of low S-Ft of 25% and of low S-Zn of 35% in the high phytate group, and an estimated prevalence of low S-Ft of 5% and low S-Zn of 15% in the low phytate group. With 100 infants per group we could allow for a 20% dropout rate ($\alpha=0.05$, power 80%) to show a significant difference in the prevalence of low S-Ft and low S-Zn between groups.</td>
<td>Primary outcomes of physical growth (knee-heel difference, 2 mm in 6 months), psychomotor development (Bayley scales of development, 5 points difference) and diarrheal disease morbidity (relative risk 0.65 in comparison with placebo). With a dropout rate of 20%, 170 per group ($\alpha=0.05$, power 80%) would detect significant differences between groups.</td>
</tr>
<tr>
<td>Outcomes</td>
<td>Major outcomes:</td>
<td>Major outcomes:</td>
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<tr>
<td></td>
<td>Iron status (Hb, MCV, S-Ft, S-TfR and S-Fe)</td>
<td>Iron status (Hb, S-Ft, S-TfR)</td>
</tr>
<tr>
<td></td>
<td>Zinc status (S-Zn)</td>
<td>Zinc status (S-Zn)</td>
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<tr>
<td></td>
<td>Prevalence of Hb $&lt;110$ g/l, S-Ft $&lt;12$ $\mu$g/l and S-Zn $&lt;10.7$ $\mu$mol/l</td>
<td>Prevalence of Hb $&lt;110$ g/l, S-Ft $&lt;12$ $\mu$g/l and S-Zn $&lt;10.7$ $\mu$mol/l</td>
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<tr>
<td>Secondary outcomes:</td>
<td>Major functional outcomes</td>
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<td>Somatic and linear growth</td>
<td>Somatic and linear growth</td>
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<td>Attained infant development</td>
<td>Attained infant development</td>
<td></td>
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<tr>
<td>Incidence of diarrheal disease and lower respiratory infections</td>
<td>Incidence of diarrheal disease and lower respiratory infections</td>
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Methods

Infants in ZINAK.
Table 6. Data collection in SINUS and ZINAK.

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<td>Intervention</td>
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<tr>
<td>Blood sample</td>
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<td>Anthropometry</td>
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</tr>
</tbody>
</table>

1Socioeconomic status.
Table 7. Analytical methods used in SINUS and ZINAK.

<table>
<thead>
<tr>
<th>Study site</th>
<th>SINUS</th>
<th>ZINAK</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin</td>
<td>Sysmex Sulfolyser automated hemoglobin reagent</td>
<td>Hemocue photometer</td>
</tr>
<tr>
<td>concentration</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum iron</td>
<td>Ferrozine method</td>
<td>NA</td>
</tr>
<tr>
<td>Serum ferritin</td>
<td>Immunoturbidometric technique</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>Serum transferrin receptor</td>
<td>Enzyme-linked immunosorbent assay</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>Serum zinc</td>
<td>Atomic absorption spectrophotometry</td>
<td>Atomic absorption spectrophotometry</td>
</tr>
<tr>
<td>Serum copper</td>
<td>Atomic absorption spectrophotometry</td>
<td>Atomic absorption spectrophotometry</td>
</tr>
<tr>
<td>Weight</td>
<td>Seca 835 digital baby scale</td>
<td>Seca 835 digital baby scale</td>
</tr>
<tr>
<td>Length</td>
<td>Harpenden infantometer</td>
<td>Locally produced wooden board</td>
</tr>
<tr>
<td>Knee-heel length</td>
<td>Infant knemometer BK5</td>
<td>Infant knemometer BK5</td>
</tr>
<tr>
<td>Head circumference</td>
<td>Non-stretchable plastic measuring tape</td>
<td>Non-stretchable plastic measuring tape</td>
</tr>
<tr>
<td>Mid upper arm</td>
<td>Non-stretchable plastic measuring tape</td>
<td>Non-stretchable plastic measuring tape</td>
</tr>
<tr>
<td>circumference</td>
<td></td>
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<tr>
<td>Infant development</td>
<td>Bayley Scales of Infant Development</td>
<td>Bayley Scales of Infant Development</td>
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<tr>
<td></td>
<td>Mental development (MDI)</td>
<td>Mental development (MDI)</td>
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<tr>
<td></td>
<td>Psychomotor development (PDI)</td>
<td>Psychomotor development (PDI)</td>
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<tr>
<td></td>
<td>Behavior (BRS)</td>
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Table 8. The energy and nutrient content in the infant cereals and infant formula per 100 g ready-to-feed product.

<table>
<thead>
<tr>
<th></th>
<th>Milk cereal drink/Formula</th>
<th>Porridge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6 – 7 mo.</td>
<td>8 – 11 mo.</td>
</tr>
<tr>
<td>Energy (kcal)</td>
<td>CC</td>
<td>PR</td>
</tr>
<tr>
<td>66</td>
<td>66</td>
<td>65</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>2.3</td>
<td>2.3</td>
</tr>
<tr>
<td>Fat (g)</td>
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<td>2.4</td>
</tr>
<tr>
<td>Carbohydrate (g)</td>
<td>8.6</td>
<td>8.6</td>
</tr>
<tr>
<td>Iron (mg)</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td>Zinc (mg)</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Calcium (mg)</td>
<td>93</td>
<td>93</td>
</tr>
<tr>
<td>Vitamin C (mg)</td>
<td>17</td>
<td>17</td>
</tr>
<tr>
<td>Phytate (μmol)</td>
<td>30.0</td>
<td>9.9</td>
</tr>
<tr>
<td>% IP 6(^{7})</td>
<td>65</td>
<td>87</td>
</tr>
<tr>
<td>Phytate:iron molar ratio</td>
<td>1.4:1</td>
<td>1.2:2</td>
</tr>
<tr>
<td>Phytate:zinc molar ratio</td>
<td>6.5:1</td>
<td>2.2:1</td>
</tr>
</tbody>
</table>

1Commercial MCD and porridge. 2Phytate-reduced MCD and phytate-reduced porridge. 3Infant formula and porridge with regular phytate level. 4Iron pyrophosphate. 5Iron sulfate 6Iron orthophosphate (EKA-Fer). 7Percentage myo-inositol hexaphosphate [IP\(_6\)] of total phytate.
Methods

ZINAK

In the Indonesian study, infants were randomly assigned to one of 4 supplementation groups: iron (Fe group), zinc (Zn group), iron + zinc (Fe + Zn group), or placebo. The four supplements provided the infants with a daily dose of either 10 mg of iron as ferrous sulfate (Fe group), 10 mg of zinc as zinc sulfate (Zn group), 10 mg each of iron and zinc (Fe + Zn group), or placebo, in a sweet-tasting syrup. All supplements included, per dose (1.6 ml, i.e. 2 measuring pipettes), 30 mg of ascorbic acid, sugar and water. The supplements would then contain approximately the recommended daily intake of iron and vitamin C and between 1-2 times the recommended daily intake of zinc for infants 6-11 mo of age (9, 117).

PT Konimex, Solo, Indonesia, manufactured the supplements in association with the Department of Pharmacology, Gadjah Mada University. The supplements were administered by the parents/caretakers once daily from 6 to 12 mo of age (180 days of supplementation). Field workers oversaw and administered the daily dose every third day and monitored the intake the previous two days by means of parent recall. Empty bottles were replaced every 2 weeks and the remaining syrup, if any, was measured and registered.

Primary outcomes

Iron and zinc status

In both studies, venous blood was collected in zinc-free vacuum systems. In the Swedish study iron status was analyzed at the Department of Clinical Chemistry, Umeå University Hospital by use of a Sysmex SE 9000 Autoanalyzer (Tillqvist, Kista, Sweden). Hb was analyzed using Sysmex™ Sulfolyser automated hemoglobin reagent (Toa Medical Electronics Co, Los Alamitos, CA, USA) and MCV was automatically calculated from the erythrocyte particle concentration and hematocrit. S-Fe was analyzed by the ferrozine method (Iron kit 1553712 and UIBC kit 1030600, Boehringer Mannheim, Scandinavia AB, Bromma, Sweden). S-Ft was analyzed by an immunoturbidimetric technique (BM/Hitachi 704/717/911, Boehringer Mannheim) calibrated against WHO standard 80-602.

In ZINAK, Hb was analyzed using a portable Hemocue® photometer (Hemocue AB, Angelholm, Sweden) and S-Ft was analyzed by radioimmunoassay (Diagnostic Products, San Diego,
Methods

CA). In both studies S-TfR was analyzed by enzyme-linked immunosorbent assay (ELISA) (Ramco, Houston, TX), and S-Zn and S-Cu were assessed by atomic spectrometry (188).

Anthropometry

In both studies, anthropometrical measurements were performed on a monthly basis from 6 to 12 mo of age, and in SINUS, bi-monthly from 14-18 mo of age. Naked weight was measured to the nearest 0.02 kg using a Seca 835 digital baby scale (Seca, Hamburg, Germany). Recumbent length was in the Swedish study measured using a Harpenden infantometer (CMS Weighing Equipment, London, UK), and in the Indonesian study using a locally produced wooden board. Knee-heel length was measured in both studies using an infant knemometer (Infant Knemometer BK5, FORCE Institutet, Brøndby, Denmark). Head circumference and mid-upper arm circumference were measured using a non-stretchable plastic measuring tape.

Infant development

Both studies used the Bayley Scales of Infant Development (BSID) (189) to assess development, recording the mental development index (MDI) and the psychomotor development index (PDI). In ZINAK the behavioral rating scale (BRS) was also recorded. In SINUS developmental testing was done at ages 7, 13 and 18 mo, whereas in ZINAK it was assessed at 6 and 12 mo of age.

Baseline and follow-up data collection

In SINUS, background data including family size, parental education and occupation were collected at 12 mo and then again at 18 mo, with birth weight and birth length collected retrospectively from birth records. In ZINAK, socio-economic information was retrieved from the CHN-RL surveillance system at baseline. CHN-RL field workers or community midwives measured birth weight at the time of delivery. After the end of the supplementation period, the Indonesian families were interviewed on perceived side-effects of the supplements (abdominal pain, decreased appetite, vomiting, diarrhea, constipation, increased crying or fussiness) as well as the general health condition of the child before and after supplementation.
Morbidity registration

Parents recorded daily symptoms of fever, coryza, cough, fast or difficult breathing, ear discharge and change in stool consistency in the Swedish study. Records were collected at monthly intervals and checked for consistency.

