NON-ANEMIC IRON DEFICIENCY AND HEALTH OUTCOMES IN PRE-SCHOOL CHILDREN

By

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Abstract

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Doctor of Philosophy, Graduate Department of Health Policy, Management and Evaluation
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There is a gap in evidence on the epidemiology, diagnosis and management of non-anemic iron deficiency (NAID) in young children. Using the data from a community practice-based research network (TARGet Kids!) four studies were conducted to enrich the evidence-base related to NAID in Canadian pre-school children.

The prevalence of NAID was found to be 7% (95% CI, 5.95%-8.05%). Risk factors significantly associated with NAID included younger age (OR 1.08; 95% CI: 1.06, 1.11), higher zBMI (OR 1.22; 95% CI: 1.01, 1.48), longer duration of breastfeeding (OR 1.05; 95% CI: 1.01, 1.08) and volume of cow’s milk intake (OR 1.13; 95% CI: 1.01, 1.26). Practice patterns associated with the management of NAID by community physicians showed substantial variation. Follow-up laboratory tests showed 65.5% had resolution, 25.9% had persistence and 3.4% had progression of NAID to IDA.

To identify clinically important thresholds for serum ferritin (SF) in diagnosing iron deficiency in children a restricted cubic spline regression analysis was performed. A spline curve showing the association between hemoglobin and SF identified: a threshold for SF (17.9 µg/L) where the clinical impact of iron deficiency may not come into effect until values lower than this cut-off has been reached; and a SF cut-off (4.6 µg/L) that may have clinical impact on the neurodevelopment of children.

To assess the effectiveness of iron interventions in children with NAID, the protocol for a multi-site, pragmatic, placebo controlled, superiority randomized trial was described (OptEC trial). The protocol included the methods of an internal pilot study, performed to recalculate the sample size and assess the adherence rate in children enrolled in the OptEC trial. Using internal pilot study data, the initial sample size (N_a =112-198) was recalculated to range between 32-56 subjects. Adherence rate ranged between 14% -100% and 44% of the children had an adherence ≥ 86%.
Dedication

This dissertation is dedicated
to the loving memory of my grandmother, Nazmir Begum
Acknowledgments

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Abbreviations

NAID  non-anemic iron deficiency
IDA   iron deficiency anemia
HO1   haem oxygenase 1
Tf    transferrin
TfR   Tf receptors
DMT1  divalent metal transporter
RNA   ribonucleic acid
mRNA  messenger RNA
IRE   iron responsive elements
IRPs  iron-responsive proteins
SF    serum ferritin
Hb    hemoglobin
EP    erythrocyte protoporphyrin
RWD   red cell distribution width
CRP   C-reactive protein
CHr   reticulocyte hemoglobin
ROC   receiver operating characteristic curve
LR    likelihood ratio
SD    standard deviation
WHO   World Health Organization
AAP   American Academy of Pediatrics
USPSTF US Preventive Services Task Force
AUC   area under the curve
BSID  Bayley’s scale of infant development
PDI   psychomotor development index
MDI   mental development index
CCTR  Cochrane controlled trials register
OR    odds ratio
SMD   standardized mean difference
MD    mean difference
RR    relative risk
TARGGet Kids! The applied research group for kids
CPS   Canadian Pediatric Society
PBRN  practice-based research network
AHRC  applied health research centre
VIF   variance inflation factor
RCS   restricted cubic spline
ECD   early child development
MSEL  Mullen scales of early learning
ELC   early learning composite
ANCOVA analysis of covariance
SES   socio-economic status
Chapter 1 : Introduction

The purposes of this chapter are to:

1. Present the thesis aim (general and specific), hypotheses and study questions.
2. Provide the reader with an introduction to the biology, metabolism and homeostasis of iron in the human body.
3. Define the different stages of iron deficiency and introduce the stage of non-anemic iron deficiency (NAID)
4. Review the epidemiology, etiology and diagnosis of the different stages of iron deficiency
5. Review the various interventions used to treat and prevent iron deficiency
6. Provide an overview of iron deficiency in the current Canadian context
1.1 Research aims, hypotheses and study questions

1.1.1. General aim & Hypotheses

The overarching goal of this thesis is to create an evidence base for the non-anemic stage of iron deficiency which we term NAID. The hypotheses was that the prevalence of NAID is high in young Canadian children and due to the lack of screening with appropriate iron indicators and cut-offs, this disorder remains undetected and untreated and may cause negative impact on child development. It was hypothesized that children diagnosed with NAID receiving four months of oral iron plus dietary advice will have better developmental outcomes than those who receive placebo plus dietary advice. Furthermore, methods based research, such as performance of an internal pilot study can refine the sample size and protocol of randomized clinical trials aimed to investigate the effectiveness of iron interventions and increase the validity of the evidence.

1.1.2. Specific aims

The specific aims of this thesis were:
1) To evaluate the prevalence and risk factors associated with NAID in urban Canadian pre-school (12-60 months) children;
2) To describe the physician practice patterns associated with the management of NAID in Canadian primary health care settings;
3) To describe the longitudinal hematological outcome of children (12-60 months) identified with NAID in Canadian primary health care settings;
4) To identify clinically important thresholds/cutoffs for serum ferritin to diagnose iron deficiency in young children (12-36 months) by examining its relationship with recommended hemoglobin concentration cut-offs;
5) To assess the effectiveness of four months of oral iron plus dietary advice versus placebo plus dietary advice, in children with NAID aged 12 to 40 months, to improve their developmental and hematological outcomes;
6) To perform an internal pilot study in order to recalculate the sample size and refine the conduct of a randomized controlled trial aimed at evaluating the effectiveness of oral iron plus dietary advice versus
placebo plus dietary advice in children (12-40 months) with NAID, in improving their developmental and hematological outcomes;

1.1.3. Study Questions

The study questions that follow from the stated hypotheses are as follows:
1) What is the prevalence rate and risk factors associated with NAID in urban Canadian pre-school (12-60 months) children?
2) How are children (12-60 months) identified with NAID in Canadian primary health care settings, managed (treatment and follow-up)?
3) For children (12-60 months) identified with NAID in Canadian primary health care settings, what are their longitudinal hematological outcomes?
4) What is the relationship between serum ferritin and hemoglobin concentration in diagnosing iron deficiency in young children (12-36 months)?
5) In young children 12 to 40 months of age, identified with NAID, is four months of oral iron treatment plus dietary advice better than placebo plus dietary advice, to improve developmental and laboratory outcomes?
6) Can the data from an internal pilot study be used to recalculate the sample size and refine the conduct of a randomized controlled trial aimed at evaluating the effectiveness of oral iron plus dietary advice over placebo plus dietary advice in children (12-40 months) with NAID in improving their developmental and hematological outcomes?
1.2 Physiology, epidemiology, diagnosis and treatment of iron deficiency

1.2.1 Importance of Iron in the body:

To physicians and clinical researchers “iron” usually refers to an important micronutrient, essential for the sustenance of the human body. Very few of us pause to consider that iron is a reactive metal with complex biochemical properties (atomic weight 55.85, atomic number 26) (1, 2). It is these chemical properties of iron that drive and regulate how it interacts with the human body (1).

Iron is an essential component of virtually all living cells, specifically human cells. Under physiological conditions the ability of iron to convert between two thermodynamically stable oxidation states, the ferric (Fe\(^{3+}\)) form and the ferrous (Fe\(^{2+}\)) form, makes it ideally suited to the catalysis of biochemical reactions. A large number of enzymes depend on iron for their biological function (3). These include electron transfer, the transport, storage, and activation of oxygen, nitrogen fixation, detoxification of activated oxygen species, and deoxyribonucleotide synthesis from ribonucleoside diphosphates (1, 3). These very characteristics that make iron so valuable for living systems also mean that the metal is able to catalyze reactions leading to the production of toxic oxygen radicals, particularly when it is present in excess. To deal with this dual nature of iron, individual cells and the body as a whole have evolved sophisticated mechanisms for regulating iron influx and efflux (3).

During the processes of iron absorption and distribution, it is almost always tightly bound to proteins, leaving an extremely low concentration of free intracellular iron. The regulation of intracellular iron is very important, because even low concentrations of “free iron” can result in severe damage to a number of cellular constituents including membranes and DNA (1, 4-7). Therefore, to maintain normal iron homeostasis specific carriers have evolved which function not only to keep iron in a soluble and bioavailable form but to keep it in a benign state (1, 3).

1.2.2 Metabolism and homeostasis of Iron in the body (body distribution, absorption, transport, and cellular processing)

Advanced biomedical, physiological and genetic studies have elucidated the main pathways of iron metabolism, namely iron absorption, distribution, usage, storage and recycling. A large number of co-
regulated proteins and receptors interplay to maintain intra- and extracellular iron balance through these processes (3, 8-10). Deregulation of iron homeostasis can result in severe imbalance in cellular functions, which can in turn lead to diseases such as iron deficiency or iron overload (8, 11). Thus, in order to begin the process of designing novel treatments for various iron related diseases, it is important to understand the various iron metabolic pathways.

Absorption of iron in the body:

About 1-2 mg of dietary iron is absorbed per 24 h, and the total iron balance is maintained by a daily loss of ~1 mg via nonspecific mechanisms (mostly cell desquamation) (12). Iron absorption consists of three main processes: uptake of iron into the intestinal mucosal cell, movement through the intestinal cell and release from the cell in the circulation (6, 13, 14). Each of these processes is determined by the activities of specific iron transport and storage proteins (13). These three sites are also the regulatory sites where the absorption of iron are regulated by various dietary and physiological factors shown in Table 1 (6, 13, 14).
Table 1: Factors influencing iron absorption

<table>
<thead>
<tr>
<th>Dietary factors</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Physio-chemical form</strong></td>
<td>Iron in foods exists in two main forms: haem (animal source) and non-haem (non-animal source) iron. About 20-30% of haem iron is absorbed and its absorption is unaffected by other dietary variables. Non-haem iron absorption is influenced by dietary and physiological variables that either enhance or inhibit absorption.</td>
</tr>
</tbody>
</table>
| **Dietary constituents affecting non-haem iron absorption** | **Enhancers:** Food containing ascorbic and citric acid (orange, pear, apple, pineapple, plum, rhubarb); cysteine-containing peptide (beef, lamb, pork, liver, chicken, fish); citric, malic and tartaric acid (carrot, potato, beetroot, cauliflower, broccoli, tomato, cabbage, turnip); ethanol and lactic acid (sauerkraut, beer, wine)  
**Inhibitors:** Food containing phytate (wheat bran, soy protein, oats, maize, rice); polyphenol (tea, coffee, oregano) and oxalic acid (spinach) |
| **Dose**                        | A negative relationship exists between iron dose and percentage absorption. However, the upper limit to iron absorption is determined by various host factors |

<table>
<thead>
<tr>
<th>Physiological factors</th>
<th></th>
</tr>
</thead>
</table>
| **Internal**                    | Iron content of mucosal cells: Exposure to iron reduces subsequent absorption  
Body iron stores: Low stores causes marked increase in absorption and vise-versa  
Rate of erythropoisis: Positive correlation  
Tissue hypoxia: Increased absorption |
| **Physiological state**         | Increased absorption during growth and pregnancy |
| **Other physiological factors** | GI secretions (gastric acid, bile, pancreatic secretions and mucus): Increased absorption |
Mechanism of iron absorption:

Absorption of iron by the epithelial cells of the proximal gastrointestinal tract is a highly regulated process that maintains body iron homeostasis (13, 15). Dietary iron, broadly classified into haem and non-haem iron, each have a separate and distinct mode of uptake by the enterocytes (16). Haem iron is taken up by a haem-receptor located in the apical brush border membranes and subsequently endocytosed in pits at the base of the microvilli, before transit into lysosomes. Absorbed haem is catabolised by haem oxygenase 1 (HO1) to release inorganic iron that enters the cytosolic pool. Non-haem iron must first be reduced to the ferrous form for it to be taken up at the apical surface of the enterocytes. The ferrous ion is driven by proton-coupled electrogenic transport into the cytosol of enterocytes. Transferrin receptors in the basolateral membrane of the enterocytes mediate the transfer of cytosolic iron into the portal circulation where it binds with transferrin (Tf), the protein that transports iron to different body compartments (13, 14). Figure 1 depicts the mechanism of iron absorption.

Figure 1: Mechanism of iron absorption (14)

Transport and distribution of iron in the body:

Iron is transported within the body between sites of absorption, storage, and utilization by the plasma glycoprotein transferrin (Tf), which binds iron very tightly but reversibly (12). Transferrin is recognized by specific cell membrane Tf receptors (TfR), which are crucial for cellular iron acquisition. Following its intra-cellular release from Tf-TfR complexes, iron enters functional compartments or is stored as ferritin (12, 17). Table 2 depicts the distribution of iron in the functional and storage compartments of the body.