In ZINAK, field workers visited the families every third day, recording compliance to supplementation as well as symptoms of illness for the day of visit, and by parental recall, for the two days preceding the visit. Symptoms of fever (mother’s own definition); coryza, cough, difficult or fast breathing, ear-discharge, diarrhea and vomiting were recorded.

Dietary intake

Much emphasis was put on collecting dietary data in the Swedish study. Each month, starting from 6 mo of age, parents or caregivers recorded the type and amount of each food item consumed by the infant during five consecutive days. Household measures were used for quantities, and the participating family was encouraged to serve the study child all meals from a standardized plate. The families were also given a booklet with photos of different portion sizes of common infant foods, using the standardized plate, to facilitate registration (Figure 7).

Breast-feeding was recorded as ‘meal’, equivalent to a full meal, or ‘snack’, i.e. a short feed mainly for comfort or other non-nutritive purposes, according to the mother’s own perception. Daily energy and nutrient intake was calculated using the MAT’s software (Rudans Lättdata, Västerås, Sweden), which uses the food composition tables of the Swedish National Food Administration (190). The database was complemented with baby foods, formulas and other recipes not originally included according to information from the participating families and the manufacturers.

Intakes of breast milk were set to 134 g per meal up to 8 mo of age, to 102 g per meal beyond 8 mo, and to 25 g per snack at all ages (191). Nutrient intake from breast milk was calculated from Jensen (192) and Tsang et al (193). Nutrient and energy intake from breast milk was added to the total daily nutrient and energy intake of the study subjects for the 6-11 mo period, but not in the subsequent period.
Figure 7. Pictures from the booklet of different portion sizes of infant foods used in the recording of dietary intake in the SINUS study.

In the Indonesian study, 24-h recalls of dietary intake were collected monthly, but analyses of that data were not included in this thesis.

**Statistical methods**

SPSS for Windows 11 (SPSS Inc, Chicago, IL) was used for all statistical computations. Non-normally distributed variables, e.g. S-Ft and S-Tfr values, were log-transformed. Main outcomes are shown as means and standard deviations, geometric means, when applicable, and proportions. Anthropometrical data are shown as z-scores compared to the WHO/NCHS reference population (194). Prior to analysis, the anthropometrical data were interpolated to correspond to each completed month of age. Conversion to anthropometrical z-scores was done using Epi Info 2000 version 1.1.1 (Centers for Disease Control and Prevention, Atlanta, GA) using the 2000 CDC reference growth data (194). Development was assessed using the BSID.

This scale measures three facets of infant development, mental development using the MDI, psychomotor development using the PDI and behavior using the BRS. Developmental data are shown as mean MDI and PDI, and for the Indonesian study, also median BRS. BRS data were highly skewed so in order to achieve normal distribution, rank was used as outcome in the analyses. Morbidity was analyzed with Poisson regression using Stata 6.0 (Stata Corp, College Station, TX) and is shown as incidence and incidence rate ratios (95% confidence interval; CI) for diarrhea and acute
respiratory infections (ARI) with the control groups, i.e. in SINUS the CC group and in ZINAK the placebo group, as reference. In both studies, an episode of illness was defined as the symptom for at least one day, followed by at least three disease-free days. Diarrhea was defined as three or more loose or watery stools in 24 hours, while ARI was defined as fever in combination with cough and/or fast or difficult breathing.

Factor analysis was used on the monthly diet registrations. The Swedish dietary data are shown as mean or geometric mean daily intakes of energy and selected nutrients during the 6-8, 9-11 and 12-17 months periods, respectively. When comparing proportions, Chi²-test and/or Fisher’s exact test were used in both studies. Statistical significance was set at p <0.05.

In SINUS, one-factor ANOVA was used in the intention-to-treat analysis for the continuous variables comparing the study groups. To adjust for effects over time, analysis of co-variance (ANCOVA), using initial values at 6 mo as covariates for results at 12 mo was used. Significant differences between study groups were Bonferroni corrected to allow for multiple comparisons.

In paper III the combined data from all three SINUS groups was used. The rationale behind this was that although the three groups, i.e. CC, PR and IF differed in which study products they consumed, the group allocation had little effect on the main outcomes. For Hb, there was a significant difference between PR and IF, but this became non-significant when adjusting for mean daily iron intake, indicating that it was iron intake and not group allocation per se that had an impact.

The associations between dietary iron intake, iron status variables and background variables were analyzed with linear regressions. Multivariate regression models were constructed, using variables with biologically known or plausible relevance to the main outcomes and dietary iron intake and those with a univariate p-value <0.20, i.e. gender, birth weight, growth (i.e. relative weight change since birth), intake of cow’s milk and breast milk, baseline values of main outcomes, study group and intakes of energy, protein, calcium, ascorbic acid and phytate, testing for interaction and confounding.

In ZINAK, two-factor ANOVA was applied to examine main effects and interactions between iron and zinc supplementation for the continuous outcome variables. When a significant interaction (p
<0.10) was found, follow-up test using Bonferroni adjustment was performed. To adjust for possible confounding, the covariates gender, birth weight, initial values for the main outcomes, compliance measured as volume of supplement consumed, presence of vomiting, both as reported in the follow-up interview on side-effects and as recorded in the morbidity registrations, mother’s education and as a proxy for socioeconomic status, the location of the household’s water source were included in the analysis.

Lowess smoothed plots, which uses an iterative locally weighted least-squares method to fit a curve to a set of points (195), were used to visualize the dose-effect relationship between daily iron intake and the main outcomes in paper III, and the dose-effect relationship between volume taken and outcome for the different treatment groups in paper IV. In the former paper, this was followed by construction of multivariate regression models, as discussed above. In the latter paper, the Lowess smoothed plots were followed by multivariate linear regression analyses to statistically assess the associations between iron and/or zinc supplementation, and S-Ft and S-Zn. Adjustments were made for potential confounding of the dose-effect. In the regression analysis, presence of vomiting reported as a sideeffect at the follow-up interview with the mother, educational level of the mother, water source of the household, and birth weight were identified as possible confounders and thus included in the model.
FINDINGS

BASELINE CHARACTERISTICS

This thesis reports the main findings of the two studies until the end of the interventions at 12 mo of age. However, in the Swedish study some functional findings of the follow-up until 18 mo of age are also reported. Figure 8 summarizes the flow of infants in SINUS. There were no significant differences between infants who completed the trial and those who did not with regard to birth weight, birth length, breast-feeding duration, parental level of education, family size, baseline biochemistry, anthropometry or development, nor were there any statistically significant differences at baseline between the three study groups for any of the socio-economic, anthropometrical, hematological or biochemical variables, except for low S-Zn being more common in the CC group than in the other groups (31% vs. 16% and 19% in the PR and IF groups, respectively, p=0.04).

Figure 9 summarizes the flow of participating infants in ZINAK. Baseline biochemistry (Hb, S-Ft, S-TfR, S-Zn), anthropometry, psychomotor development and socio-demographic characteristics did not differ between those who completed and did not complete the trial, nor were there differences between the four treatment groups in baseline characteristics.

Two study sites differed significantly in several of the background characteristics (family size, mother’s educational level, birth weight and number of infants breast fed at study start) and baseline measurements, including S-Ft, S-TfR, S-Zn, and prevalence of anemia, low S-Ft, IDA, low S-Zn, and the anthropometrical measurements (Table 9). However, baseline Hb did not differ significantly between sites and there were no statistical differences in mental or psychomotor development index.
Figure 8. Flow of infants in the SINUS study. CMA – cow’s milk allergy
Figure 9. Flow of infants in the ZINAK study.
Table 9. Baseline characteristics of infants in SINUS and ZINAK.

<table>
<thead>
<tr>
<th></th>
<th>SINUS (n=267)</th>
<th>ZINAK (n=549)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Persons per household (n)¹</td>
<td>3.7 (0.84)</td>
<td>4.2 (1.35)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mother’s education</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;9 years (%)</td>
<td>94</td>
<td>44</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Girls (%)</td>
<td>52</td>
<td>48</td>
<td>0.33</td>
</tr>
<tr>
<td>Birth weight (kg)¹</td>
<td>3.62 (0.483)</td>
<td>3.21 (0.462)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Breast fed at 6 mo (%)</td>
<td>75</td>
<td>97</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hb (g/L)¹</td>
<td>115 (8.2)</td>
<td>114 (13.3)</td>
<td>0.084</td>
</tr>
<tr>
<td>S-ferritin (µg/L)²</td>
<td>44.6 (2.49)</td>
<td>33.6 (2.58)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>S-TfR (mg/L)²</td>
<td>6.17 (1.43)</td>
<td>7.08 (1.39)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>S-zinc (µmol/L)¹</td>
<td>13.2 (3.69)</td>
<td>9.3 (2.52)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hb &lt;110 g/L (%)</td>
<td>28</td>
<td>41</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>S-ferritin &lt;12 µg/L (%)</td>
<td>9</td>
<td>15</td>
<td>0.018</td>
</tr>
<tr>
<td>IDA (%)</td>
<td>3</td>
<td>8</td>
<td>0.008</td>
</tr>
<tr>
<td>S-zinc &lt;10.7 µmol/L (%)</td>
<td>22</td>
<td>78</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MDI¹</td>
<td>99.1 (5.66)</td>
<td>99.7 (8.08)</td>
<td>0.35</td>
</tr>
<tr>
<td>PDI¹</td>
<td>95.6 (10.2)</td>
<td>95.7 (11.67)</td>
<td>0.94</td>
</tr>
<tr>
<td>WLZ¹</td>
<td>0.12 (1.01)</td>
<td>-0.09 (1.13)</td>
<td>0.009</td>
</tr>
<tr>
<td>WAZ¹</td>
<td>0.48 (0.93)</td>
<td>-0.41 (0.99)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HAZ¹</td>
<td>0.69 (0.77)</td>
<td>-0.31 (0.84)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Data are ¹ mean (SD), ² geometric mean (antilog SD).
Findings

**Deviation from protocol**

In SINUS, there were no differences in consumption of the study products between study groups (Table 10), nor were there any significant difference between study groups in the number of infants who left the study before 12 mo (5%, 7% and 12%, p=0.17 for the CC, PR and IF groups, respectively). No adverse effects of the different diets were reported.

In ZINAK, vomiting was more common in the Fe + Zn group (Table 11). Compared to the placebo group, the Fe + Zn group had a 4.1 (95% CI 3.4-4.9) higher risk for vomiting, and the Zn group a 1.9 (95% CI 1.5-2.5) higher risk, respectively. Other perceived side effects (abdominal pain, diarrhea, constipation, poor appetite, increased crying, and fuzziness) did not differ between treatment groups (data not shown). Vomiting was negatively associated with total volume of given supplement (r = -0.387, p<0.001), which implies the possibility of loss of supplement after the daily dose. For consumption of supplement, main effects for both iron treatment and zinc treatment were significant, but interaction was not.

**IRON AND ZINC STATUS**

At baseline, overall prevalence of Hb <110 g/L, S-Ft <12 µg/L and S-Zn <10.7 µmol/L was 28%, 9% and 22%, respectively among the Swedish infants. Six infants could be classified as having IDA (Hb <110 g/L, S-Ft <12 µg/L and MCV <73 fL). After 6 mo of consuming the diets with different phytate content, neither iron status (Hb, S-Ft, S-Fe, and S-TfR) nor S-Zn differed significantly between the groups consuming lower amounts of phytate (PR and IF) and the CC group (Table 11).

Mean Hb was slightly but significantly lower in the IF group than in the PR group (117 vs. 120 g/L, p=0.015) (Table 12) at 12 mo of age. The diet in the IF group had a lower iron content than the other two groups, and total iron intake was significantly lower in the IF group than in the other groups. When adjusting the intention-to-treat analysis for total iron intake, the group differences in effect on Hb between the PR and IF groups became non-significant (p=0.48) and the effect estimates were reduced from a difference of 3.3 g/L to 0.8 g/L for Hb.
Table 10. Mean daily consumption of study formula/milk cereal drink and porridge, frequency of breast feeding, and intake of breast milk, energy and selected nutrients at 6–8 and 9–11 months of age (n=267).

<table>
<thead>
<tr>
<th></th>
<th>6 – 8 months</th>
<th>9 – 11 months</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CC (n=94)</td>
<td>PR (n=90)</td>
</tr>
<tr>
<td>Formula/MCD (g/d)²</td>
<td>329 (275.5)</td>
<td>321 (268.2)</td>
</tr>
<tr>
<td>Porridge (g/d)²</td>
<td>94 (50.9)</td>
<td>85 (51.4)</td>
</tr>
<tr>
<td>Breast feeding</td>
<td>3.9 (2.5)</td>
<td>4.1 (2.4)</td>
</tr>
<tr>
<td>frequency (times/d)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breast milk intake</td>
<td>413 (276)</td>
<td>424 (254)</td>
</tr>
<tr>
<td>(g/d)²</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Energy (kcal/kg/d)²</td>
<td>89.9 (15.27)</td>
<td>89.8 (18.50)</td>
</tr>
<tr>
<td>Protein (g/kg/d)²</td>
<td>2.4 (0.67)</td>
<td>2.4 (0.82)</td>
</tr>
<tr>
<td>Fat (g/d)²</td>
<td>30.9 (6.03)</td>
<td>31.5 (6.49)</td>
</tr>
<tr>
<td>Carbohydrates (g/d)²</td>
<td>98.0 (17.34)</td>
<td>95.8 (22.15)</td>
</tr>
<tr>
<td>Iron (mg/d)²</td>
<td>7.5 (3.69)</td>
<td>7.6 (4.27)</td>
</tr>
<tr>
<td>Zinc (mg/d)²</td>
<td>2.8 (0.78)</td>
<td>2.8 (0.98)</td>
</tr>
<tr>
<td>Vitamin C (mg/d)²</td>
<td>124 (48.9)</td>
<td>121 (54.0)</td>
</tr>
<tr>
<td>Calcium (mg/d)²</td>
<td>661 (228.5)</td>
<td>649 (261.1)</td>
</tr>
<tr>
<td>Phytate (µmol/d)²</td>
<td>124 (82.4)</td>
<td>48 (31.0)</td>
</tr>
</tbody>
</table>

¹Commercial MCD and porridge. ²Phytate-reduced MCD and phytate-reduced porridge. ³Infant formula and porridge with regular phytate level. ⁴Mean (SD). ⁵Geometric mean for 9-12 mo. period. ⁶Significantly different from other groups 6-8 mo. (ANOVA p<0.05, Bonferroni corrected). ⁷Significantly different from other groups 9-12 mo. (ANOVA p<0.05, Bonferroni corrected). ⁸Phytate from study products only. ⁹Significantly different from Commercial cereal (ANOVA p<0.05, Bonferroni corrected). ¹⁰At 6-8 mo. CC n=70, PR n=67, IF n=62. At 9-12 mo. CC n=34, PR n=35, IF n=33.
Findings

Table 11. Details of follow-up at the end of supplementation.

<table>
<thead>
<tr>
<th>Study group</th>
<th>Main effect</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P-value</td>
<td>P-value</td>
</tr>
<tr>
<td>Placebo</td>
<td>Fe</td>
<td>Zn</td>
</tr>
<tr>
<td>(n=164)</td>
<td>(n=163)</td>
<td>(n=162)</td>
</tr>
</tbody>
</table>

**Treatment**

Total supplement volume (ml)¹

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Fe</th>
<th>Zn</th>
<th>Fe+Zn</th>
<th>Fe</th>
<th>Zn</th>
<th>Fe × Zn</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>242 (51)</td>
<td>218 (54)</td>
<td>232 (57)</td>
<td>202 (70)</td>
<td>&lt;0.001</td>
<td>0.004</td>
<td>0.60</td>
</tr>
</tbody>
</table>

**Health**

Breast fed at 12 months [n (%)]

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Fe</th>
<th>Zn</th>
<th>Fe+Zn</th>
<th>Fe</th>
<th>Zn</th>
<th>Fe × Zn</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>134 (94)</td>
<td>154 (94)</td>
<td>155 (95)</td>
<td>148 (92)</td>
<td>0.12</td>
<td>0.04</td>
<td>0.99</td>
</tr>
</tbody>
</table>

Illness within 2 weeks prior to endpoint [n (%)]

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Fe</th>
<th>Zn</th>
<th>Fe+Zn</th>
<th>Fe</th>
<th>Zn</th>
<th>Fe × Zn</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>40 (26)</td>
<td>43 (28)</td>
<td>43 (29)</td>
<td>40 (26)</td>
<td>0.82</td>
<td>0.90</td>
<td>0.53</td>
</tr>
</tbody>
</table>

**Side-effects**

Any perceived side effect [n (%)]

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Fe</th>
<th>Zn</th>
<th>Fe+Zn</th>
<th>Fe</th>
<th>Zn</th>
<th>Fe × Zn</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>94 (57)</td>
<td>96 (59)</td>
<td>102 (63)</td>
<td>112 (70)</td>
<td>0.001</td>
<td>0.34</td>
<td>0.88</td>
</tr>
</tbody>
</table>

Vomiting [n (%)]²

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Fe</th>
<th>Zn</th>
<th>Fe+Zn</th>
<th>Fe</th>
<th>Zn</th>
<th>Fe × Zn</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>44 (27)a</td>
<td>49 (30)a</td>
<td>56 (35)a</td>
<td>84 (53)b</td>
<td>0.004</td>
<td>&lt;0.001</td>
<td>0.043</td>
</tr>
</tbody>
</table>

Data are ¹ mean (SD), ²defined as reported vomiting as side effect during supplementation at follow-up interview. Means not sharing a common superscript letter are significantly different at P<0.05 based on 2-factor ANOVA (Bonferroni adjusted).

S-Cu was significantly higher in the CC group compared to the PR group (17.0 vs. 15.6 µmol/L, p=0.004). Overall, from 6 to 12 months of age, the prevalence of Hb <110 g/L decreased significantly (28% vs. 15%, p=0.001), the prevalence of S-Ft <12 µg/L increased (9% vs. 16%, p=0.012) and the prevalence of S-Zn <10.7 µmol/L remained unchanged (23% vs. 29%, p=0.09). The proportion of infants with Hb <110 g/L at 12 months tended to be higher in the IF group compared to the other two groups (23% vs. 11% and 13%, in the CC and PR groups, respectively, p=0.06).
There were no differences between study groups in prevalence of low S-Ft or low S-Zn at 12 months.

Among the Indonesian infants, 41% had Hb <110 g/L, 15% had S-Ft <12 µg/L and 78% had S-Zn <10.7 µmol/L at baseline. After 6 mo of supplementation, there was a significant negative interaction between iron and zinc treatment for Hb (main effects: iron treatment p=0.020, zinc treatment p=0.56; iron × zinc interaction p=0.021) (Table 13). The Fe group had a significantly higher Hb concentration at the endpoint compared to placebo and Fe + Zn. The prevalence of Hb <110 g/L in the Fe group was significantly lower than in the placebo and Fe + Zn groups (25%, 36%, 38%, and 44% in the Fe, Zn, Fe + Zn and placebo groups, respectively, p<0.05).

For ln S-Ft, the interaction was also negative and significant (main effects: iron treatment p<0.001, zinc treatment p=0.13; iron×zinc interaction p=0.023). S-Ft was significantly higher in the Fe group compared to the Fe + Zn group. For S-TfR, the main effects (iron treatment p<0.001, zinc treatment p=0.003) were significant, but there was no significant interaction (iron × zinc interaction p=0.76).