Table 2: Distribution of iron in the body

<table>
<thead>
<tr>
<th>Functional Iron</th>
<th>60-70%</th>
</tr>
</thead>
<tbody>
<tr>
<td>As hemoglobin in circulating erythrocytes</td>
<td></td>
</tr>
<tr>
<td>As myoglobin, cytochromes and other iron-containing enzymes</td>
<td>10%</td>
</tr>
<tr>
<td>Stored Iron</td>
<td></td>
</tr>
<tr>
<td>As ferritin and hemosiderin in macrophages</td>
<td>20-30%</td>
</tr>
<tr>
<td>of the liver, bone marrow and spleen</td>
<td></td>
</tr>
<tr>
<td>Transport Iron</td>
<td>&lt;0.1%</td>
</tr>
<tr>
<td>As plasma transferrin</td>
<td></td>
</tr>
</tbody>
</table>

Although iron bound to Tf is < 0.1% (~3 mg) of the total body iron, dynamically it is the most important iron pool with the highest rate of turnover. The turnover of Tf iron is roughly 25 mg/24 h. Transferrin-bound iron enters target cells – mainly erythroid cells, but also immune and hepatic cells (8).

Acquisition and utilization of iron by the cell:

The main sites of these metabolic processes are within the erythroid cells which require sufficient quantities of iron for the synthesis of hemoglobin, which eventually contains virtually all erythrocyte
Iron (18). Other than erythroid cells these metabolic processes occur and are maintained within other specialized tissues such as macrophages, heart, skeletal muscles, and cells of blood-testis barrier, blood-brain barrier, brain, intestine and placenta (12).

Transferrin-bound iron enters the cell through a highly specific process of receptor-mediated endocytosis (8). Once the endosome (siderosomes) carrying the Tf-TfR complex detaches from the cell membrane it undergoes a conformational change promoting the release of Fe$^{3+}$ from transferrin. Fe$^{3+}$ is then reduced to Fe$^{2+}$ by ferrireductase and transported to the cytoplasm by a carrier protein (DMT1). At the same time TfR is recycled to the cell membrane and transferrin is shed back into the circulation (8, 19, 20). Figure 2 depicts the transferrin cycle.

Figure 2: The transferrin cycle (21)


Iron that enters the cell is then transported to intracellular sites of use (synthesis of protein, electron transfer and catalysis of enzymes), and/or stored within ferritin, or may contribute to the regulation of
cellular iron metabolism by influencing other intracellular factors (3, 12). A metabolically and kinetically active intra-cellular pool of iron is maintained by sensitive control mechanisms that monitor iron levels in the labile pool and prevents the expansion of this pool, while still making the metal available for iron-dependent proteins and enzymes (12).

Recycling of iron:

Normally ~80% of absorbed iron is transported to the bone marrow for hemoglobin synthesis in developing erythroid cells. From these sites, reticulocytes are released into the circulation where, within about 24 hours, they develop into mature erythrocytes that circulate in the blood for about 120 days (humans). Senescent erythrocytes are phagocytosed by a specialized population of macrophages in the bone marrow, liver and spleen (reticuloendothelial system) where the heme moiety is split from hemoglobin and catabolized enzymatically via heme oxygenase. The liberated iron is released back to plasma transferrin at a rate that normally matches the rate of iron transport for erythropoiesis. (4, 17, 22-24). The amount of iron required for daily production of 300 billion red blood cells (20-30 mg) is provided mostly by macrophage iron recycling (21). Because daily absorption just balances daily loss, this internal turnover of iron is essential to meet the bone marrow requirements for erythropoiesis (8). Also small amounts of the daily plasma iron turnover are exchanged with nonerythroid tissues, namely, the liver. Iron taken up by the hepatocytes is mainly in the form of ferritin and it protects the hepatocytes against iron deficiency (17).

Storage of iron:

All cells are capable of storing iron; however, principal storage sites in humans are the liver, spleen and bone marrow. In the cell, iron can be stored in two forms: in the cytosol as ferritin and after breakdown of ferritin, in the lysosomes as hemosiderin (8, 17). The liver contains about 60% of the ferritin in the body with the remaining amount present in muscle tissues and cells of the reticuloendothelial system (macrophages). Macrophages act as a storage depot for iron recovered from senescent erythrocytes (8, 25). Figure 3 shows the distribution and metabolism of iron within the body.
Iron homeostasis:

Human beings are unable to excrete iron actively, so its concentration in the body must be regulated at the site of iron absorption in the proximal small intestine (8, 9, 21). The absorption of iron by duodenal enterocytes is dependent on diet, body iron stores, marrow erythropoietic activity, blood hemoglobin
concentration, blood oxygen content and the presence of systemic inflammation. Iron absorption decreases in the presence of iron overload and inflammation and increases in response to iron deficiency, accelerated erythropoiesis and hypoxia (6, 8, 26).

Cellular and systemic iron homeostasis:

Intracellular iron homeostasis involves the regulation of protein synthesis and actions related to iron acquisition, utilization and storage. Much of this process is regulated at the gene level by specific regulatory elements (IRE: Iron Responsive Elements). IREs are found on messenger RNAs (mRNA) of various forms of iron storage and transport structures (ferritin, TfR, DMT1, ferroportin). IREs regulate proteins involved in iron metabolism by interacting with iron-responsive proteins (IRPs) (27).

Humoral or systemic regulatory mechanisms for iron absorption include the dietary regulator, the stores regulator and the erythropoietic regulator (27). The stores regulator mechanism senses iron levels in the body and responds to total body iron level (21). After ingestion of foods or iron supplements, higher nonheme iron absorption occurs when iron stores are depleted and lower absorption occurs when stores are replete or enlarged. An inverse linear relationship has been described between iron absorption and serum ferritin (a measure of iron stores). It has been suggested that once iron stores are replete and a steady state has been achieved, beyond this point no further increase in iron absorption occurs (28, 29).

Body iron stores are also regulated by the iron regulatory hormone hepcidin which plays a role in determining the amount of iron absorbed by the gut and release from storage sites (30). Hepcidin is an antimicrobial peptide that is secreted by hepatocytes in the circulation. Ferroportin, a transmembrane protein acts as the receptor for hepcidin. It carries hepcidin across the basolateral membrane of enterocytes for regulation of apical iron absorption. The production of hepcidin is inversely related to iron stores, erythropoietic activity, hemoglobin, oxygen content and inflammation. Hepcidin expression is decreased when body iron requirements are high, such as when iron stores decrease, during iron deficiency, accelerated erythropoiesis, and hypoxia (31).

1.2.3 Deficiency of Iron in the body
Iron deficiency is a state in which there is insufficient iron to maintain the normal physiological function of tissues such as the blood, brain, and muscles (32). It is the result of long-term negative iron balance. Iron stores in the form of hemosiderin and ferritin are progressively diminished and no longer meet the needs of the body (32). As a result, an array of systemic effects of iron deficiency becomes evident. Symptoms of iron deficiency are subtle and non-specific, and often become apparent only in its severe stages (32, 33). Hence, it is important to know the stages of iron deficiency in order to fully recognize effects caused by this disorder.

1.2.4 Stages of iron deficiency

Iron status can be considered as a continuum from iron deficiency with anemia (IDA), to iron deficiency without anemia, to normal iron status, and finally iron overload (33). Iron deficiency without anemia again has two stages: iron deficient erythropoiesis and iron depletion (33). In iron depletion, the amount of stored iron is reduced but the amount of functional iron may not be affected, hence persons who have iron depletion have no iron stores to mobilize if the body requires more iron. In iron-deficient erythropoiesis, stored iron is depleted and transport iron is reduced further; the amount of iron absorbed is not sufficient to replace the amount lost or to provide the amount needed for growth and function. Thus is both of these stages the amount of stored iron is reduced and together we are terming this stage Non-Anemic Iron Deficiency (NAID). In IDA, the most severe form of iron deficiency, the shortage of iron (storage and transport) leads to underproduction of iron-containing functional compounds, such as hemoglobin, myoglobin and various cytochromes (Table 3) (33).
Table 3: Spectrum of body iron content (33)

<table>
<thead>
<tr>
<th>Stages</th>
<th>Sub-stages</th>
<th>Stored iron</th>
<th>Transport iron</th>
<th>Functional iron</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron deficiency</td>
<td>Iron deficiency anemia (IDA)</td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td></td>
<td>Non-anemic iron deficiency</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>(NAID)</td>
<td>Low</td>
<td>Low</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>Iron deficient erythropoiesis</td>
<td></td>
<td></td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>Iron depletion</td>
<td>Low</td>
<td>Normal</td>
<td>Normal</td>
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<tr>
<td>Iron sufficiency</td>
<td></td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Iron overload</td>
<td></td>
<td>High</td>
<td>High</td>
<td>Normal</td>
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1.2.5 Epidemiology of iron deficiency: A global overview

Nutritional iron deficiency is considered a major form of malnutrition throughout the world (34). Children from developed and developing countries alike are affected by this nutritional disorder (9). In 1993, a WHO/United Nation’s International Children’s Emergency Fund (UNICEF)/United Nations University (UNU) consultation advocated that iron deficiency not iron deficiency anemia be labeled as the core problem (35). Despite this recommendation, estimates from developing countries are often based only on measurement of anemia from restricted regions and target populations (9). WHO estimates that 39% of children younger than 5 years in developing countries are anemic with approximately 50% of anemia caused by iron deficiency (9, 32, 35).

In contrast, the occurrence of iron deficiency in developed countries is derived from nationally representative samples with specific indicators of iron status (9). Data from the widely quoted third National Health and Nutrition Examination Survey (NHANES III) in the United States report a 9% prevalence of iron deficiency in toddlers 1 to 2 years of age (36). However, the last NHANES survey was conducted during the period of 1999-2000 which collected iron status data. Table 4 compares the


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<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>1-2</td>
<td>1339</td>
<td>9</td>
</tr>
<tr>
<td>3-5</td>
<td>2334</td>
<td>3</td>
</tr>
<tr>
<td>6-11</td>
<td>2813</td>
<td>2</td>
</tr>
</tbody>
</table>


The values shown in table 4 suggest that the highest prevalence of iron deficiency during both the survey periods is among children less than three years of age. Children of this age group have also been shown to have higher risk of developing iron deficiency (37-39). Risk factors include history of prematurity or low birth weight, exposure to lead, exclusive breastfeeding beyond 4 months of age without supplemental iron, weaning to whole milk or complementary food that do not include iron-fortified cereals or food naturally rich in iron, poor growth and low socio-economic status especially children of Mexican American descent (40). Another study found toddlers who were overweight (OR: 3.4; 95% CI: 1.1–10.1) and those not in day care (OR: 1.9; 95% CI: 1.0–3.3) at high risk for developing iron deficiency (41).

Similar values for prevalence and risk factors of iron deficiency have been identified in European countries. The European Society for Pediatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN) Committee on Nutrition has reported the prevalence of iron deficiency in children aged 1-3 years to vary between 5% and 20% and the prevalence of IDA to be 3-9% in the same age group (42). Children in this age group were shown to have the highest prevalence of iron deficiency. Risk factors for iron deficiency and IDA in European infants and toddlers include low birth weight, early cord clamping, male sex, low socio-economic status, low meat intake, low intake of iron-fortified products (including infant formula and follow-on formula) and high intake of cow’s milk (42).
In these reports the prevalence of iron deficiency is a combination of the prevalence of IDA and NAID. The NHANES and the European data do not report the prevalence or risk factors specifically associated with NAID (36). Considering the low prevalence of IDA in the developed parts of the world, it can be assumed that the rest of the prevalence of iron deficiency is due to the non-anemic stage (NAID). Despite the high prevalence of NAID in children of developed countries and the potential for NAID to progress to IDA, the epidemiology specific to NAID is sparse and speculative and necessitates investigation.

1.2.6 Etiology of ID in children

The causes of iron deficiency among young children can be multifactorial (33). Need of increased iron due to rapid rate of growth coincident with inadequate intake of dietary iron places children aged less than 24 months, at the highest risk of any age group for iron deficiency (9, 40). Hence feeding practices adopted during this period greatly influence children’s iron status. In a study of infants aged 6 months, frequency of iron deficiency anemia was lowest in infants fed iron-fortified formula (about 1%) but occurred in 15% of breastfed infants and 20% of infants fed cow’s milk or non-fortified formula (43).