At the endpoint, the prevalence of S-Ft <12 µg/L was 5%, 35%, 10%, and 37% in the Fe, Zn, Fe + Zn and placebo groups, respectively. The Fe and Fe + Zn groups had significantly lower prevalence of IDA (Hb <110 g/L and S-Ft <12µg/L) as compared to placebo (2%, 16%, 3% and 21% in the Fe, Zn, Fe + Zn and placebo groups, respectively, p<0.001). The prevalence of IDA increased significantly from 6 to 12 mo of age in the placebo group (p=0.002).

Two-factor ANOVA for S-Zn showed significant main effects (iron treatment p=0.01, zinc treatment p<0.001), but no significant interaction (iron × zinc interaction p=0.15). In the Zn and Fe + Zn groups, significantly fewer infants had low S-zinc levels as compared to placebo and the Fe group (87%, 47%, 54%, and 78% in the Fe, Zn, Fe + Zn, and placebo groups, respectively, p<0.001).
GROWTH

The Swedish infants grew at the level of or above Swedish national (196) and international reference populations (194) for height and weight throughout the study period (Figure 10). The overall mean WAZ decreased significantly from 6-18 mo, both compared to Swedish national data (WAZ 0.34 vs. -0.17, p<0.001) and an international reference population (WAZ 0.49 vs. 0.18, p=0.001). Neither during the intervention, i.e. from 6 to 12 mo of age, nor during the subsequent follow-up until 18 mo of age, were there any significant differences among study groups in any of the anthropometrical measures (Table 12).

Overall, anthropometrical status deteriorated significantly in the Indonesian infants from 6 to 12 mo of age (Figure 10). The prevalence of wasting increased from 4% to 16% (p<0.001), of underweight from 4% to 38% (p<0.001) and of stunting from 4% to 10% (p<0.001). Between study groups, the proportion of wasting at 12 mo was significantly higher in the Fe group compared to the Zn group (21.5 vs. 11.1%, p=0.044).

At the end of the 6 mo supplementation period there was a significant negative interaction between iron and zinc treatment for WAZ (main effects: iron treatment p=0.046, zinc treatment p=0.11; iron×zinc interaction p=0.020). In the Zn group, WAZ was significantly higher compared to the placebo and Fe + Zn groups (Table 13).

Further, two-factor ANOVA showed significant negative interaction between iron and zinc treatment for knee-heel length (main effects: iron treatment p=0.65; zinc treatment p=0.59; iron×zinc interaction p=0.001). Knee-heel length was higher in the Zn and Fe groups compared to placebo. Controlling for potential confounders, such as consumption of supplement and occurrence of vomiting, did not significantly change the results. For WHZ, there was a significant main effect of zinc treatment (p=0.004), but no effect of iron treatment and no interaction (data not shown). HAZ, head circumference, or mid-upper arm circumference were not significantly different among the groups.
Figure 10. Weight-for-age and height-for-age z-scores in the ZINAK and SINUS studies from 6 to 12 mo, and 6 to 18 mo of age, respectively.

Mental and psychomotor development

Of the 300 infants in the Swedish trial, 276 were ever tested and 194 (65%) had measurements at 7, 13 and 18 mo of age. There were no significant differences in mean BSID MDI or PDI between study groups at any time point (Table 12), nor were there any significant associations between iron or zinc status and developmental scores at any age.

In ZINAK, there was significant negative interaction between iron and zinc treatment for BSID PDI at 12 mo (main effects: iron treatment \( p=0.39 \); zinc treatment \( p=0.82 \); iron\( \times \)zinc interaction \( p=0.009 \)). In the Fe group, attained PDI at 12 mo was significantly higher than in the placebo group (difference in mean 2.86, \( p=0.042 \)) (Table 13).
Table 12. Outcomes at 12 mo of age in the SINUS study.

<table>
<thead>
<tr>
<th>Study group</th>
<th>CC</th>
<th>PR</th>
<th>IF</th>
<th>P-value&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Biochemistry</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemoglobin (g/L)&lt;sup&gt;2&lt;/sup&gt;</td>
<td>119 (8.0)&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>120 (8.4)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>117 (9.5)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.039</td>
</tr>
<tr>
<td>MCV (fL)&lt;sup&gt;2&lt;/sup&gt;</td>
<td>76 (3.4)</td>
<td>76 (2.8)</td>
<td>75 (3.8)</td>
<td>0.065</td>
</tr>
<tr>
<td>Serum iron (µmol/L)&lt;sup&gt;2&lt;/sup&gt;</td>
<td>9.1 (4.62)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.2 (4.34)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.7 (4.16)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.005</td>
</tr>
<tr>
<td>Serum ferritin (µg/L)&lt;sup&gt;3&lt;/sup&gt;</td>
<td>25.3 (1.99)</td>
<td>21.3 (1.92)</td>
<td>25.2 (1.97)</td>
<td>0.145</td>
</tr>
<tr>
<td>Serum TfR (mg/L)&lt;sup&gt;2&lt;/sup&gt;</td>
<td>6.88 (1.44)</td>
<td>7.11 (1.41)</td>
<td>6.78 (1.41)</td>
<td>0.66</td>
</tr>
<tr>
<td>Serum zinc (µmol/L)&lt;sup&gt;2&lt;/sup&gt;</td>
<td>12.2 (2.56)</td>
<td>12.5 (2.80)</td>
<td>12.4 (2.68)</td>
<td>0.79</td>
</tr>
<tr>
<td>Serum copper (µmol/L)&lt;sup&gt;2&lt;/sup&gt;</td>
<td>17.1 (3.53)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15.6 (3.40)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16.4 (3.25)&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.014</td>
</tr>
<tr>
<td><strong>Anthropometry</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WAZ&lt;sup&gt;2&lt;/sup&gt;</td>
<td>0.16 (0.96)</td>
<td>0.13 (0.97)</td>
<td>0.19 (0.96)</td>
<td>1.00</td>
</tr>
<tr>
<td>HAZ&lt;sup&gt;2&lt;/sup&gt;</td>
<td>0.69 (0.66)</td>
<td>0.61 (0.86)</td>
<td>0.57 (0.66)</td>
<td>0.59</td>
</tr>
<tr>
<td>WHZ&lt;sup&gt;2&lt;/sup&gt;</td>
<td>0.17 (1.10)</td>
<td>0.23 (0.90)</td>
<td>0.26 (1.03)</td>
<td>0.85</td>
</tr>
<tr>
<td>Mid-upper arm circumference (cm)&lt;sup&gt;2&lt;/sup&gt;</td>
<td>15.7 (1.20)</td>
<td>15.6 (1.01)</td>
<td>15.7 (1.06)</td>
<td>0.81</td>
</tr>
<tr>
<td>Knee-heel length (cm)&lt;sup&gt;2&lt;/sup&gt;</td>
<td>20.74 (0.860)</td>
<td>20.67 (0.919)</td>
<td>20.73 (0.790)</td>
<td>0.96</td>
</tr>
<tr>
<td><strong>Development</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDI&lt;sup&gt;2&lt;/sup&gt;</td>
<td>108.2 (7.49)</td>
<td>108.7 (8.59)</td>
<td>108.4 (9.54)</td>
<td>0.81</td>
</tr>
<tr>
<td>PDI&lt;sup&gt;2&lt;/sup&gt;</td>
<td>100.3 (12.44)</td>
<td>99.5 (12.45)</td>
<td>99.9 (12.65)</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Data are '<sup>1</sup>ANOVA P-value for difference between groups, '<sup>2</sup>mean (SD), '<sup>3</sup>geometric mean. Means not sharing a common superscript letter are significantly different at P<0.05 (Bonferroni adjusted).
Table 13. Outcome of supplementation on biochemical indicators of iron and zinc status, anthropometry and development at 12 months of age.

<table>
<thead>
<tr>
<th>Study group</th>
<th>Main effect P-value</th>
<th>Interaction P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Placebo Fe Zn Fe+Zn</td>
<td>Fe Zn Fe × Zn</td>
</tr>
<tr>
<td><strong>Biochemistry</strong></td>
<td>n = 143 n = 136 n = 134 n = 136</td>
<td></td>
</tr>
<tr>
<td>Hemoglobin (g/L)</td>
<td>113 (16.0)a 119 (15.3)b 116 (15.2)c 115 (15.9)d</td>
<td>0.020 0.56 0.021</td>
</tr>
<tr>
<td>Serum ferritin (µg/L)</td>
<td>12.9 (3.7)a 46.5 (2.0)b 13.3 (3.6)c 32.3 (2.9)d</td>
<td>&lt;0.001 0.13 0.023</td>
</tr>
<tr>
<td>Serum TfR (mg/L)</td>
<td>9.02 (1.73) 6.71 (1.33) 9.78 (1.42) 7.56 (1.36)</td>
<td>&lt;0.001 0.005 0.87</td>
</tr>
<tr>
<td>Serum zinc (µmol/L)</td>
<td>9.06 (1.27) 8.76 (1.24) 11.58 (1.41) 10.80 (1.34)</td>
<td>0.11 &lt;0.001 0.23</td>
</tr>
<tr>
<td>Serum copper (µmol/L)</td>
<td>15.2 (5.1) 15.2 (4.8) 15.0 (5.1) 14.7 (4.5)</td>
<td>0.65 0.34 0.55</td>
</tr>
<tr>
<td><strong>Anthropometry</strong></td>
<td>n = 164 n = 163 n = 162 n = 161</td>
<td></td>
</tr>
<tr>
<td>WAZ</td>
<td>-1.72 (1.00)a -1.65 (1.08)a -1.46 (1.08)a -1.68 (1.02)a</td>
<td>0.092 0.079 0.004</td>
</tr>
<tr>
<td>HAZ</td>
<td>-0.81 (0.86) -0.66 (0.91) -0.77 (0.92) -0.90 (0.90)</td>
<td>0.84 0.16 0.16</td>
</tr>
<tr>
<td>WHZ</td>
<td>-1.01 (1.16) -1.07 (1.23) -0.70 (1.06) -0.86 (1.06)</td>
<td>0.26 0.004 0.14</td>
</tr>
<tr>
<td>Mid arm circumference (cm)</td>
<td>14.7 (1.18) 14.7 (1.12) 14.8 (1.14) 14.6 (1.04)</td>
<td>0.36 0.77 0.039</td>
</tr>
<tr>
<td>Knee-heel length (cm)</td>
<td>19.30 (0.95)a 19.45 (0.96)a 19.50 (0.94)a 19.40 (0.94)a</td>
<td>0.46 0.62 0.003</td>
</tr>
<tr>
<td><strong>Development</strong></td>
<td>n = 165 n = 163 n = 167 n = 160</td>
<td></td>
</tr>
<tr>
<td>Mental development index</td>
<td>99 (10.0) 101 (9.7) 101 (9.3) 100 (9.8)</td>
<td>0.76 0.63 0.069</td>
</tr>
<tr>
<td>Psychomotor development index</td>
<td>103 (10.8)a 106 (11.0)b 105 (10.6)ab 103 (10.3)ab</td>
<td>0.82 0.39 0.009</td>
</tr>
<tr>
<td>Behavioural rating scale</td>
<td>42 (20-62) 42 (22-69) 39 (20-66) 35 (19-53)</td>
<td>0.55 0.062 0.091</td>
</tr>
</tbody>
</table>

Data are 1 mean (SD), 2 geometric mean (antilog SD), 3 weight-for-age z-score, 4 height-for-age z-score, weight-for-height z-score, median (25-75 percentile), and main effect and interaction calculated from group ranks. Means not sharing a common superscript letter are significantly different at P<0.05 based on 2-factor ANOVA (Bonferroni adjusted).