Current WHO guidelines for infant and young child feeding recommends to practice exclusive breastfeeding from birth to 6 months of age, and introduction of complementary foods at 6 months of age while continuing to breastfeed up to two years of age and beyond (44, 45). This recommendation pertains to healthy term infants. Evidence is immensely scarce with respect to exclusive breastfeeding and iron status in infancy. Result of one randomized trial has demonstrated that exclusively breastfed infants supplemented with iron between 1 and 6 months of age had higher hemoglobin and mean corpuscular volume at 6 months of age than did their unsupplemented peers. This trial did not show any difference between the two groups in their biochemical iron indicators (46). Other non-randomized studies have reported that iron supplementation through complementary feeding before 6 months of age is not likely to be an adequate mechanism for preventing iron deficiency and anemia (47, 48). Due to the mixed evidence on breastfeeding and children’s iron status, even though WHO recommends exclusive breastfeeding for the first 6 months of children’s life, they also state that infants must still be
managed individually, so that insufficient growth or other adverse outcomes are not ignored and appropriate interventions are provided (44, 49). The American Academy of Pediatrics (AAP) however, has recommended that exclusively breastfed term infants receive oral iron supplementation (1 mg/kg per day) starting at 4 months of age and continued until appropriate iron containing complementary foods have been introduced (40). A few studies have examined the effect of breastfeeding duration beyond 6 months on children’s iron status and have identified an association between total duration of breastfeeding and iron deficiency and IDA (50, 51). Other feeding practices that may contribute to early childhood iron deficiency include excessive early consumption of cow’s milk and day time bottle feeding (52, 53).

After 24 months of age, the growth rate of children slows and the diet becomes more diversified, the risk for iron deficiency decreases. After 36 months of age, dietary iron and iron status are usually adequate (9, 33). However, dietary iron bioavailability is low in populations consuming monotonous plant-based diets with little meat (54). In plant-based diets most dietary iron is non-haem iron, and its absorption is often less than 10% (compared with 15-35% for haem iron e.g. meat) (54, 55). The absorption of non-haem is increased by meat and ascorbic acid, but inhibited by phytates, polyphenols and calcium (55). Hence, other contribute dietary constituents to iron deficiency in children.

1.2.7 Laboratory indicators and diagnosis of iron deficiency

1.2.7.1 Laboratory indicators of iron status

Iron status can be assessed using iron indicators through several laboratory tests. Because each indicator assesses a different aspect of iron metabolism, measurement of these indicators enable iron status to be characterized in detail (figure 4).
Figure 4: Various iron indicators for different stages of iron deficiency (56)

<table>
<thead>
<tr>
<th>Stages of iron deficiency</th>
<th>NAID†</th>
<th>IDA†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron depletion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iron deficient erythropoiesis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iron deficiency anemia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low serum ferritin (≤12µg/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low transferrin saturation (≤10%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- Low Hb† (≤110 g/L)
- Low MCV† (<70 fL)
- High EP† (≥ 35 mcg/dl of whole blood)
- High RWD† (varies, depending on electronic cell counter)


Hematological tests based on characteristics of red blood cells (i.e., hemoglobin concentration, hematocrit, mean cell volume, and red blood cell distribution width) are generally more available and less expensive than biochemical tests (i.e., serum ferritin concentration, serum iron, erythrocyte
protoporphyrin concentration, total iron-binding capacity, transferrin saturation, and serum transferrin receptors). However, biochemical tests detect earlier changes in iron status (32, 33).

Indicators are used for different purposes in clinical screening and diagnosis vs. public health assessment at a population level, and different indicators may perform best for different tasks (57).

1.2.7.2 Diagnosis of iron deficiency

The most reliable way of diagnosing iron deficiency is the bone marrow histopathology; however, this is an invasive procedure that is not applicable to usual clinical practice (32). A wide variety of biomarkers exist for the assessment of iron status as used in a variety of clinical and population-level settings (see section 1.2.5.1). Since each indicator assesses a different aspect of iron metabolism, no single test is considered appropriate for diagnosing iron deficiency (58). In establishing the iron status of an individual, it is desirable to use multiple biomarkers that will accurately reflect iron status. These tests must be performed in addition to hemoglobin concentration. Hemoglobin determines the adequacy of the circulating red cell mass and indicates the severity of iron deficiency (38, 40, 59). Which iron indicators should be used in combination with hemoglobin, depend on the prevalence of iron deficiency in that population, available resources, diagnostic characteristics of the indicators, technical consideration, need for venipuncture and cultural practices (32, 38).

Assessment of Hemoglobin (Hb) concentration:

The concentration of the iron containing protein hemoglobin (Hb) reflects the amount of functional iron in the body (8, 33). Because changes in Hb concentration occur only at the late stage, it is considered a late indicator of iron deficiency. However, assessment of hemoglobin is considered essential for determining iron deficiency in children (33).

In children 6 to 24 months of age Hb concentration <110 g/L (ie, more than 2SDs below the mean for age and sex) indicates the presence of anemia in the body (32, 33, 38, 40). Up to the age of 5 years the same cut-off is used to diagnose anemia (32, 33). Anemia is an important indicator of health status (in individuals and populations) but the extent to which it is an appropriate indicator of iron status has many caveats. In populations where the prevalence of iron deficiency and IDA is low, and other causes
of anemia, such as hemolytic anemia, anemia of chronic disease, and anemia attributable to other nutrient deficiencies, have become proportionately more common, a simple measure of Hb is not sufficient to make a presumptive diagnosis of anemia attributable to iron deficiency (40, 60). This limitation of using Hb concentration as a measure of iron status is reflected in its lack of sensitivity to diagnose iron deficiency. The diagnostic value of <110 g/L to diagnose iron deficiency has been found to have a sensitivity of 25% and specificity of 92% in children aged 1-5 years (61). In case of detection of iron deficiency, this lack of sensitivity is largely attributable to the marked overlap in Hb concentration between populations with iron sufficiency and iron deficiency (33, 62). Thus to identify iron deficiency in its various stages, Hb concentration must be combined with other measures of iron status.

The World Health Organization (WHO), various international, regional and national health organizations, specific to the pediatric population have recommended various indicators to diagnose iron deficiency (32, 33, 63). The American Academy of Pediatrics (AAP) currently recommends universal screening for anemia with determination of hemoglobin concentration at approximately 1 year of age. However, AAP also recommends that children identified with low Hb and in high risk children, specific iron indicators should be assessed in addition to hemoglobin concentration (40). These include: serum ferritin and C-reactive protein (CRP) measurements; or reticulocyte hemoglobin (CHr) measurement. This thesis will mainly concentrate on these indicators recommended by the AAP (40).

Serum ferritin (SF):

SF has become one of the most widely used biomarkers of iron status in populations of all ages. SF concentration is an early indicator of the status of iron stores and is the most specific indicator available of normal and depleted iron stores (38). Hence, it is one of the few indicators that can be efficiently used to diagnose iron deficiency, starting from its early stages without anemia (NAID) to its most severe form, IDA (33, 38).

In adults a comprehensive review of the literature concerning the laboratory diagnosis of iron deficiency found SF radioimmunoassay to be the most powerful test with an area under the receiver
operating characteristic curve (ROC) of 0.95 using bone marrow staining as the gold standard. It also showed a SF value of ≤ 15 µg/L to have a very high likelihood ratio (LR) for diagnosing iron deficiency (LR 51.85, 95% CI: 41.53-62.27) (64). However, these diagnostic characteristics were based on the adult population and similar studies have not been conducted in children.

The laboratory diagnosis of iron deficiency in infants and children requires special attention to the use of age-specific thresholds (62). In clinical practice and in research, commonly used SF cut-off value for identifying iron deficiency in children range between <10-12 µg/L (32, 36, 39, 40). However, diagnostic thresholds for iron deficiency in children are not universally agreed upon. Furthermore, the sources of evidence for these recommendations are not well described.

The World Health Organization (WHO) in 2001 recommended a SF cut-off value of <12 µg/L for children aged less than 5 years (32). A review of the literature identified a consultation in 1987 by the International Nutritional Anaemia Consultative Group (INACG) that concluded that at all ages a SF value of less than 10-12 µg/L was indicative of a depletion of iron stores (65). Later in 1993 another consultation convened by WHO and United Nations International Children’s Emergency Fund (UNICEF), together with key partners revised these cut-offs. Separate cut-offs were provided for individuals less than five years of age and five years of age or older, for males and females, and for individuals less than five years of age with concurrent infection (63). This consultation provided the basis for the resulting 2001 WHO document - *Iron deficiency anaemia: assessment, prevention and control, a guide for programme managers*, that reported the current recommendations for SF cut-off for children, mentioned above (32). Then in 2004 a second consultation and resulting document reinforced these cut-offs and emphasized the utility of SF as an indicator of the iron status of population (66). Reviewing these documents revealed that the source of the evidence for these SF cut-offs was a review by Dallman et al. (62). Interestingly, the American Academy of Pediatrics (AAP) in 2010 recommended a SF cut-off value of 10 µg/L for the diagnosis of iron deficiency in children aged 0-3 years (40). The AAP referenced this cut-off value from the same review mentioned above by Dallman et al. (62).

In the review by Dallman et al. the characteristics of iron metabolism and nutrition in infants and children was evaluated and the application of this information to the prevention of iron deficiency was
discussed. Based on a literature review of studies investigating the distribution of SF either in the iron deficient or healthy population the authors of this review stated that “… at all ages a SF value below 10 or 12 µg/L indicates depletion of iron reserves” (67-70). Among these studies only one (Siimes et al.) included children aged 0-15 years recruited from three hospitals and a pediatric practice in California, USA (68). The aim of this study was to determine the usefulness of SF radioimmunoassay in reflecting iron stores during normal development and in the diagnosis of iron deficiency. It reported healthy children (n=486) to have a range of SF, 7 - 142 ng/ml (this is equivalent to µg/L) and children with IDA (defined by a hematocrit less than 33% and serum iron saturation less than 16%) had a range of SF, 1.5 - 9 ng/ml. This range was based on a total number of 13 children with IDA. In this study, overlap of SF with normal and iron deficient population was small and no conditions were found to give false low values (68).

While the evidence related to the diagnostic accuracy of SF cut-offs for diagnosing iron deficiency in the adult population is established, the same cannot be stated for children. Especially for the 1-3 years old pediatric group which has been shown to have the highest prevalence rate of iron deficiency in North America (40, 71). From the above evidence, it is evident that currently used cut-off of SF (<10-12 µg/L) for young children originated from a review by Dallman et al. published in 1980 and that the Siimes et al. study published in 1974 was the source of evidence for suggesting a SF cut-off of <10 µg/L for children of all ages. The samples sizes of these studies were meager and the methodologic approaches used were dated. More recently, the cut-off of SF was evaluated in a reference population of the NHANES III survey (72). In this study the fifth percentile of the distribution of SF (<10 µg/L) was selected as the threshold for defining iron deficiency. Another study (N=827) using the same approach in children aged 18 months, however, identified 12 µg/L as the cut-off for iron deficiency (73).

In conclusion, it is clear that the threshold values for SF in young children are not well delineated. These thresholds of SF were determined using population distributions of SF either in the iron deficient or in the healthy population. Considering the effect of iron deficiency on children’s neurodevelopment, it is imperative to identify thresholds for SF that is associated with important clinical impact in young children. Furthermore, the diagnostic accuracy of these thresholds need to be
evaluated in this population (12-36 months) using modern research methods such as Receiver Operating Characteristics (ROC) analysis (74).

C-reactive protein (CRP):

Despite the diagnostic and methodological benefits associated with assessing SF in diagnosing iron deficiency, it is also subject to certain errors. Because SF is an acute-phase reactant, concentration of SF may be elevated in the presence of chronic inflammation, infection, malignancy or liver disease (40). Hence, combining SF concentration with determination of another acute-phase reactant such as C-reactive protein (CRP) or alpha 1-acid glycoprotein has been suggested (40, 75). CRP values ranging between 10-30 mg/L invalidate the use of serum ferritin to diagnose iron deficiency (9). Also in times of infection, SF value of <30µg/L has been suggested as the cut-off value for iron deficiency (38).

Reticulocyte hemoglobin content (CHr):

The reticulocyte hemoglobin content (CHr) provides an indirect measure of the functional iron available for new red blood cell production. It is the product of cellular volume and cellular hemoglobin concentration (76). CHr is measured during reticulocyte analysis by automated hematology analyzers. Measures of reticulocyte indices, such as CHr, have been found to be sensitive indicators of early iron deficient erythropoiesis because of the 4-day life span of a reticulocyte (76).

The CHr assay has been validated in children and standard values have been determined (40). Two studies have examined the clinical utility of CHr in pediatric patients with comparison to other markers. The optimal CHr concentration cut-off value for the diagnosis of iron deficiency differed slightly in these two studies at 26.0 pg (sensitivity 70%, and specificity 78%) and 27.5 pg (sensitivity 83%, and specificity 72%) (77, 78). In one study CHr with a cut-off value of 26.0 pg was found to be the strongest predictor of iron deficiency in children (mean age of 2.9 years) compared to serum ferritin, MCV, MCH, RWD and Zn protoporphyrin (77). This same study also compared the receiver operating characteristic curves (ROC) for CHr and serum ferritin in the diagnosis of iron deficiency, and found the area under the curve (AUC) was significantly greater for CHr than for ferritin (77).
Unlike serum ferritin, CHr concentration is not affected by inflammation (infection), malignancy, anemia of chronic disease or any other causes of acute-phase reaction (40, 79). Hence, this biomarker of iron status can properly identify functional iron deficiency and diagnose iron deficiency in the presence of an acute-phase response (79). Despite the advantages associated with using CHr as the biomarker of choice for diagnosing iron deficiency, it is not widely available and its assessment requires specific automated hematology analyzers (40, 76).

Therapeutic trial:

In 2004, a WHO and US Centre for Disease Control (CDC) joint consultation recommended that the most efficient indicators to assess the iron status of populations are those that can detect change in iron status of a population in response to iron interventions, using the fewest and simplest tests (57, 80). In 2010 the AAP recommendations included a therapeutic trial with iron supplementation as an approach to diagnosing iron deficiency in children (40). Studies have shown that an increase in Hb concentration of 10g/L after 1 month of therapeutic supplementation to signify the presence of IDA (40, 81, 82). A therapeutic trial can be particularly informative in randomized controlled trials when both placebo and iron treatment groups are used (62). A comprehensive review of nine randomized trials depicted hemoglobin and SF to be the most efficient combination of indicators for monitoring change in iron status of a population (57). Furthermore, CHr has been shown to have an early response to iron therapy and can be used to measure its effectiveness (83).

Differentiation between NAID and IDA using iron indicators:

For this thesis the diagnosis of NAID followed the same principle as diagnosing iron deficiency, that is, using multiple indicators in addition to assessing Hb (38, 40). For my thesis, NAID is defined as low serum ferritin (≤12 µg/L) level with CRP < 10 mg/L and a normal hemoglobin level (≥110 g/L), whereas IDA is defined as, both a low serum ferritin (≤12 µg/L) with CRP < 10 mg/L and low hemoglobin level (<110 g/L).
1) NAID (non-anemic iron deficiency): hemoglobin $\geq 110$ g/L plus serum ferritin $\leq 12$ µg/L with CRP $< 10$ mg/L

2) IDA (Iron deficiency anemia): hemoglobin $< 110$ g/L plus serum ferritin $\leq 12$ µg/L with CRP $< 10$ mg/L

3) IS (Iron sufficiency): hemoglobin $\geq 110$ g/L plus serum ferritin $> 12$ µg/L with CRP $< 10$ mg/L

1.2.8 Prevention and treatment of iron deficiency

1.2.8.1 Prevention of iron deficiency

The problem of iron deficiency can be addressed through primary prevention efforts or through secondary prevention efforts of screening, early detection and subsequent therapy (33). World Health Organization sponsored and national guidelines in various countries have been developed to prevent and treat iron deficiency in both developed and developing nations (32, 84). For the current thesis we will mainly focus on those created for children of developed countries. Among these the guidelines sponsored by the American Academy of Pediatrics is one that has played an instrumental role in the prevention and treatment of iron deficiency in children of United States and has been considered in the development of guidelines by other developed countries (40). Recently another review was sponsored by the US Preventive Services Task Force (USPSTF) which investigated the benefits and harms of routine iron supplementation and screening for IDA in children aged 6-24 months (85). The following sections will describe the findings from these reviews.

Primary prevention of iron deficiency:

The AAP guideline for primary prevention of iron deficiency and IDA in toddlers (1-3 years) hinges on healthy feeding practices with consumption of naturally iron- rich food and foods that aid in iron absorption and avoid foods that reduce iron absorption (40). Given the possible adverse effects of iron in certain children, universal food fortification has not been recommended as an option to prevent iron deficiency in this age group. An alternative for toddlers who do not eat adequate amounts of iron-
containing food is iron supplementation in the form of iron sulfate drops and chewable iron tablets or as a component of either liquid or chewable multivitamins (40). However, supplementation with therapeutic iron has many barriers that need to be overcome before this intervention can be implemented to prevent iron deficiency in young children (40). Other nutrition interventions that were not recommended in the AAP guideline, but have been recommended by other groups for primary prevention of iron deficiency in toddlers include restricting cow’s milk consumption, iron supplementation of infants breastfed beyond 12 months, and avoiding excessive juice intake by young children (33, 56).

The review sponsored by USPSTF, identified some improvements in hematological values (incidence of iron deficiency and changes in SF level) and limited evidence indicated no benefit in neurodevelopmental test scores with routine iron supplementation (as oral iron drops, iron-fortified formula and as iron-fortified milk, foods or meat) in children aged 6-24 months (85). Studies that reported a benefit in hematological values (a total of 7 trials), found higher rates of iron deficiency in the control group, suggesting baseline risk to be important in determining who will benefit from supplementation. Harms of routine iron supplementation in children were rarely reported and supplementation did not result in higher rates in studies reporting harms. However, majority of these studies were conducted in developing countries (85).

Secondary prevention of iron deficiency:

Secondary prevention involves screening for iron deficiency followed by treatment (33). The AAP has concluded that universal screening for anemia should be performed with determination of hemoglobin concentration at approximately 1 year of age (40). Universal screening should also include an assessment of risk factors associated with iron deficiency/IDA: prematurity, low birth weight, exposure to lead, exclusive breastfeeding beyond 4 months of age without supplemental iron, weaning to whole milk or complementary foods that do not include iron-fortified cereals or foods naturally rich in iron, feeding problems, poor growth, and low socioeconomic status. Selective screening should be performed at any age when these risk factors for iron deficiency and IDA have been identified (40). Children identified with low Hb (<110 mg/L) or significant risk factors are recommended to be further tested with laboratory measures of iron status using serum ferritin with CRP, or CHr in addition to Hb concentration. Children with positive screening should be treated for iron deficiency with effective iron
interventions (86). The AAP does not recommend inclusion of iron specific biomarkers in the screening of iron deficiency/IDA. Hence, the AAP does not recommend screening for NAID, the early latent stage of iron deficiency. Nor does the AAP recommend which interventions are most effective in treating iron deficiency after screening.

The USPSTF review was commissioned to update the previous recommendation by USPSTF (2006) for the prevention of iron deficiency in children aged 6 to 24 months in the US and for populations relevant to the US (87). In this systematic review of evidence, no randomized controlled trials evaluating the benefits or harms of screening programs for children aged 6-24 months was identified (85). The conclusion of this review stated that high quality research is needed to assess the benefits and harms of both routine iron supplementation and screening to prevent IDA in young children in developed countries.

Challenges associated with screening for iron deficiency in young children:

Screening for iron deficiency presents some substantial challenges (86). The first challenge is the limitation of hemoglobin as the optimal screening test for detecting iron deficiency (33, 40, 56, 86). This approach does not fulfil several of the principles for effective screening programs presented by World Health Organization (84, 88). These principles include the ability to recognize the latent or early symptomatic stage. Therefore, for iron deficiency, the ideal screening test should be capable of identifying iron deficiency in the pre-anemic stage (e.g. NAID stage), thus preventing the progression to IDA and its associated mental, motor and behavioral effects (56). Most studies and programs have relied on hemoglobin as an index of iron status. Screening for anemia with hemoglobin determination neither identifies children with NAID nor specifically identifies those with IDA (40). Furthermore, in the developed world and where the bulk of the burden of anemia is due to causes other than iron deficiency, anemia assessment as a diagnostic test for iron deficiency has been called into question (84). The second challenge to screening iron deficiency relate to diagnostic follow-up monitoring. Evidence has found there is poor follow-up testing and poor documentation of improved hemoglobin concentration after screening positive for iron deficiency (86). Steps to improve this approach include the implementation of technology-based reminders for screening and follow-up of toddlers with a diagnosis of iron deficiency or IDA (40). Another challenge relates to timing of screening. The
prevention of iron deficiency has focused on the first 12 months of life. Current AAP guideline recommends universal screening for iron deficiency at 12 months of age (40). At 1 year, breastfeeding or iron-fortified formula is often replaced with cow’s milk, non-fortified cereals and juices. Evidence shows 1-3 years olds have the lowest daily iron intake of any age group across the life course (89). Two large-scale studies, the Third National Health and Nutrition Examination Survey and the Third Report on Nutrition Monitoring in the United States, reported the prevalence of iron deficiency anemia in 1-2 years old to be 3%, and in 1-3 years olds to be 15% (56, 72). Hence, the optimal timing to detect iron deficiency in toddlers need to be further investigated.

1.2.8.2 Treatment of iron deficiency

There are three main methods for treating iron deficiency: therapeutic iron supplementation, education combined with dietary modification or diversification, and fortification of foods with iron (9). Based on consensus and evidence these interventions have been recommended in various national and international iron deficiency treatment guidelines (32, 33, 40). However, the efficacy and effectiveness of these nutrition interventions need to be understood in order to develop a comprehensive program to control iron deficiency that includes an appropriate mix of interventions designed to best address local conditions (90, 91).

Supplementation with therapeutic iron:

Therapeutic supplementation aims at correcting established iron deficiency (32). Global guidelines for iron supplementation published by the Nutritional Anemia Consultative Group/World Health Organization/UNICEF provide guidelines for iron supplementation both to prevent and to treat iron deficiency (91). In the case of prevention the dose of iron recommended for children 6-24 months and 24-60 months of age is 12.5 mg/d elemental iron and 20-50 mg/d elemental iron respectively. In the case of treatment of established iron deficiency in children < 2 years, 25mg of elemental iron for 3 months have been recommended (91, 92). Older children can be given a powder or crushable tablet, whereas, children younger than 2 years need a liquid supplement that can be dropped into their month. The most common form of iron that children are supplemented with is ferrous sulfate; however, fumarate and gluconate are also used. These compounds have low cost and have high bioavailability
Supplemental iron as drops has been recommended for pre-term infants who are fed breast milk and those who have been diagnosed with IDA (33, 40). Evidence has shown that therapeutic iron in prescribed doses to be efficacious and safe for infants and children (see below). However, there is a lack of data on whether non-anemic stages of iron deficiency (i.e. NAID) should be treated with therapeutic iron.

Evidence of the efficacy of therapeutic iron in treating iron deficiency:

(This evidence is described in detail in chapter 2 and 3, please refer to these chapters)

Education combined with dietary modification or diversification:

Health education informs mothers/caregivers regarding ways to increase dietary consumption of iron through the consumption of iron-rich foods and foods that increase absorption of iron and avoid consumption of food that decrease iron absorption (9, 93). The use of dietary guidelines has become a universal component of all nutrition education programs (92). Dietary guidelines can direct health educators on what specific elements of iron nutrition need to be disseminated. These guidelines usually reflect the iron deficiency management and prevention guidelines followed by a particular country or community. They can be sponsored and endorsed by national health organizations for nation-wide use and distribution or simply developed by local health professionals and researchers to provide community based health education. Furthermore, these guidelines may not only provide recommendations specific to iron, but also deliver recommendations for the overall improvement of nutrition in children (example: Canada’s Food Guide) (94).

Although the reduction of iron deficiency through health education is the most sustainable approach, change of dietary practices and preferences is difficult and foods that provide highly bioavailable iron are expensive (9). Small-scale health education interventions have been successful in the UK where dietary education to treat iron deficiency was introduced (95, 96). A study in pre-school children reported a fall in the prevalence of IDA from 25% to 8% in 2 years due to implementation of health education on the importance of dietary iron and the consequences of iron deficiency (95). Larger programs have not been that successful. A study of dietary intake of pre-school-aged children showed
that those who were provided supplemental iron and nutrition education through the Women Infant and Child (WIC) program in United States had significantly higher intakes of energy, ascorbic acid and iron than nonparticipants. However, there was no residual long-term impact on dietary intake resulting from earlier enrollment in the program (92, 97).

There is an urgent need to test the efficacy of dietary strategies in children with NAID in developed countries. Currently there is an absence of data on the effectiveness of dietary education in children with NAID, especially in developed countries. However, a randomized trial in adult women showed that an intensive dietary program has the potential to improve the iron status of non-anemic iron deficient British women [serum ferritin increased in the diet group by 26% (p = 0.068) in comparison to the placebo group] (98).

Fortification of foods with iron:

The reduction in iron deficiency in infants in developed countries has been attributed to iron fortification of infant formulas and weaning foods (infant cereal) (9, 99). Three British studies have shown iron deficiency was less common in those infants (>6 months to 24 months) receiving an iron-fortified formula, more common in those receiving an unfortified formula and most common in those on pasteurized cow’s milk (100-102). Despite beneficial impact of fortification on iron deficiency in infants in the first year of life, universal food fortification for all age groups is problematic in developed country settings, given the possible adverse effects of iron in certain sub-sets of children (103, 104). Furthermore, consumption of a diverse diet by toddlers restricts them from eating enough of any one type of food that can serve as a vehicle for iron fortification (40).