Controlling for potential confounders, such as volume consumed of supplement, initial iron status, vomiting and mother’s education, did not significantly change the result. There were no significant differences for MDI or BRS between groups.
INCIDENCE OF DIARRHEA AND RESPIRATORY INFECTIONS

Among the Swedish infants, we recorded an overall incidence of 0.97 episodes of diarrhea per person year and 1.55 episodes of ARI per person year from 6 to 11 mo, and from 12-17 mo, the incidence of diarrhea and ARI was 0.70 and 0.89 per person year, respectively. The decreases in both diarrhea and ARI incidences from 6-11 to 12-17 mo were significant. There were no differences in incidence of diarrhea or ARI among the study groups during the 6-11 mo. period. However, the IF group had significantly higher incidence rate ratio (RR) for diarrhea during the 12-17 mo period compared to the PR group (RR=1. 77, 95% CI 1.05 – 2.97), but not to the CC group. There was a trend, albeit non-significant, in the relative risk for diarrhea between the IF and PR groups; during the 10-11, 12-13, 14-15 and 16-17 mo periods RR was 1.12 (95% CI 0.66-1.90), 2.56 (0.90-7.27), 1.66 (0.75-3.69) and 1.41 (0.56-3.58), respectively.

Among the Indonesian infants, the incidence of diarrhea (2.9, 3.0, 2.7 and 2.8 episodes/person year for the Fe, Zn, Fe + Zn and placebo groups, respectively) and ARI (3.5, 3.6, 3.4 and 3.7 episodes/person year for Fe, Zn, Fe + Zn and placebo, respectively) did not differ significantly between treatment groups, nor did duration of diarrhea or ARI (data not shown). Compared to the Swedish infants during the 6-11 mo period, the incidence was three times higher for diarrhea and more than two times higher for ARI (Figure 11). Anthropometrical status was not associated with incidence of infectious disease among the Indonesian infants, nor was there any significant interaction between treatment group and level of nutritional status in respect to morbidity.

DIETARY INTAKE

Among the Swedish infants, 75% of the participating infants were breast-fed at baseline. Median duration of breast-feeding was 8.9 mo. Total duration and daily frequency of breast-feeding, as well as daily intake of breast milk was similar between the three groups. Energy intake from the total diet was similar in all groups throughout the study, but intake of protein, iron, vitamin C, calcium and phytate differed, basically mirroring the differences in nutrient content between the infant cereals and infant formula (Table 10).
Zinc intake also differed, but only during the 6-8 mo period. Of the total energy intake, the study products contributed with on average 41% during the 6-8 mo period and 44% during the 9-11 mo period. A significant proportion of the daily intake of iron and zinc came from MCD or infant formula and porridge. On average, 62% (4.5 mg) and 59% (5.6 mg) of the daily iron intake came from the study products during the 6-8 mo and 9-11 mo periods, respectively. For zinc, 45% (1.5 and 2.0 mg) of the daily intake came from the study products during the 6-8 mo and 9-11 mo periods, respectively.

Figure 11. Incidence of diarrhea and acute respiratory infections (episodes/person year) during 6-12 mo of age in the SINUS and ZINAK studies.
IRON INTAKE AND IRON STATUS

In the Swedish infants, mean daily iron intake during 6-8 mo was significantly correlated to Hb at 9 mo (r=0.27, p<0.001), and iron intake during 9-11 mo was significantly associated with Hb at 12 mo (r=0.21, p=0.001). However, 12-17 mo iron intake was not correlated to Hb at 18 mo (r=0.082, p=0.22) (Figure 12). Daily iron intake during 6-8 mo, or 9-11 mo was not associated with S-Ft at 9 or 12 mo, respectively. However, the 12-17 mo iron intake was significantly correlated to S-Ft at 18 mo (r=0.14, p=0.032) (Figure 13). Iron intake during 6-8 mo was significantly positively correlated to S-Fe at 9 mo (r=0.16, p=0.011) and the 9-11 mo intake was correlated to S-Fe at 12 mo (r=0.20, p=0.001). However, the 12-17 mo iron intake was not associated with S-Fe at 18 mo (r=-0.03, p=0.65). Dietary iron intake was also not associated with MCV or S-TfR at any time point.

In a multivariate linear regression model, the relationship between dietary iron intake and Hb was explored further. At 9 mo, Hb was significantly associated with daily iron intake during 6-8 mo (β=4.0, p<0.001), adjusting for baseline Hb and relative weight change from birth to 9 mo, explaining 36% of the variation in Hb at 9 mo. Hemoglobin at 12 mo was significantly associated with daily iron intake from 9-11 mo (β=5.3, p<0.001), adjusting for initial Hb and relative weight change from birth to 12 mo. Adjusted \( r^2 \) for this model was 0.32. Hemoglobin concentration at 18 mo was not associated with iron intake during 12-17 mo period.

The association between dietary intake and S-Ft was also tested in a multivariate model. No association was found between ln S-Ft at 9 or 12 mo and dietary iron intake during 6-8 mo or 9-11 mo, respectively. However, ln S-Ft at 18 mo was significantly associated with dietary iron intake from 12-17 mo (β=0.44, p=0.010), adjusting for ln S-Ft at 12 mo and relative weight increase from 12 to 18 mo. This model explained 16% of the variation of ln S-Ft at 18 mo of age.
Figure 12. Lowess curves of the effects of iron intake [mg/(kg body weight \times d)] during 6-8 mo, 9-11 mo and 12-18 mo on Hb (g/L) at 9 mo, 12 mo, and 18 mo of age. At 9 mo, the dose-response relationship was linear between iron intake 6-8 mo and Hb (regression coefficient $\beta=3.3$, $p=0.005$), without evident difference in slope below or above a daily intake of 0.8 mg/(kg body weight \times d). At 12 mo, the Hb-slope was significant up to an iron intake 9-11 mo of 1.2 mg/(kg body weight \times d) ($\beta=8.8$, $p<0.001$). At 18 mo, Hb was not significantly associated with iron intake 12-18 mo ($\beta=1.8$, $p=0.41$).
Findings

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Figure 13. Lowess curves of the effects of iron intake [mg/(kg body weight x d)] during 6-8 mo, 9-11 mo and 12-18 mo on S-Ft (µg/L) at 9 mo, 12 mo, and 18 mo of age. At 9 and 12 mo, iron intake and ln S-Ft was not significantly associated (iron intake 6-8 mo: β=-0.1, p=0.36 and iron intake 9-11 mo: β=-0.06, p=0.59, respectively). At 18 mo, ln S-Ft was significant associated with iron intake 12-18 mo (β=0.38, p=0.034), but without evident difference for intakes < or > 1.0 mg/(kg body weight x d).

We assessed the relationship between total amounts of iron consumed from 6-8 mo, from 6-11 mo, and from 6-17 mo, and Hb and ln S-Ft at 9, 12 and 18 mo of age, respectively. These analyses confirmed the significant associations between total iron intake at 6-8 mo and Hb at 9 mo (p<0.001) and the 6-11 mo total iron intake and Hb at 12 mo (p<0.001). However, there was no association between total iron intake during 6-8 , 6-11, or 6-17 mo, and subsequent ln S-Ft, whereas the 12-18 mo total iron intake was significantly associated with ln S-Ft at 18 mo (p=0.015).

We also stratified the multiple linear regressions of Hb and ln S-Ft for initial iron status, i.e. those infants with S-Ft in the lowest quartile (S-Ft <23 µg/L) and highest quartile (S-Ft >80 µg/L). This did not significantly change the results. Neither did inclusion or exclusion of the 6 infants who at 6 mo fulfilled the WHO criteria for IDA (Hb <110 g/L, S-Ft <12 µg/L and MCV <73 fl).
In the Indonesian study, we explored the association between supplemental iron and S-Ft. To evaluate the efficacy of the Fe and Fe + Zn regimens on S-Ft at the endpoint, the dose-effect over the range of total supplement intake during the treatment period was studied (Figure 14). Lowess curves for the Fe and Fe + Zn groups indicated an initial dose-effect, followed by a plateau with no further increased effect. In the ordinary least square regression model, the dose effect up to 200 ml was compared with that above 200 ml of total supplementation.