1.2.9 The Canadian context

In Canada, there are no national data of iron deficiency in infants and toddlers (71). Several small studies targeting different Canadian population groups have shown the prevalence of iron deficiency to range from approximately 12% to 64% (71). Certain Canadian Aboriginal populations have very high prevalence of iron deficiency (14% to 50%) (105). Other risk factors include low socioeconomic status, low birth weight, and excessive cow’s milk intake (71). The Canadian Health Measures Survey (CHMS) provides population-representative national data on many important public health indicators.
In 2012 they updated estimates of the iron status of Canadians. Although CHMS primarily reported on prevalence rates associated with iron sufficiency of Canadians, they also reported prevalence of deficient hemoglobin (<110 g/L, 0.5%) and low serum ferritin (<12 µg/L, 3.2%) separately for 487 children 3 to 5 years of age (107). CHMS did not collect data on children under 3 years, the group that has been found to have greatest risk of developing iron deficiency. Hence, the prevalence associated with iron deficiency in this younger age group was not reported.

Guidelines for screening for iron deficiency in the Canadian pediatric population are dated. The Canadian Task Force on Preventive Health Care concluded almost 20 years ago that there was insufficient evidence to recommend screening for infants between 6 and 12 months of age (108). However, for all infants in high-risk groups, physicians were recommended to consider screening between 6 and 12 months of age. However, groups of urban Canadian infants who were considered to be normal risk had a prevalence of IDA ranging between 1.5% and 8.4%, which is over the limit for labeling iron deficiency as a public health problem in a specific country (32, 109). Furthermore, evidence from a large group of children aged 12-59 months in Alberta found the peak age for IDA was 19 months, with prevalence of 7.69% (110). Hence, the appropriate age at which screening for iron deficiency should be recommended requires further investigation.

A 2013 joint statement by Health Canada, Canadian Paediatric Society, Dietitians of Canada and Breastfeeding Committee for Canada provides nutritional guidelines for healthy, term older infants and young children aged 6 to 24 months of age (111). With respect to iron, these guidelines follow Canada’s Food Guide recommendations and include a few food based recommendations targeting the prevention of iron deficiency in children (111, 112). These recommendations include –

- Continue to recommend a variety of iron-rich foods. Ensure that foods such as meat and meat alternatives and iron-fortified cereal are offered a few times each day.

- If parents and caregivers introduce cow’s milk, advise them to delay until 9 to 12 months of age. Recommend limiting cow’s milk intake to no more than 750 ml per day.
In spite of recommendations by the WHO, Canada does not have a national strategy to address infant and childhood iron deficiency (113). However, these strategies can only be recommended when appropriate evidence regarding the prevalence, risk factors and the efficacy of interventions for treating and preventing iron deficiency in children are available. An effective surveillance program is essential for gathering evidence on any child health and nutrition related disorders (114). Surveillance of iron deficiency would require an ongoing process of recording and assessing iron status in an individual or a community (32).
Chapter 2: Current evidence on outcomes associated with IDA

The purposes of this chapter are to:

1. Describe the various outcomes associated with iron deficiency in young children
2. Describe the mechanism of how iron deficiency affects child neurodevelopment (pathophysiology)
3. Review the evidence of the efficacy of therapeutic iron supplementation in children aged 1-5 years identified with IDA
4. Review the evidence to show the association between IDA and child’s neuro-developmental, hematological outcomes and adverse effects
2.1 Outcomes associated with iron deficiency in children

Iron deficiency is a systemic condition that adversely affects the cognitive performance, behavior and hematological outcomes of children. It also depresses immune function that increases morbidity from infection in children of all age groups (32, 115). Other long term effects of iron deficiency include physical capacity and work performance and the negative consequences of impaired mental development on human capital formation (32, 116). Adverse outcomes associated with iron deficiency are dependent on its stage and severity. The different stages of iron depletion lead to an incremental involvement of different systems in the body (117). It is now recognized that even without anemia, mild to moderate iron deficiency has adverse functional consequences (35, 40, 118). Despite this understanding, the majority of the evidence is focused on the most severe stage of iron deficiency, IDA (119). However, to have a comprehensive understanding of the effects of iron deficiency, the spectrum of physiological changes that occur at different stages of iron depletion need to be elucidated.

2.2 How iron deficiency affects child development (the mechanistic link)

At the cellular level iron is involved in a variety of processes and its deficiency affects virtually every organ system, including the nervous system. Neurological problems implicated with deficiency of iron in children include developmental abnormalities, ischemic stroke, venous thrombosis and breath-holding episodes. (120).

Iron is abundant in the brain. It is distributed unevenly in both gray and white matter of the nervous system. It is most commonly located in oligodendrocytes of human brains. Oligodendrocytes are the myelin-producing cells in the brain. Iron is required for myelin production as a cofactor for cholesterol and lipid biosynthesis. In addition to myelin production; it has been proposed that oligodendrocytes have the additional function of storing and mobilizing iron in the central nervous system of humans. Iron deficiency causes delayed myelination and hypomyelination through the direct effect of decreased
synthesis of nervonic acid (120-122). Delayed myelination and/or hypomyelination may cause irreversible cognitive and motor impairment in children (123).

Decreased brain iron stores may impair the activity of iron-dependent enzymes necessary for the synthesis, function and degradation of neurotransmitters, such as dopamine, serotonin, GABA (gama-aminobutyric acid), phenylalanine and noradrenaline. (120, 122). As a consequence, longer central conductive time (CCT), indicating slower nerve conduction velocity, was found at age 12 and 18 months in children who were identified with IDA at 6 months of age. This implied that the effect of iron deficiency on biological neural functioning may be irreversible (124).

### 2.3 Evidence of the efficacy of therapeutic iron in treating iron deficiency

A Cochrane systematic review with meta-analysis was performed to investigate the efficacy of therapeutic iron to improve neurodevelopment in children under the age of three. Initially published in 2001, this review was updated in 2013, however, the conclusion remained the same and no new trials were identified (125, 126). No convincing evidence of beneficial effect was identified of short term iron therapy (≤30 days) on the mental and psychomotor development of children [Bayley Scale, Psychomotor Development Index (PDI) between iron treated and placebo groups, OR -1.25 (95% CI, -4.56 to 2.06) and in Mental Development Index (MDI), OR 1.04 (95%CI, -1.30 to 3.39)] in five randomized trials (126). Whereas, the effect of long term iron therapy (>30 days) in two randomized trials remain unclear. An extremely large effect was identified from one randomized trial [Bayley Scale PDI, between iron treated and placebo groups, OR 18.40 (95% CI, 10.16, 26.64) and in MDI, OR 18.80 (95% CI, 10.17, 27.43] (127). The other trial used a screening tool rather than a measure for the assessment of psychomotor development, and did not depict substantial beneficial effect on the psychomotor development of children (128). Due to these mixed results, the conclusion of the review was that there is no definite evidence to suggest that treatment of IDA with therapeutic iron will result in positive changes in cognitive function and an urgent need for large randomized controlled trials of iron therapy in young children with iron deficiency was declared (126). Very similar evidence related to the efficacy of therapeutic iron on children’s neurodevelopment has been depicted in another systematic review with iron deficient children aged between 24 and 60 months (129). This review also assessed the efficacy of daily iron supplementation on the hematological outcomes in children which
showed a significant increase in Hb (p<.00001) and SF (p<.0001) level. A small benefit [294 subjects, SMD = 0.25 (95% CI 0.06-0.45, p = 0.01] from iron on cognitive outcomes was identified, although the combination of the different scales limited the validity of the meta-analysis (129). This review also highlighted a lack of data on the effect of iron supplementation on clinically important pediatric outcomes and has addressed an immediate need for well conducted and clearly reported interventional studies. A systematic review in low birth weight/premature infants also found significant hematological effects of therapeutic iron supplementation. Similar to previous reviews, due to insufficient evidence on children’s neurodevelopment no definitive recommendation was made and the need for large scale randomized trials was reiterated (130). One limitation of this review was that the baseline iron status of all children was not reported; in most trials iron was provided on a prophylactic basis.

Evidence from the above reviews suggests a benefit of daily iron supplementation on Hb and iron stores in 2- to 5-year-old children. A small benefit from iron on cognitive parameters is suggested, although the number of studies is small. The evidence also highlights a concerning lack of high quality data on the effect of iron supplementation on the clinically important outcomes of anemia, iron deficiency and cognitive development in preschool children in the developed country setting. There remains a need for well conducted and clearly reported interventional studies in this age group that evaluate important clinical and functional outcomes, side effects, and adherence.

### 2.4 Association between IDA and child development

The highest level of evidence to suggest an association between IDA and various outcomes in children can be identified from reports of randomized controlled trials or systematic reviews that evaluate the efficacy of iron interventions (131). Several extensive reviews have been published on the association between IDA and child development (125, 132, 133). These reviews suggest a significant association between IDA and poor child development. However, whether this condition is reversible by treatment with iron has been inconclusive. Of 6 randomized controlled trials in children less than two years, only one showed a significant impact. Of eight double-blinded randomized controlled trials of iron therapy in children older than two years, four reported a significant association between IDA and child development.
development (132). However, many of these studies suffered from lack of statistical power and very few trials followed the children after the treatment had stopped (125, 132). It is possible that the relationship between iron deficiency and development is confounded by other factors like low socio-economic status, poor maternal education, low birth weight, early weaning and parasitic infection (37, 41, 134-136). Hence the impact of iron deficiency on child development is either irreversible or cofounded by factors mentioned above.

Furthermore, Lozoff and colleagues have extensively studied the neurodevelopment of iron deficient children from infancy through a longitudinal study conducted in Costa Rica (137). They assessed the development of children identified with IDA during infancy at multiple time points, up to 25 years (134, 138-140). The significant developmental difference between children with IDA and iron sufficiency persisted throughout the longitudinal study and at 25 years, those with IDA in infancy had substantially higher rates of high-school non-completion and poor emotional health (140). This evidence suggests that children having IDA in early childhood may be associated with developmental adversity in later life.

### 2.5 Association between IDA and children’s hematological outcomes

In most trials hemoglobin (Hb) and serum ferritin (SF) have been used as the indicators to identify iron deficiency and also as outcomes to determine the effect of interventions. Other hematological outcomes include the incidence of iron deficiency, IDA and/or anemia. Important systematic reviews such as the Cochrane review (Logan et al. and Wang et al.) and the review by Sachdev et al. despite using these indicators to define iron deficiency and IDA, did not investigate the effect of iron interventions on these hematological outcomes (125, 126, 133). More recent reviews investigating the efficacy of iron interventions have found children aged 2-5 years receiving iron supplementation had a mean Hb of 6.97 g/L (9 studies; 2154 subjects; 95% CI: 4.21,9.72; p<00001) greater than controls, whereas mean SF was 11.64 mg/L (5 studies; 1407 subjects; 95% CI: 6.02,17.25; p<0001) greater, thus indicating significant association (129). In these trials children who were identified as anemic or iron deficient at baseline resulted in a greater improvement in Hb and SF level. Similar effects of iron supplementation on hematological outcomes (Hb and SF) were reported in two other systematic
reviews where the effect of iron supplementation was investigated in low birth weight infants and school-aged children (130, 141). Unlike the review investigating children 2-5 years of age, randomized trials included in the latter two reviews reported significant reduction in the incidence of IDA as a result of treatment with iron.

2.6 Adverse effects associated with iron supplementation

Side effects associated with therapeutic iron supplementation in children include constipation, nausea, vomiting, diarrhea, black stool and staining of teeth (142). The frequency of these side-effects is directly related to the dose of iron (142). Furthermore, there is no evidence demonstrating that side effects are the major cause of non-adherence in oral iron supplementation programs. However, most of these trials were performed in developing countries (129, 141). Concerns have also been raised in developing countries about a possible association with increased infections and iron supplementation (such as malaria, respiratory infection, diarrhea); however trial evidence has not supported this relationship (129, 141, 143).

2.7 Conclusion:

The above evidence clearly presents an association between IDA and children’s developmental and hematological status. The current controversy regarding iron deficiency in children, however, relates to the degree of iron deficiency that impairs child development and the efficacy of iron therapy in correcting developmental deficits (132). In the next chapter, I present the evidence on the association between early stages of iron deficiency (NAID) and children’s hematological and developmental status through the investigation of the efficacy of iron intervention on these important outcomes.
Chapter 3: Efficacy of oral iron therapy in improving the developmental outcome of pre-school children with NAID: a systematic review

The purposes of this chapter are to:

1. Describe a systematic review of randomized controlled trials investigating the association between NAID and children’s neuro-development and hematological outcomes through the evaluation of the efficacy of therapeutic iron supplementation in children aged 1-5 years.

Published in manuscript form:

This evidence synthesis was performed as part of course work (HAD5308H: Evidence Synthesis: Systematic Reviews and Meta-Analysis) performed by the candidate and is apart from the original research performed for this thesis.
3.1 Abstract

Objective: To systematically review the efficacy and safety of oral iron therapy in pre-school children (1–5 years) with NAID, determined by children’s developmental and hematological status and the incidence of reported side-effects.

Design: A random-effects model was used to show mean differences with 95 % confidence intervals of developmental and hematological scores between iron-treated and non-treated groups.

Setting: MEDLINE, EMBASE, Cochrane library and bibliographies of identified articles were searched up to September 2011. Randomized and observational studies were assessed by two reviewers independently. Quality of the trials was assessed on the basis of concealment of allocation, method of randomization, masking of outcome assessment and completeness of follow-up.

Subjects: From the titles of 743 articles, full text review was completed on forty-six and two randomized trials of acceptable quality met the inclusion criteria. The two trials included a total of sixty-nine children.

Results: One study showed a statistically significant difference in the post-treatment Mental Developmental Index score among children who received oral iron therapy v. no therapy (mean difference = 6.3, 95 % CI 1.5, 11.0, P value not provided). Both studies showed significant improvement in SF level (mg/l: mean difference = 51.1, 95 % CI 33.6, 68.6, P <0.01 and mean difference = 17.1, 95 % CI 7.5, 26.6, P value not provided, respectively) in children who received iron therapy.

Conclusions: Evidence is insufficient to recommend oral iron therapy to children with NAID. There is urgent need of conducting adequately powered, randomized trials examining the efficacy of oral iron therapy in pre-school children with NAID.
3.2 Background

Iron deficiency is the most common and widespread nutritional disorder in the world (144). Iron deficiency represents a spectrum ranging from non-anemic iron deficiency (NAID: normal Hb, low iron status) to iron deficiency with anemia (IDA: low Hb, low iron status). Because iron is involved in many central nervous system processes, its deficiency may adversely affect the cognitive performance and motor development in children (32, 120, 145).

Summarized in two systematic reviews, numerous studies have established a relationship between IDA and poor cognitive and motor development in infants and children (125, 132). However, there is conflicting evidence as to whether this delay can be reversed following treatment with oral iron. Most randomized trials have shown that children treated with the recommended dose and duration of iron have corrected anemia but biochemical evidence of iron deficiency as well as poor cognitive and motor development persist (137, 146, 147). These findings suggest that iron deficiency, when it reaches its most severe stage, may have irreversible effects.

Mild to moderate iron deficiency has also been associated with adverse developmental consequences. Observational studies suggest that, compared with children who are iron sufficient, children with NAID tend to have lower developmental scores, verbal competency, comprehension and intelligence quotient (146, 148, 149). However, studies focusing on the efficacy of iron therapy in children with IDA and also including children with NAID and iron sufficiency as comparison groups did not show developmental difference between the latter two groups (137). Thus, the severity of iron deficiency that may impact the development of children remains unknown. Current WHO and American Academy of Pediatrics guidelines do not recommend screening for NAID (32, 40). Therefore, children with this condition are less likely to be identified and treated. This further adds to the insufficient evidence base relating NAID as a cause of poor development in children and the efficacy of iron therapy in this population. Furthermore, it is possible that the relationship between iron deficiency and development is confounded by other factors like low socio-economic factors, poor maternal education, low birth weight, early weaning and parasitic infection (134, 136, 150, 151).
Oral iron is the treatment of choice for IDA because of its effectiveness, safety and cost-effectiveness (152, 153). When taking iron preparations orally, side-effects occur occasionally in the form of staining of teeth, vomiting, heartburn, darkening of stools, constipation or loose stools (154, 155). Rates of non-compliance attributed to side effects range from 0 % to 6 % (156).

The current controversy regarding iron deficiency in children relates to the degree of iron deficiency that impairs child development and the efficacy of iron therapy in correcting developmental deficits (157). The overall aim of the present evidence synthesis was to determine whether there is enough evidence to suggest that NAID is causally associated with poor development in children and whether oral iron therapy is effective in improving development in pre-school children with NAID. Considering the irreversible developmental impact of IDA, we hope that this systematic review will focus attention on NAID, the early stage of iron deficiency, in order to build an evidence base for screening and treatment of NAID to prevent progression to IDA.

3.3 Methods

3.3.1 Criteria for considering studies for review

Types of studies
Randomized or quasi-randomized controlled trials and observational studies with prospective longitudinal design were considered for the current review. Separate meta-analysis for observational studies and randomized controlled trials were planned to avoid methodological heterogeneity.

Types of participants
The participants were iron deficient (SF <12mg/l) but non-anemic (Hb >110 g/l) children who were otherwise healthy and aged 1–5 years. Studies on children with developmental disorders, chronic disease, congenital or genetic disorder and iron deficiency were excluded.

Types of interventions
The dose and duration of iron therapy have been established for IDA but not for NAID. According to guidelines for the prevention of IDA and the opinion of experts, it was decided to select studies with a
minimal dose of 2 mg elemental iron/kg body weight per d once daily, administered for a minimum duration of 3 months (32, 35). The treatment group included children who received oral iron therapy (≥2mg elemental iron/kg body weight per day, administered for ≥3 months) with or without other interventions aimed at improving iron level (such as dietary counselling, vitamin C, folic acid). The control group included children receiving placebo or no treatment.

Types of outcome measures
Primary outcome for the current review was the change or the end-of-study scores of children’s development, measured using any standardized scale that can be converted to standard scores expressed by mean and standard deviation. Bayley’s Scale of Infant Development (BSID) is one such scale which has a population mean of 100 and an SD of 15 (158). Secondary outcomes included the change or the end-of-study levels of Hb and SF. Safety outcomes included the rate of any reported side-effects of Fe treatment including vomiting, heartburn, constipation, loose stools, staining of teeth and darkening of stools.

3.3.2 Search methods for identification of studies

A comprehensive search strategy was performed to identify all relevant studies, including searching the electronic literature and hand searching. We searched the following electronic databases and updated results as of 13 January 2011: MEDLINE (1950 to the present), EMBASE (1980–2011 Week 1) and the Cochrane Controlled Trials Register (CCTR; Cochrane Library issue 4, 2010). We scanned reference lists of identified trials and important review articles for published studies which may have been missed by the literature search. We included only published data in English language. However, we planned to contact key authors of those articles where we expected to find data not published but relevant to our review.

Search terms
Under the supervision of an expert librarian (E.U.), the following key search terms were used: ‘anemia/hypochromic anemia’, ‘iron/blood’, ‘iron-deficiency’ and ‘ferritin’ combined with concepts of child development. Appropriate truncations and possible misspellings were included. Where appropriate, a trials search filter was applied. The described search strategy (detailed search strategy
shown in the Appendix) was used for MEDLINE. For use with EMBASE and the Cochrane Library this strategy was adapted slightly.

3.3.3 Data collection and analysis

Selection of studies
Titles and abstracts of studies identified on searches of electronic databases were read to determine whether they might meet the inclusion criteria. Full copies of those possibly meeting these criteria were assessed by two independent reviewers (K.A., T.K.). Studies that met the inclusion criteria were again reviewed by the same reviewers for quality assessment. Differences of opinion about inclusion of studies and quality assessment of studies were resolved by discussion. Arbitration by a third reviewer (P.C.P.) was kept open.

Assessment of risk of bias in included studies
For randomized controlled trials, two reviewers independently assessed the methodological quality of the studies using a predefined checklist, as suggested for the Cochrane Database of Systematic Reviews (159). We performed an overall assessment of risk of bias, based on responses from criteria used to assess the quality of the studies. Studies that reached A or B score were intended to be included in the meta-analysis. For studies with longitudinal designs we intended to score the quality of the studies using the Newcastle–Ottawa Scale (NOS) (160). We planned to include studies that had at least one star in each category of the scale. Assessment was intended to be carried out independently by two reviewers.

Data extraction and synthesis
We planned to use the RevMan software version 5.0 to generate meta-analyses and show summarized effect size if appropriate data were available. Data regarding the stated outcomes of the review [standardized mean difference (SMD) or mean difference (MD) with 95 % CI for continuous outcomes and relative risk (RR) with 95 % CI for binary outcomes] were extracted and entered into the RevMan software. The original data were not modified but for the purpose of data synthesis for meta-analysis, calculations were required from available data. Pooled results were to be presented as forest plots. Statistical heterogeneity was assessed using the Cochran Q test and by calculating I² values. We
expected methodological, clinical and statistical heterogeneity among the studies. Thus, we intended to use the random-effect model for meta-analyses. Subgroup analysis for children aged <2 and ≥2 years was planned. The robustness of the results were intended to be checked by performing sensitivity analysis showing the influence of study quality as well as the influence of large-scale studies. In the case of duplicate publications and companion papers of a primary study, the original publication (usually the oldest version) obtained priority. We intended to assess publication bias using the funnel plot method.

3.4 Results

3.4.1 Literature search

Excluding duplicates, 743 articles were identified in first scan; 697 were excluded after reading the titles and abstract (Figure 5). Full text review was completed on forty six articles and we identified two randomized clinical trials of oral iron treatment in which children with NAID were randomized to a treatment or control group (127, 161). For both studies, the primary objective was to study children with IDA; however, both included children with NAID as a comparison group, and these data were available for the review. No studies with observational design met the inclusion criteria. The reason for exclusion of forty-four studies is reported in appendix 1.
3.4.2 Study participants and interventions

The baseline characteristics of the children in the two included studies and the dosage and duration of oral iron treatment are reported in Table 5. Both studies used the same inclusion criteria: birth weight greater than 2500 g; singleton; no major congenital anomalies; no jaundice treated by phototherapy; no hospital admission or supplementation with micronutrients during the 6 months before enrollment; no
clinically identified neuromotor delay; no chronic illness or folic acid deficiency; no signs of abnormal haemoglobinopathy or thalassemia; and weight, length and head circumference within ±2SD of reference standard.
<table>
<thead>
<tr>
<th>Author, date, country</th>
<th>Study design</th>
<th>Participants</th>
<th>Exclusion criteria</th>
<th>Interventions, dosage, duration</th>
<th>Outcome measures</th>
<th>Side effects</th>
<th>Adjusted co-variates</th>
<th>Dropout</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Akman et al., 2004, Turkey</td>
<td>Single-blind, randomized, controlled trial</td>
<td>N total = 108, children aged 6–30 month. NAID (n = 40). n = 21 (treatment) n = 19 (control)</td>
<td>Subjects with pervasive developmental disorder or severe mental and motor disability</td>
<td>Oral ferrous-glisine-sulphate therapy (3 mg/ kg, twice daily) 3-months</td>
<td>Bayley Scales of Infant Development BSID-I (MDI and PDI)</td>
<td>Not reported</td>
<td>Unclear</td>
<td>Among the total participants the parents of 4 subjects declined to participate and 2 others dropped out during follow-up</td>
<td>MDI score differences between the NAID treatment and control groups were found to be significant after 3 months of oral iron treatment. Similar findings were not found for the PDI score.</td>
</tr>
<tr>
<td>Idradinata et al., 1993, Indonesia</td>
<td>Double-blind randomized-controlled trial</td>
<td>N total =141 children aged 12 to 18 months NAID (n=29) n = 14 (treatment) n = 14 (control)</td>
<td>Infants with Hb between 105 and 120 g/L</td>
<td>Oral ferrous sulphate (3 mg/kg per day) 4 months</td>
<td>Bayley Scales of Infant Development BSID (MDI and PDI)</td>
<td>Not reported</td>
<td>Mothers’ maximum school grade</td>
<td>Among the total participants the parents of 15 infants declined to participate</td>
<td>Pre-treatment to post-treatment changes in the two intervention subgroups within the NAID were not significantly different.</td>
</tr>
</tbody>
</table>

** Both the studies had the same inclusion criteria
Akman et al. (2004, Turkey) included 108 children aged 6–30 months (average age 17 months) (28). Of these, forty (37 %) had NAID; twenty-one received oral iron treatment and nineteen received no treatment. Oral iron was given for 3 months at a dose of 3 mg elemental Fe/kg body weight per day, twice daily. The Hb and SF cut-offs were: IDA (Hb,<11 g/dl, serum ferritin ≤12mg/l, mean corpuscular volume,<70 fl) and NAID (Hb ≥11 g/dl, SF ≤12mg/l, mean corpuscular volume ≥70 fl).

Idjradinata et al. (1993, Indonesia) included 141 children aged 12–18 months (27). Of these, twenty-nine (21%) had NAID; fourteen received oral iron treatment and fifteen received no treatment. The oral iron was given for 4 months at a dose of 3 mg of elemental iron/kg body weight per day. The Hb and SF cut-offs were: IDA (Hb <105 g/l, transferrin saturation ≤10 % and SF ≤12mg/l) or NAID (Hb ≥120 g/l, transferrin saturation ≤10 % and SF ≤12mg/l). Children whose Hb level was between 105 and 120 g/l were excluded.

3.4.3 Methodological quality

The results of the assessment of the risk of bias of the two included studies are reported in Table 6. Both studies showed moderate risk of bias (B quality). Both studies provided insufficient information regarding allocation concealment. Akman et al. (2004, Turkey) was a single blind trial; mothers were not blinded because placebo was not used. Further, although the two groups differed in a number of variables, for example socio-demographic variables, it was not clear whether the analysis was adjusted for these differences. Idjradinata et al. (1993, Indonesia) did not report on the method of laboratory analysis. Both studies reported child development using the BSID. This scale reports development using two indices, the Mental Developmental Index (MDI) and the Psychomotor Developmental Index (PDI), with the standardized score having a mean of 100 and an SD of ±15.