In the Fe group there was a significant dose-effect up to the level of 200 ml (corresponding to a total dose of 1250 mg of iron over a period of 6 months, or 7 mg per day). At higher total doses no further increase in effect was seen. Above 200 ml of total supplement, the effect on the Fe group was significantly higher than on the Fe + Zn group ($\beta = 0.171$, $P = 0.003$), but there was no significant interaction between treatment group and volume. In spite of the appearance of the Lowess curves, no difference in effect for a given dose and no interaction in dose-effect between the groups for supplementation amounts below 200 ml could be shown between Fe and Fe + Zn (data not shown).
Figure 14. Lowess curves of the effect of the volume of supplementation on serum ferritin (log scale) at 12 mo of age. In the Fe group there was a significant dose effect up to 200 ml, after which no further increase in serum ferritin was seen. Above 200 ml of consumed supplement the effect on serum ferritin was significantly higher in the Fe group compared to the Fe + Zn group, without significant treatment group × volume interaction. Below 200 ml there were no significant difference in serum ferritin between the Fe and Fe + Zn groups.
DISCUSSION

In the present thesis the effects of two different approaches to improve iron and zinc nutriture in infants, i.e. phytate-reduction of commonly consumed infant cereals, and iron and zinc supplementation are reported. The results were obtained from two intervention trials. Both studies used a randomized, double-masked design and included infants from 6 mo of age. In evaluating these interventions, multiple outcomes were measured, and several of the same variables, i.e. Hb, S-Ft, S-TfR, S-Zn, growth, development and incidence of diarrhea and respiratory infections were assessed at both sites using similar or identical methods.

The common challenge to improve iron and zinc nutriture in infants and the many similarities in design and execution of the trials allow for some comparisons of the two studied cohorts. However, the several differences between study sites also call for separate discussions of the two studies, the practical implications of the findings at the different settings and their consequences, both at the local level and in a broader perspective.

In the Indonesian study a factorial design was used. This is a cost-effective study design when evaluating the effects of several treatments and several outcomes at once. It also allows for testing of interactions between different treatments. The latter characteristic is especially important because of the plausible interactions between iron and zinc.

In the Swedish study, where the main problem was if the phytate content of complementary foods caused the unexpected high prevalence of low S-Ft and low S-Zn (166), a more traditional trial design was used with three study groups, although a factorial design with different levels of e.g. both phytate and iron could have been reasonable. However, the primary objectives were to compare the present Swedish infant feeding practice to one with reduced phytate-levels, but also compare current Swedish infant feeding practices, i.e. phytate-containing infant cereals with the internationally common usage of iron-fortified formula and porridge. Thus, the design using three groups was deemed the most appropriate.
The Swedish cohort was recruited from well-baby clinics and could be viewed as a healthy volunteer sample, whereas the recruitment of the Indonesian sample was community-based, although from a cohort of women followed during pregnancy. The Swedish cohort may therefore differ in some characteristics as compared to the infant population in the study area. However, using double-masked randomization to the various study groups should have handled much of this bias in the interpretation of the results of the actual interventions. However, generalization of the results to populations with nutritional and health status very different from the populations studied should be made with caution.

Baseline randomization was successful in both studies, although there was a significant difference between randomization groups in the prevalence of low S-Zn in the Swedish study. However, adjusting for initial zinc status in SINUS did not affect the main outcomes of S-Zn or prevalence of low S-Zn. Compliance was measured in both studies. In SINUS, the dietary intake was recorded, including the intakes of the study products.

These records show that the intake of MCD and formula was approximately two servings per day, and for porridge ≤1 serving per day. These intakes are at the level of previous Swedish studies (169) (J Svahn, personal communication 2002) and indicate that the study products were consumed as part of a mixed diet. However, a higher than average intake of these iron-fortified study products can not be ruled out completely, as the prevalence of low S-Ft and low S-Zn at 12 mo was lower in the CC group compared to what was reported in a previous study (166).

In ZINAK, the intake of the study supplements was measured both through records of self-reported compliance and measurement of actual consumed supplement. Further, careful follow-up of possible sideeffects of the supplementation with both day-to-day recordings of symptoms, and a comprehensive interview at the end of the supplementation, were done. Analysis of these data revealed that there were substantial differences in the sideeffects of the different supplements.

The group consuming the combined iron and zinc supplement had a substantially higher prevalence of vomiting, which could potentially reduce the effect estimates of the intervention. However, adjusting for these factors in the analysis modified the results only slightly. Adverse effects in the combined group have been reported.
from other studies of similar design and indicate one level of possible interaction between iron and zinc in the combined supplement. Dijkhuizen et al reported a higher drop-out rate in the combined Fe+ Zn group (197), and Penny et al reported that vomiting within 30 min of receiving supplement was significantly more common in the combined zinc, iron and vitamins group (198). Also, Baqui et al, when supplementing Bangladeshi infants from 6 mo of age with either iron, zinc, iron + zinc, a mix of iron, zinc and vitamins, or placebo found higher drop-out rate in the group consuming the mineral and vitamin mix, and higher drop-out rates due to vomiting in the groups receiving either the mineral and vitamin mix or iron + zinc (199).

At baseline, there were significant differences between the study sites for several background and nutritional parameters, e.g. household size, maternal education, S-Ft, S-TfR, S-Zn, prevalence of anemia and IDA, and anthropometrical measurements such as WAZ and HAZ. These differences were not surprising given the large socio-economical differences between the study sites. It is noteworthy, though, that there were no differences in Hb or development. In addition, mean WAZ and HAZ at 6 mo of age in the Indonesian cohort were higher than those reported from other low-income Asian countries (178). Both studies dispute the common notion that the prevalence of ID is several times higher than the prevalence of IDA (200).

A striking although not unexpected difference in the development of health indicators was seen between the Swedish and Indonesian infants during the 6-12-mo period. Whereas the Swedish infants remained stable or improved in the measures of nutritional status, the Indonesian infants deteriorated from a fairly low degree of malnutrition to high prevalence of anemia, IDA, low S-Zn, low weight, and wasting. Further, morbidity from both diarrhea and ARI were several times higher in the Indonesian cohort compared to their Swedish peers.

Whereas several of these differences can be attributed to socioeconomic differences, e.g., the maternal level of education, hygienic conditions, accessibility to health care, etc., many can be attributed directly to dietary factors, which could be addressed in appropriate food interventions programs. The deterioration in anthropometrical status was considerable among the Indonesian infants with the prevalence of stunting doubling from 6-12 mo, the prevalence of wasting becoming 4 times greater in the same time
Discussion

period, the prevalence of underweight increasing more than 9-fold. Compared to other studies from the same area (176, 177), we report higher prevalence of both underweight and wasting, with particularly wasting being very high, i.e. >8%. The reasons for this are unclear. Indonesia was struck by a severe economic crisis beginning in late 1997.

Studies on food consumption among pregnant women in the CHN-RL surveillance area before and during the crisis show that the women, especially poor women, decreased their intake of most nutrients (201). For infants or children no similar analyses are available. Although it seems plausible that the economic crisis also affected the nutrient intakes of infants and children, directly or indirectly, we could not show any differences in the prevalence of malnutrition in infants born before and during the economic crisis.

EFFECTS OF PHYTATE REDUCTION

Though phytate has been shown to reduce iron and zinc bioavailability in several single-meal studies (202, 203), phytate-reduction of infant cereals in this long term follow-up had no effect on iron and zinc status, growth, development or incidence of diarrhea or respiratory infections in Swedish infants until 12 mo of age.

Phytate, in the absence of ascorbic acid, inhibits iron absorption in a dose-dependent manner above a phytate:Fe molar ratio of 1:7 (135). However, ascorbic acid counteracts the effect of phytate when the ascorbic acid:Fe molar ratio exceeds 4:1 (73, 74). In SINUS, all infant cereals except PR, had phytate:Fe molar ratios >1:7, implying that, theoretically the phytate could bind all available iron, except in the phytate-reduced MCD. On the other hand, ascorbic acid:Fe molar ratios were high, in all cereals >4:1.

Thus, the ascorbic acid content may have limited the phytate effect, ensuring similar long-term iron absorption from the regular and phytate-reduced cereals. Davidsson et al (74) showed that increasing the ascorbic acid content from an ascorbic acid:Fe molar ratio of 2:1 to 4:1 increased Fe incorporation into red blood cells in a similar fashion as completely removing the phytate from regular, phytate-containing soy formula.

Studies have shown that a phytate: Zn molar ratio above 15:1 may be associated with increased risk of zinc deficiency (131). In
SINUS, the phytate: Zn molar ratios were generally below 10:1 and a further reduction in phytate content had no effect on S-Zn. However, the PR group had significantly lower S-Cu than the CC group. A possible explanation is that zinc absorption may have been higher in the phytate-reduced group although not evident from measurement of S-Zn, causing lower copper absorption. In a study on infant rhesus monkeys, zinc absorption was higher, plasma zinc was higher, and plasma copper concentration was lower in the group fed phytate-reduced soy formula than in the group fed regular soy formula (204).

Comparing the internationally more common feeding pattern of using iron-fortified infant formula and cereals (the IF group) to the Swedish regime with phytate-containing infant cereals (the CC group) revealed no significant differences. This indicates that the present Swedish practice of feeding does not compromise iron or zinc status compared to an infant formula with 4 mg iron/L when the phytate-containing infant cereal has a sufficiently high ascorbic acid: Fe molar ratio.

EFFECTS OF IRON AND ZINC SUPPLEMENTATION

In low-income settings, where complementary foods are mostly home-prepared from primary products with low iron and zinc bioavailability, supplementing these minerals may be a feasible and effective way of improving iron and zinc nutriture during infancy (9). However, in ZINAK we found significant negative interactions when combining iron and zinc in, both for the biochemical results, i.e. Hb and S-Ft as well as the functional outcomes, i.e. WAZ, knee-heel length and psychomotor development.

In a recently published evaluation of the efficacy and effectiveness of iron supplementation as prevention for anemia in infancy, it is stated that iron supplementation to infants usually improves Hb and S-Ft (205). In the present study, iron given alone had a significant positive effect on Hb and S-Ft compared to both placebo and the Fe + Zn group. For Hb, the group mean differences to the placebo and Fe + Zn groups were 5.9 g/L (p=0.001) and 3.7 g/L (p=0.042), respectively. A difference of >5 g/L usually indicates public health significance. For S-Ft the group, mean differences to the placebo and Fe + Zn groups were 34 µg/L (p<0.001) and 10 µg/L (p=0.007), respectively. There was also a significant main effect of iron treatment on S-TfR, with TfR decreasing significantly
compared to placebo. This indicates that the iron supplement was consumed by the participating infants, and that iron supplementation is effective in preventing anemia and IDA during the 6-12 mo age period in Indonesian infants. However, the Fe group had a significantly higher prevalence of S-Zn <10.7 µmol/L (87% vs. 78%, p=0.033), indicating that iron supplementation may have adversely affected zinc absorption.

Zinc treatment had a significant main effect on S-Zn with no evidence of interaction between iron and zinc, suggesting that zinc supplementation is effective in improving zinc status measured as S-Zn in Indonesian infants. The finding is in agreement with a recent meta-analysis showing that supplementation of zinc to infants and young children has been associated with significant increases in S-Zn (110).

In the present study, iron supplementation had a positive, small, but significant effect on knee-heel length compared to placebo (difference in means 1.9 mm), but it had no effect on body weight or overall length. From a public health point of view, the small difference in knee-heel length between the Fe and placebo groups is negligible, but indicates that iron may be one of several growth-limiting factors in this population. Iron deficiency has been implied to contribute to growth retardation, but different studies have given conflicting results, where some have showed an effect of iron supplementation on growth, while others have failed to demonstrate any difference.

Aukett et al (206) showed an increased weight gain in anemic, British toddlers receiving iron, and supplementation to Indonesian preschoolers resulted in increased linear growth (207). In the former study, the weight gain was greatest in the group with the greatest increase in Hb, and in the latter the positive results on linear growth were associated with decreased morbidity in the iron group. Studies in school children have also shown effects of iron supplementation on growth (208-210). A recent randomized trial in Ethiopian children assessing the effects of consuming foods cooked in iron pots showed improved iron status and growth (211).

On the other hand, Moffatt et al (212) in a carefully designed prospective study did not find any differences in growth between infants given iron fortified and unfortified formula until 15 mo of age, despite differences in both hematological parameters and psychomotor development. Similarly, neither Rosado et al (213),
nor Rahman et al (214) or Sunghong et al (215) found any effect of iron supplementation on growth compared to placebo.

A recent meta-analysis on the effects of zinc supplementation on growth has shown positive effects on both height and weight (110). The meta-analysis shows that the positive effect on the HAZ was almost two times greater if the child was stunted at enrollment, and that the effect on WAZ was more than two times greater if the child was underweight at inclusion. Likewise, the effect on growth was greater in those with S-Zn <12.2 µmol/L. However, neither the differences between stunted and non-stunted nor underweight and non-underweight or those with S-Zn below or above 12.2 µmol/L were statistically significant.

Zinc supplementation to the infants in the ZINAK study significantly improved growth (WAZ and knee-heel length) compared to placebo. The differences in means between the zinc and placebo groups were 0.26 for WAZ and 2.0 mm for knee-heel length, which agree with earlier results [referred in (110)]. Again, and as with the effects of iron supplementation, the public health significance of this difference may be limited, but point toward that zinc is one of several growth-limiting factors in this population.

Iron supplementation in combination with zinc had no effect on growth. These results are thus in line with several other studies which have failed to show growth-benefits of simultaneous iron and zinc supplementation (197, 213, 216), but contrary to Perrone et al (217), who showed improved growth in the combined iron and zinc group. However, in that study the iron and zinc supplementation was separated by 12 h and the subjects were older (4-11 years) and all were stunted as compared to the present study. The separation in time between the administration of iron and zinc is likely to have eliminated interactions at the intestinal absorption level, allowing zinc to act independently as growth-promoting agent.

In the last three decades, a large body of evidence has accumulated on the association between iron deficiency and impaired neurodevelopment in infants and children (49, 50, 52-55, 206, 218-239). The effects of iron on brain development have been reviewed recently (240). Animal studies indicate that monoamine, especially dopamine function and the myelination process seem especially vulnerable to iron depletion. Early iron depletion in rats, i.e. from birth to 21 d of age, induced irreversible changes in brain
Discussion

In human infants such "window of vulnerability" to iron deficiency may lie between ages 3 and 23 mo, ages where iron deficiency is most common world-wide (59, 240).

Early studies by Oski et al (221) and Walter et al (222) indicated that even mild iron deficiency, i.e. without anemia, was correlated with poor cognitive development, but later studies (224, 225) have indicated that anemia in combination with iron deficiency is needed to cause developmental effects. Some studies implicate that at least part of the effect on mental and psychomotor development is irreversible, even after treatment with iron and an excellent hematological response (49, 50), although others have disputed this (228).

However, iron deficiency has in many of the studies been associated with other socioeconomic risk factors of poor development, and though attempts to adjust for these have been made, confounding of the causal relationship between ID and development by these other factors can not be ruled out (232). Martins et al conclude that though few of the studies fulfill stringent scientific criteria to allow us to draw strong inferences on the association between ID and infant development, the data presented could imply significant positive neurodevelopmental effects of iron supplementation in infants with IDA (238).

Several animal studies have shown that zinc deficiency induces mental and behavioral changes. Similar to iron deficiency, the effects are clearly manifest in overt zinc deficiency. However, studies in young rhesus monkeys show that even marginal zinc deficiency can induce behavioral changes (242). Studies on zinc deficient adult humans have implicated impaired short-term memory and changes in food preferences (243). Studies of zinc on infant development and behavior in infants and children have been inconclusive (244-250).

In ZINAK, there was a small but significant positive effect of iron supplementation compared to placebo on psychomotor development measured with the Bayley Scales of Infant Development. The difference was 3 points, which may be insignificant from a public health point of view. No effect was seen on cognitive development or behavior. However, the results point in the same direction as those published by Idradinata and Pollitt (228) and Moffatt et al (212), which showed significant effects of iron supplementation on psychomotor development in iron-
deficient children or infants at high risk of developing IDA. In ZINAK, combining iron and zinc supplements had no effect on psychomotor development compared to placebo, nor was there any effect of zinc supplementation on either development or behavior.

There was no decline in psychomotor development with age in the infants not treated with iron, although anthropometrical status declined and the prevalence of IDA increased significantly, which is contrary to other studies (212, 234). This, together with a low baseline prevalence of IDA, possibly diminishing the differences in psychomotor development between iron treatment and placebo, may imply that other environmental or nutritional factors, e.g. long breast-feeding duration and a high degree of formal education among mothers may have moderated the effects of iron deficiency in the non-iron treated groups, as has been described in the case of protein-energy malnutrition and psychomotor development (251).

Several in vitro studies have shown that iron deficiency impairs immune function, and that iron administration restores function but clinical studies in children, with few exceptions, have not been able to demonstrate significant positive effects of iron supplementation. In a review on the effects of iron on immunity, Oppenheimer (252) concludes that: a) parental iron treatment to newborns can cause sepsis and meningitis, and should not be used; b) iron-fortified infant foods do not reduce infectious disease morbidity compared to breastfeeding, but has other beneficial effects; that there is some, although not very compelling evidence that oral iron supplementation in non-malarious, disadvantaged populations may be of benefit; and c) that high doses of oral iron (>2 mg/kg/d) to children in malaria-endemic populations carries increased risk of both clinical malaria and other infections, such as pneumonia.

In a recent meta-analysis, Gera and Sachdev examined the effects of iron supplementation on infectious disease morbidity in children (48). They found no benefits of iron supplementation, and though it did not significantly increase the overall risk of infection, there was a small, but significant increase in diarrheal disease (incidence rate ratio 1.11, 95% CI 1.01-1.23). Iron supplementation increased the odds ratio for malaria parasitemia (1.43, 95% CI 1.08-1.91). However, in the meta-regression analysis, adjusting for baseline parasitemia, iron supplementation was not significantly associated with malaria-positive blood smears (p=0.076).
Adults fed a zinc-restricted diet display signs of increased susceptibility to infections (253). In urban slum children in India, low plasma zinc (8.4 µmol/L) significantly increased the risk of diarrhea and acute lower respiratory infections, with boys being more vulnerable than girls (254). A recent meta-analysis has shown that zinc supplementation significantly reduces diarrheal morbidity and pneumonia in children in low-income settings (111). Diarrheal disease has also been found to induce zinc deficiency through massive fecal losses of zinc (102).

In ZINAK, neither iron, nor zinc supplementation, alone or in combination had any effect on the incidence of diarrhea or respiratory infections. One possible reason may be that the preventive effect of zinc on diarrheal disease has been more pronounced among children ≥12 mo than in younger children (111, 255). However, Baqui et al found that combined iron and zinc supplementation reduced the incidence of severe diarrhea and acute lower respiratory infections, particularly in less well-nourished infants, i.e. WAZ <-1, when given once weekly to infants from 6 mo of age, although a combination of iron, zinc and minerals increased the risk of diarrhea and significantly contributed to vomiting (199).

Further, the diarrheal incidence in ZINAK was lower than in some other studies (199, 255, 256). Also, the high proportion of infants in this study who were still being breast-fed together with high accessibility to safe water as well as family factors, such as the relatively high educational level of the mothers, might have added a protective effect. Taken together, these factors may have made it difficult to achieve further reductions in the morbidity incidences through zinc supplementation in this population.

**IRON AND ZINC INTERACTIONS**

The results in ZINAK indicate that interaction between iron and zinc is possible for several outcomes, biochemical as well as functional, when combining the two minerals in the same supplement. Several possible levels of iron-zinc interactions can be identified. First, compliance to the various supplements was different, with vomiting in relation to the supplements being reported more often from the combined Fe + Zn group. We adjusted for this as well as consumption of supplements in the analysis and the interactions remained significant. However, it is
not know what proportion of the different supplements was lost through vomiting and thus the possibility cannot be ruled that vomiting, i.e. losing an unknown proportion of the ingested supplement before absorption, may have affected the results. As discussed earlier, decreased compliance or increased adverse effects of combined iron-zinc supplementation has been reported by others (197-199).

A second level of interaction is intestinal absorption. Several studies have shown that giving iron and zinc together in a water solution affects absorption of both minerals (137, 139-141). However, when the minerals were given as part of infant foods, no effect of high iron:zinc molar ratios on iron or zinc absorption was seen (140, 143-145). Common iron-zinc pathways have been identified (146), but the substrate specificity and how these transporters act \textit{in vivo}, particularly in human infants is not known.