3.4.4 Outcomes

Meta-analyses were not performed due to high level of heterogeneity (see ‘Heterogeneity and publication bias’ section) between the two studies. Considering the primary outcome, comparison of the MDI and PDI in the iron-treated and non-treated NAID groups, Akman et al. (2004, Turkey) showed a statistically significant difference in the post-treatment MDI score (MD=6.3, 95 % CI 1.5, 11.0, P value not provided); and comparison of the post treatment PDI score showed no statistically
<table>
<thead>
<tr>
<th>Author, date, country</th>
<th>Sequence generation</th>
<th>Allocation concealment</th>
<th>Blinding of participants, personnel and outcome assessors</th>
<th>Incomplete outcome data</th>
<th>Selective outcome reporting</th>
<th>Other bias</th>
<th>ABC Cochrane score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Akman et al., 2004, Turkey</td>
<td>adequate (table of random numbers)</td>
<td>unclear (no information was provided)</td>
<td>Inadequate (a trained psychologist, unaware of each child’s hematological status, administered the BSID-I to all subjects before and after the 3-mo follow-up; mothers were not blinded due to placebo not being used)</td>
<td>adequate (number and reasons for dropouts and withdrawals were described)</td>
<td>adequate (the IDA group was the main focus)</td>
<td>unclear (adjustment for other biases)</td>
<td>B - moderate risk of bias</td>
</tr>
<tr>
<td>Idjradinata et al., 1993, Indonesia</td>
<td>adequate (table of random numbers)</td>
<td>unclear (no information was provided)</td>
<td>adequate (placebo was a syrup similar in appearance to the ferrous sulphate, and both had a sweet, cherry flavour)</td>
<td>adequate (number and reasons for dropouts and withdrawals, the procedure with compliance were described)</td>
<td>adequate (the IDA group was the main focus)</td>
<td>adequate (adjustment for other biases)</td>
<td>B - moderate risk of bias</td>
</tr>
</tbody>
</table>
significant difference (MD=20.2, 95 % CI 27.0, 6.6; Figures 6 and 7). Idjradinata et al. (1993, Indonesia) showed no statistically significant difference in either the post-treatment MDI score (MD=21.6, 95 % CI 29.4, 6.2) or the post-treatment PDI score (MD=1.2, 95 % CI 26.0, 8.4; Figs 6 and 7).

Considering the secondary outcomes, namely the comparison of Hb and SF levels in the iron-treated and non-treated NAID groups, Idjradinata et al. (1993, Indonesia) reported a statistically significant increase in the post-treatment Hb level (g/l: MD=11.5, 95 % CI 5.1, 17.9, P<0.01) and the post-treatment SF level (mg/l: MD=51.1, 95 % CI 33.6, 68.6, P<0.01; Figures 8 and 9). Akman et al. (2004, Turkey) reported no statistically significant increase in post-treatment Hb level (g/l: MD=2.7, 95 % CI 21.7, 7.1); but the SF level showed a significant increase (mg/l: MD=17.1, 95 % CI 7.5, 26.6, P value not provided; Figures 8 and 9). Neither of the studies intended to report side-effects.

3.4.5 Heterogeneity and publication bias

Clinical heterogeneity between the studies is described in Table 5. Some of this heterogeneity can be explained by the difference in the range of age of the children in the two studies. The children in Akman et al. were relatively older compared with Idjradinata et al. (mean age, months: 18 (SD 6.1) v.14 (SD 0.51)). Other potential causes of heterogeneity include the exclusion criteria and duration of iron treatment (3 months v. 4 months). The drop-out rates for the two studies were approximately 6 % and 11 % (of the total participants). Neither study reported the characteristics of the children who declined to participate. Only Idjradinata et al. reported the results after adjusting for mothers’ maximum school grade and no clear information regarding adjustment for covariates was reported in Akman et al.’s study. When we attempted to combine the results of the two studies, evidence of high statistical heterogeneity was observed. The combined result for the MDI score showed a Q value of 3.51 (P=0.06) and an I² value of 72 %, indicating significant statistical heterogeneity. For the PDI score the Q value was 0.08 (P=0.8) and the I² value 0 %, indicating less heterogeneity. However we were not able to estimate the between-study variance with precision with only two studies because the π² (variability) as well as I² become 0 when Q ≤ k-1 (df =1). Due to the lack of homogeneity between the studies we decided not to show the combined effect size of the results. We are unable to comment on the publication bias issue with only two included studies.
Figure 6: Forest plot of comparison: Developmental scores of non-anemic iron deficient pre-school children on iron supplementation versus no treatment/placebo, outcome: Mental Developmental Index (MDI). Study results are not combined.

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>Mean</th>
<th>SD</th>
<th>Total</th>
<th>Mean</th>
<th>SD</th>
<th>Total</th>
<th>Mean Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Akman 2004</td>
<td>101.52</td>
<td>8.76</td>
<td>21</td>
<td>95.26</td>
<td>6.4</td>
<td>19</td>
<td>6.26 [1.54, 10.98]</td>
</tr>
<tr>
<td>Idjradinata 1993</td>
<td>107.7</td>
<td>10.5</td>
<td>14</td>
<td>109.3</td>
<td>10.5</td>
<td>14</td>
<td>-1.60 [-9.38, 6.18]</td>
</tr>
</tbody>
</table>

Figure 7: Forest plot of comparison: Developmental scores of non-anemic iron deficient pre-school children on iron supplementation versus no treatment/placebo, outcome: Psychomotor Developmental Index (PDI). Study results are not combined.

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>Mean</th>
<th>SD</th>
<th>Total</th>
<th>Mean</th>
<th>SD</th>
<th>Total</th>
<th>Mean Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Akman 2004</td>
<td>97.71</td>
<td>8.56</td>
<td>21</td>
<td>97.94</td>
<td>12.85</td>
<td>19</td>
<td>-0.23 [-7.07, 6.61]</td>
</tr>
<tr>
<td>Idjradinata 1993</td>
<td>107.8</td>
<td>9.7</td>
<td>14</td>
<td>106.6</td>
<td>9.7</td>
<td>14</td>
<td>1.20 [-5.99, 8.39]</td>
</tr>
</tbody>
</table>
Figure 8: Forest plot of comparison: Hematological outcome of non-anemic iron deficient pre-school children on iron supplementation versus no treatment/placebo, outcome: Hemoglobin (g/L). Study results are not combined

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>Experimental</th>
<th>Control</th>
<th>Mean Difference</th>
<th>Mean Difference</th>
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<td></td>
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<td>SD</td>
<td>Total</td>
<td>Mean</td>
</tr>
<tr>
<td>Akman 2004</td>
<td>126.4</td>
<td>6.5</td>
<td>21</td>
<td>123.7</td>
</tr>
<tr>
<td>Idjradinata 1993</td>
<td>134.6</td>
<td>9.7</td>
<td>14</td>
<td>123.1</td>
</tr>
</tbody>
</table>

Figure 9: Forest plot of comparison: Hematological outcome of non-anemic iron deficient pre-school children on iron supplementation versus no treatment/placebo, outcome: Serum ferritin (µg/L). Study results are not combined

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>Experimental</th>
<th>Control</th>
<th>Mean Difference</th>
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<td>Mean</td>
</tr>
<tr>
<td>Akman 2004</td>
<td>33.49</td>
<td>20.05</td>
<td>21</td>
<td>16.43</td>
</tr>
<tr>
<td>Idjradinata 1993</td>
<td>63.1</td>
<td>32.6</td>
<td>14</td>
<td>12</td>
</tr>
</tbody>
</table>
3.4.6 Subgroup and sensitivity analyses

Since only two studies met the inclusion criteria, this restricted us from performing any predefined subgroup or sensitivity analyses.

3.5 Discussion

3.5.1 Major findings

In the current systematic review, two randomized controlled trials were identified for children of preschool age with NAID treated with oral iron treatment v. no treatment. This limited number of identified studies on NAID shows the need to carry out more research on this very important topic. Furthermore, due to high level of clinical, methodological and statistical heterogeneity we were unable to combine the results of the trials. One study demonstrated a significant difference in the BSID MDI post oral iron treatment (161). Neither study demonstrated a significant difference in the post-treatment BSID PDI. However, neither study adjusted for pre-treatment development score. Therefore, the efficacy of oral iron therapy in children with NAID to improve developmental outcome remains in question.

For the hematological outcomes, both studies demonstrated a significant improvement in the post-treatment SF levels. One demonstrated a significant improvement in the post-treatment Hb level (127). The other study, despite demonstrating a significant improvement in post-treatment SF level, did not demonstrate a significant difference in post-treatment Hb level (161). A possible explanation for this finding is that these children may have had very mild iron deficiency. It has been shown in therapeutic trials of iron treatment that children with greater iron deficiency respond to iron treatment at a higher rate (increase of Hb 10 g/l is indicative of iron deficiency) (56).

3.5.2 Limitations

Several methodological and statistical issues lead to the finding that both studies had moderate risk of bias. These issues include: no information regarding allocation concealment (both studies), inadequate
blinding of participants (Akman et al.), no clear statement of adjustment for biases (Akman et al.) and no clear statement on adjustment for various confounders. Another important limitation to these studies was the lack of power to demonstrate a difference in children with NAID. Idjradinata et al. stated: ‘If the developmental effects of NAID were smaller than those of IDA then the sample size needed to detect such differences would have to be larger than the sample included in the present study’ (127). In addition, a comprehensive review on the effect of iron deficiency on the development of children by Grantham McGregor and Ani (2001) specifically emphasized the importance of power and sample size (132).

3.5.3 Relation of findings to those of similar studies

A recently published review of prophylactic administration of iron to healthy infants and pregnant mothers showed no improvement in MDI (162). Meta-analysis of three of the five included studies revealed a 4-point increase in PDI; however, the numbers of infants and studies included in the review were too small to make any conclusive recommendation for screening for iron deficiency in young children. The current review is fundamentally different from the above-mentioned one where the effect of prophylactic administration of iron to non-iron deficient children was examined. We focused our review on healthy children with NAID.

To date, most of the attention regarding iron deficiency has been focused on the impact of iron treatment in children with IDA (125, 132). Studies focusing on iron treatment for children with IDA are unable to provide relevant data for children with NAID. The reasons for this gap in knowledge include study groups not being categorized according to the different levels of iron deficiency; no control group for the subsets of iron deficiency; most studies compared IDA with iron-sufficient children or children with lower level of iron deficiency. This underscores the importance of studies specifically aimed at children with NAID, which is a highly prevalent and under-recognized condition in young children both in developing and developed nations.

3.5.4 Implication on practice

Evidence is insufficient to recommend iron treatment to children with NAID.
3.5.5 Implication on research

There is an urgent need for research examining the effectiveness of oral iron treatment in children of pre-school age with NAID in respect to their developmental outcome. In order to determine if NAID is causally associated with poor development and to determine the efficacy of oral iron treatment, adequately powered (to identify a significant difference between iron-treated and not treated groups of children with NAID) and well-designed blinded, randomized controlled trials must be conducted. In addition, reporting of side-effects of iron treatment should be emphasized in these trials.

3.6 Conclusions

Our findings suggest that data regarding developmental outcomes of children with NAID following treatment with oral iron abstracted from trials specifically aimed at understanding the relationship between iron and development in children with IDA are few and inconclusive. NAID as a cause of poor development and the efficacy of oral iron therapy to reverse or prevent iron-related developmental impact in this population can only be achieved by randomized trials specifically targeting children with NAID. It is imperative that these trials have adequate sample size to detect significant differences in the NAID population. Current evidence indicating the irreversible nature of IDA further strengthens the need to identify and treat children with iron deficiency while they are still in the non-anemic stage.

Acknowledgements

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Chapter 4: Introduction to TARGet Kids!

The purposes of this chapter are to:

1. Describe the research platform (TARGet Kids!) used to conduct the studies included in this thesis and present the guiding principles behind its establishment.
2. Describe the objectives and framework of the TARGet Kids! research initiative.
3. Describe the cohort and recruitment strategy and present published statistics related to the TARGet Kids! initiative.
4. Provide an overview of the variables measured, captured and saved in an online data repository.
5. State the research priorities and the funding support of the TARGet Kids! research initiative.
All four studies selected for this thesis are conducted using data from a community practice-based research network called TARGet Kids! (The Applied Research Group for Kids). Hence, it is important to describe the structure and procedures used to run this pediatric data survey initiative. TARGet Kids! was established in 2008 by three leading child health scientists (Dr. Patricia Parkin, Dr. Catherine Birken and Dr. Jonathon Maguire) with an aim to create a collaboration between child health researchers and primary health care providers working in the community. Their ultimate goal was to establish a large child-focused primary care practice-based research network (PBRN) to advance the scientific basis for chronic disease prevention and develop innovative interventions for primary healthcare providers to overcome common health problems that limit children’s potential (163).