A third possible level of interaction is the effects and counter-effects of the two minerals on the functional outcomes. Zinc supplementation to zinc deficient infants has been shown to improve growth (110), whereas iron supplementation to iron replete infants has been shown to negatively affect growth (147-149). Given the high prevalence of low S-Zn (78%), but the low prevalence of ID and IDA (15% and 8%, respectively) at baseline, this level of interaction may also have been present in the ZINAK study.

**IRON INTAKE AND IRON STATUS**

In the analysis of the association between dietary iron intake and iron status, we expected to find little effect on variables like Hb, since all infants were well-nourished and IDA was uncommon, and infants with presumably significant IDA (Hb < 100 g/L, S-Ft < 12 µg/L and MCV < 70 fL) were excluded, but to find an increase in S-Ft, the storage form of iron. Instead the opposite was found – in infants below 12 mo of age there was a significant increase in Hb with increasing iron intake, but no effect on S-Ft.

Only at 18 mo was an association between iron intake and S-Ft found, whereas the correlation between iron intake and Hb had disappeared at this time. From 6-8 mo the relationship between dietary iron intake and Hb was linear and with no significant interaction between other nutrients and iron intake. Later, during
the 9-11 mo period, there was still a significant, but now a non-linear relationship between daily iron intake and Hb. These data suggest that the regulation of iron metabolism during the first years of life is highly dynamic, and it is tempting to speculate that dietary iron is to a higher degree channeled to erythropoiesis and incorporated into Hb during infancy, as indicated by the almost linear relationship between iron intake and Hb concentration, than later in childhood. Interestingly, S-TfR was positively associated with Hb at both 9 and 12 mo.

The concentration of transferrin receptors in serum are thought to mirror the expression of transferrin receptors on iron-requiring cells, most abundantly on cells involved in erythropoiesis, and is believed to reflect intracellular iron needs (31, 257, 258). A positive association between Hb and S-TfR may thus indicate increased erythropoiesis. Virtanen et al have described higher S-TfR in infants than in pre-pubertal boys and men, and increased erythropoiesis is one of several possible explanations (259).

Little of the dietary derived iron seems to be directed to storage, i.e. to ferritin, in infancy, as there was no correlation between iron intake and S-Ft until after 12 mo of age. Gradually less of iron obtained from the diet is incorporated into Hb during 9-11 mo, seen as a plateau in the iron intake-Hb curve at 12 mo of age. Thereafter, dietary iron seems to be channeled towards storage to an increasing extent, seen as an increase in S-Ft at 18 mo with increasing iron intake during 12-17 mo. A shift in the channeling at around 1 yr of age is also indicated by the finding that not even the total iron dose given until 11 or 17 mo of age did affect S-Ft, but only the amount of iron given after 12 mo of age.

The way iron is provided, i.e. as a food constituent or through supplementation, may modify the effects on hematological parameters. In the Indonesian study, iron intake from the supplements was more than 5.5 times lower than in the Swedish study (iron intake 0.15 mg/kg/d vs. 0.85 mg/kg/d in the ZINAK and SINUS studies, respectively). However, there were significant dose-response relationships for both iron supplementation and Hb, and iron supplementation and S-Ft in the Fe group, whereas in the Swedish study dietary iron only associated significantly to Hb but not S-Ft in infancy.

One explanation may be that iron sulfate provided as a supplement had much higher bioavailability than does the fortificant iron of
Swedish infant cereals, providing sufficient iron to increase both Hb and S-Ft. Another explanation may be that whereas the increase in Hb indicates that iron is absorbed and utilized in the erythropoiesis, the increase in S-Ft may indicate the organism’s attempt to detoxify highly reactive iron in the intestinal lumen.

**MONITORING OF IRON INTERVENTIONS**

Several authors have questioned the current definitions of ID and IDA, suggesting lower and more age-appropriate cut-offs (23, 36-38). Hence, the prevalence of ID and IDA reported in this thesis may have overestimated the initial problem. Such overestimation may also have as a consequence that true differences in iron status and effects of intervention become less clear. However, using the alternative definitions suggested did not result in significant differences between the groups. The data presented indicate that when evaluating the effects of dietary iron interventions in infancy, Hb correlates closer to dietary iron intake than does S-Ft and S-TfR, whereas the opposite may be true in the second year of life. Further, the data indicate that Hb increases regardless of iron status, at least before 9 mo of age, suggesting that the reference value of a population at that age is dependent on its iron intake, whether or not actual ID is present or not. Using reference populations with high intakes of dietary iron may thus increase the Hb distribution beyond what is “physiological” at that age, which should be considered when defining anemia in infants.
CONCLUSIONS AND RECOMMENDATIONS

Providing sufficient iron and zinc to infants after the period of exclusive breastfeeding remains a challenge. In high-income settings these problems can be partly overcome by use of fortified complementary foods. However, the composition of the complementary foods, often cereal-based and thus containing phytate may infringe on the bioavailability of iron and zinc.

The data presented show that in the high-income setting, reduction of the phytate content of commonly consumed infant cereals has little effect on iron and zinc status, growth, development or incidence of diarrhea or respiratory infections. A possible explanation to this is the high content of ascorbic acid in the commercially available infant cereals, increasing the bioavailability of iron. Given a sufficient ascorbic acid:Fe molar ratio there is no urgent need to reduce the phytate-content of infant cereals currently used in the Swedish infant population. Thus, the phytate content of complementary foods of Swedish infants does not contribute to poor iron and zinc status as feared. Instead the definitions of iron and zinc deficiency in infancy, at least before 12 mo of age, may overestimate the problem, and a change in the recommended cutoffs is suggested.

In low-income settings, where the complementary foods are more often than not home-prepared from staples such as vegetables and cereals, with low absolute amounts and low bioavailability of iron and zinc, the problem of sufficient nutrient density is considerable. Supplementation may thus be a feasible and effective alternative. However, interaction between iron and zinc is possible when they are given together.

In the low-income setting, combined supplementation with iron and zinc resulted in significant negative interaction, not only for biochemical outcomes such as Hb and S-Ft, but also for functional outcomes such as growth, i.e. weight and knee-heel length, and psychomotor development. Several levels of interaction are possible; from competition for absorptive pathways in the intestine to differences in compliance due to side effects of the combined supplement. The study also indicates that the chosen level of iron supplementation, i.e. 10 mg/d may be unnecessarily high as no further effect on iron status, i.e. S-Ft, was seen beyond 7 mg/d. From these results it is not possible to recommend routine iron-zinc
Conclusions and recommendations

supplementation at the molar concentration and mode used in this study. Instead, alternative approaches should be investigated, including intermittent supplementation with iron and zinc, e.g. weekly dosage of the two minerals, separating the supplements in time, e.g. zinc supplementation only during diarrheal episodes, the use of iron and zinc compounds with different absorptive properties than iron sulfate and zinc sulfate, administration of iron and zinc at other molar ratios than those applied here or food-based strategies where the additional iron and zinc is mixed with home-prepared complementary foods, maybe together with other vitamins and minerals as well. With the high prevalence of ID and the presumably high prevalence of ZD among infants and young children in low income countries and their various and severe consequences for child health and development, it is imperative that research efforts are focused to finding solutions to prevent these deficiency states.

Finally, studies have indicated that iron homeostasis undergoes developmental changes during infancy. In the present thesis, this theory is supported by the finding that dietary iron intake is significantly positively associated with Hb, but not serum ferritin until 12 mo, although the opposite is true after 12 mo of age. This may indicate that dietary iron is preferably channeled towards erythropoiesis during infancy, but to an increasing amount channeled towards storage in early childhood. This suggests that in evaluating dietary programs, Hb may be superior in monitoring response to dietary iron in infancy, whereas S-Ft may respond better later in childhood. However, as shown in this study, increasing Hb is not necessarily an indicator of iron deficiency, as dietary iron increased Hb regardless of iron status.
Järn- och zinkbrist bland spädbarn är mycket vanligt i ett globalt perspektiv. Dessa mineralbrister orsakar bl a anemi (blodbrist), försenad utveckling, försämrad tillväxt och ökad sjuklighet i diarré- och luftvägsinfektioner. En starkt bidragande orsak till att järn- och zinkbrist uppstår är att tilläggskosten är otillräcklig.

Vi har undersökt två sätt att förbättra intaget av järn och zink hos spädbarn i två olika befolkningar; i en svensk grupp barn minskade vi mängden fytinsyra, en hämmare av järn- och zinkupptaget i välling och gröt, medan vi i en indonesisk grupp barn undersökte effekten av dagligt järn- och zinktillskott.


Bland de svenska barnen var järnintaget under perioden 6-11 mån kopplat till Hb, men inte till serum ferritin vid 9 och 12 mån, medan det omvända gällde senare, d v s att järnintaget under
perioden 12-17 mån var kopplat till ferritin men inte Hb vid 18 mån. Detta tyder på att järn i kosten verkar gå till produktion av röda blodkroppar under spädbarnsåret, medan det senare i större utsträckning laggs i järnförråden. När man utvärderar effekten av kostinterventioner kan det därför vara bättre att följa Hb under spädbarnsåret, medan serum ferritin kan svara bättre efter 12 mån ålder. Dock behöver stigande Hb inte automatiskt betyda järnbrist, då mer järn i kosten gav ökat Hb oberoende av tidigare järnstatus.

Vi drar vidare slutsatsen att mängden fytinsyra i välling och gröt till svenska barn inte verkar bidra till försämrat järn- och zinkstatus givet tillräckliga mängder askorbinsyra. De nuvarande definitionerna av järn- och zinkbrist kan behöva justeras, då de tenderar att överdriva andelen barn med otillräckligt järn- och zinkstatus. Från den indonesiska studien drar vi slutsatsen att vi inte kan rekommendera att ge järn och zink tillsammans i de koncentrationer och på det sätt vi gjort, då detta ger upphov till negativ växelverkan, vilket motverkar syftet med tillskotten. Då både järn- och zinkbrist är vanligt är det dock av största vikt att studera hur man bäst förebygger dessa tillstånd i låginkomstländer.
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