4.1 Guiding principles behind the establishment of TARGet Kids!

The goal of primary healthcare is to ensure that children are on an optimal trajectory for health by identifying and correcting influences that are negatively affecting their health and development. Thus primary care providers are in a unique position to address common health problems faced by infants and toddlers. Furthermore, the special relationship between primary healthcare providers and their patients renders the primary healthcare setting an ideal venue for creating and disseminating knowledge generated from health research.

Pediatric health supervision or well-child care (WCC) visits were developed for the most efficient use of the primary healthcare system (as described) for the prevention of common health problems in young children (164, 165). In the USA, the American Academy of Pediatrics (AAP) has made recommendations for preventive pediatric health care in which guidelines for the frequency and content of well-child care (WCC) visits have been provided (166, 167). Similar guidelines for the health supervision/well-child visit have been recommended for the Canadian pediatric population which follows the content and periodicity schedule recommended by the Rourke Baby Record (RBR) (168). The Rourke Baby Record (RBR) has provided a structured system for preventive well-baby and well-child care for infants and children up to 5 years of age by primary care providers. Its’ periodicity schedule is based on the Canadian immunization schedule and has been endorsed by the College of Family Physicians of Canada and the Canadian Pediatric Society (CPS). The contents of the RBR are
evidence based, has undergone validation testing and is periodically updated to keep abreast of new evidence (168). Hence, the existing community-based primary health care system in Canada is well positioned to serve as a platform to engage health practitioners in a shared vision for child health, and to fill existing knowledge gaps through health surveillance and evaluation of health delivery.

Despite having a primary health care system that can be used as a platform for both delivery of health care and health research there is a lack of primary care practice-based research network (PBRN) in Canada. No network has been established in Canada to collect survey and laboratory data on healthy children. With these principles the TARGet Kids! research network was established. Currently TARGet Kids! is the only network of primary care practices in Canada collecting longitudinal data to examine growth and development trajectories of infants and pre-school children (163).

4.2 Objectives of TARGet Kids!

The overall objective of TARGet Kids! is to establish a longitudinal cohort of children recruited through primary health care settings in order to advance evidence for prevention of diseases and health promotion and to build a population level child health surveillance.

Specific objectives of TARGet Kids! include:

1. To generate evidence for chronic disease prevention and develop innovative interventions through the conduct of research using appropriate study designs and application of robust and validated research methods

2. To focus on the broad domains of healthy growth and developmental trajectories in early childhood including body mass index, physical activity, sedentary behaviors, nutrition, and cognitive-social-emotional-behavioral development

3. To consider factors related to health equity in the delivery and promotion of health care
4.3 TARGet Kids! framework

TARGet Kids! is a partnership between child health scientists from the Pediatric Outcomes Research Team at The Hospital for Sick Children Research Institute, The Applied Health Research Centre (AHRC) at the Li Ka Shing Knowledge Institute, St. Michael's Hospital, and community-based primary care pediatricians and family physicians from the Faculty of Medicine at the University of Toronto. To date, TARGet Kids! practice sites are located in Toronto, Ontario. There are currently five pediatric group practices and one large family practice unit involved in patient recruitment. Each practice has between 3 and 10 practicing physicians (see figure 10).

Figure 10: TARGet Kids! framework
TARGet Kids! Methods Centre, including research and administrative staff is situated at The Hospital for Sick Children Research Institute. TARGet Kids! Data Repository, Management and Analysis Centre is housed at The Applied Health Centre (AHRC) at the Li Ka Shing Knowledge Institute, St. Michael’s Hospital. All data generated from TARGet Kids! practice sites are entered into a secure web-based data repository (Medidata Rave®) which is controlled and managed by the TARGet Kids! data management Centre at AHRC. TARGet Kids! has established collaboration with the Mount Sinai Services laboratory which provides real-time laboratory test results to study physicians, links lab data to the central database, and long-term storage of blood sample.

### 4.4 Description of TARGet Kids! cohort

Between June 2008 and September 2013, 17173 children, under six years of age, attending scheduled health supervision/well-child visits with their primary care physician were assessed for study eligibility. Of the 13004 children who were found eligible, a total of 5062 parents consented to participate, completed all questionnaires and provided physical measures. A blood sample has been collected and analyzed for 2563 children (50.6%) (163). Exclusion criteria for this TARGet Kids! cohort include: children with associated health conditions affecting growth (e.g., failure to thrive, cystic fibrosis), children with any acute or chronic conditions (other than asthma and high functioning autism), children with severe developmental delay, and families who are unable to communicate in English. The median age of participants at the enrollment visit is 25 months (range, 0.2 - 72 months) and 2646 (52%) of participants are male. Children with blood sampling are slightly older but otherwise are similar to children who did not have blood sampling (163).

### 4.5 TARGet Kids! recruitment strategy

According to our provincial publicly funded immunization schedule, children visit their primary care physician at least 7 times at ages 2 months, 4 months, 6 months, 12 months, 15 months, 18 months, and 4-6 years (169). Many practitioners also schedule health surveillance visits at an additional 3 visits: at ages 9 months, 24 months, and 36 months. During these visits to those doctor’s offices who are participating the TARget Kids! network, parents are approached by research assistants to enroll
their child in the TARGet Kids! study. Subsequently enrolled children are followed up according to the well-child visit schedule. Out of the 5062 children enrolled, longitudinal data has been collected as follows: 3187 (3187/4149=76.8%) have had 2 or more visits, 1691 (1691/2299=73.6%) have had 3 or more visits and 628 (628/889 =70.6%) have had 4 or more visits (163).

4.6 Measured variables in TARGet Kids!

A total of 6 parent-reported questionnaires are administered throughout a planned 10-year period of longitudinal data collection. These include, the Nutrition and Health Questionnaire (NHQ) which collects data on socio-demographic information, child's dietary intake and their eating habits, and includes questions on physical activity, screen time and sun exposure ; the Nutrition Screening Tool for Every Preschooler (NutriSTEP™) assesses nutritional risk in children aged 3-5 years of age based on child's food and fluid intake, physical growth, physical activity and sedentary behavior and factors affecting food intake; the Child Behaviour Questionnaire (CBQ) – Very Short Form, provides a comprehensive assessment of reactive and self-regulative temperamental behavior patterns in young children; the Nipissing District Developmental Screen (NDDS) which is a developmental screening tool for children between 1 and 72 months of age; the Infant Toddler Checklist (ITC) is a screening tool for communication delays in children aged between 6 and 24 months; and the Parenting Stress Index (PSI) is designed to identify potentially dysfunctional parent-child systems (170-175). Other measured variables include anthropometric assessment of accompanying parent (weight and height) and child (weight, length/height and waist circumference), child blood pressure (if 2 years and older).

Non-fasting blood samples (4-7 mL) are drawn by trained pediatric phlebotomists at each practice site, and include the following measures: lipid profile, insulin, glucose, hemoglobin, serum ferritin, 25-hydroxyvitamin D, ApoA1, ApoB, CRP, ALT, adiponectin and leptin and other markers of nutritional status (163).

4.7 TARGet Kids! research priorities
1. Establish a large cohort of healthy children to be followed annually to link early health exposures to later health and disease

2. Determine prevalence, and risk factors for obesity, iron deficiency and vitamin D and develop tools needed to measure risk factors and outcomes related to these nutritional disorders

3. Implement pragmatic primary health care based randomized trials of interventions for the prevention of common nutritional disorders affecting the Canadian pediatric population

4.8 Financial support

TARGet Kids! is supported by the St. Michael’s and Sickkids Foundation and numerous Canadian Institutes of Health Research (CIHR) grants. It has also received funds from the Physician Services Incorporated Foundation, the Thrasher Fund, the Danone Institute, the Dairy Farmers of Ontario, Sun Life Financial and the University of Toronto Dean’s Fund (163).
Chapter 5 : Risk factors, practice variation and hematological outcomes of children identified with non-anemic iron deficiency, following screening in primary care setting

This chapter describes the first of four original research works of the candidate. The purposes of this chapter are to:

1. Evaluate the prevalence and risk factors associated with NAID in pre-school children.
2. Describe the physician practice patterns associated with the management of NAID in primary care settings.
3. Describe the longitudinal hematological outcome of children identified with NAID.

Published in manuscript form:

This manuscript has been peer reviewed. See responses to peer review in appendix 2

Approval for publication as thesis material has been granted to Kawsari Abdullah by the journal Paediatrics and Child Health.
5.1 Abstract

Objectives: To determine the prevalence, risk factors, physician practice patterns and the longitudinal hematological outcome of children, following screening for non-anemic iron deficiency (NAID).

Methods: A longitudinal cohort study of healthy children, aged 1-5 years. Descriptive statistics were used to describe the prevalence, risk factors, practice patterns and the hematological outcome of children identified with NAID. Association between NAID and potential risk factors was examined using a multivariate logistic regression analysis.

Results: Of 2276 children undergoing screening, 155 had NAID, for a prevalence of 7% (95% CI, 5.95%-8.05%). Risk factors significantly associated with NAID included younger age (OR 1.08, 95% CI: 1.06, 1.11), higher zBMI (OR 1.22, 95% CI: 1.01, 1.48), longer duration of breastfeeding (OR 1.05, 95% CI: 1.01, 1.08) and increased volume of cow’s milk intake (OR 1.13, 95% CI: 1.01, 1.26). An assessment of practice patterns revealed that for 37% of children an intervention for NAID was documented; and for 8.4% children a physician-ordered follow-up laboratory test was completed to re-evaluate iron status. A total of 58 (37%) children had a follow-up laboratory test, among them 38 (65.5%) had resolution of NAID, 15 (25.9%) had persistence of NAID, and 2 (3.4%) had progression of NAID to anemia.

Conclusion: NAID is common in early childhood and has association with modifiable risk factors. Substantial practice variation exists in management of NAID. Further research is necessary to understand the benefits of screening for NAID and evidence-informed practice guidelines may reduce practice variation in the management of NAID in early childhood.
5.2 Introduction

Iron deficiency represents a spectrum ranging from non-anemic iron deficiency (NAID) to iron deficiency with anemia (IDA). NAID is the early latent phase of iron deficiency (32, 40). Evidence suggests that NAID may be associated with adverse neurodevelopmental outcomes in young children (40, 118). Furthermore, in absence of treatment, NAID may progress to IDA which has been found to be associated with irreversible developmental delay in young children (134, 138, 139). These attributes of NAID suggests that the early phase of iron deficiency may meet the criteria required for screening of disease (176, 177).

The prevalence of iron deficiency and IDA among toddlers in the US has been reported to be 9.2% and 2.1% respectively (36, 40). In Canada, even though there is no data regarding the national prevalence of iron deficiency and IDA in young children, a review of several small regional studies found the prevalence of iron deficiency and IDA to range from approximately 12% to 64 % and 1.5% to 79% (71). These prevalence rates do not signify those of NAID, since NAID represents a particular stage of iron deficiency. Due to lack of screening, specifically using iron specific indicators, the true prevalence of NAID in pre-school children is not well elucidated. Most developed countries other than the United States do not recommend screening for IDA (40, 42, 108, 178). Hence, how best to screen for and manage NAID in primary care practice is unclear.

Furthermore, certain characteristics have been found to be risk factors for developing IDA in young children. These include children of families of low socioeconomic status, ethnicity, low birth weight, obesity and certain nutritional behaviors (such as, current use of bottle in pre-school children, excessive cow’s milk intake, whole cow’s milk during the first year of life and longer duration of breastfeeding) (39, 41, 53, 71, 105, 109, 135, 179, 180). Due to lack of investigation and identification of young children in their early stage of iron deficiency, there is also a gap in evidence on risk factors associated with NAID.

The current study has taken advantage of the unique opportunity presented through screening of young children for NAID using iron indicators. The overall goal of this study was to evaluate the clinical practice of screening for NAID followed by treatment and follow-up. Specific objectives were the
following: 1) To evaluate the prevalence and risk factors associated with NAID in pre-school children; 2) To describe the physician practice patterns associated with the management of NAID in primary care settings; and 3) To describe the longitudinal hematological outcome of children identified with NAID.

5.3 Methods

This was a longitudinal cohort study of healthy urban children living in Toronto, Canada (a high-income, developed country). Data was collected from June, 2008 to June, 2012. Participants included children aged 12-60 months who were screen positive for NAID. Children diagnosed with IDA or any other type of anemia, C-Reactive Protein (CRP) ≥10 mg/L, previously diagnosed developmental disorder, genetic, chromosomal or syndromic condition; and previously diagnosed chronic medical condition (exception of asthma and allergy) were excluded from the study.

Participants were recruited from the TARGet Kids! research network which is a collaboration between child health researchers at the Hospital for Sick Children and St. Michael’s Hospital, and primary care physicians within the Greater Toronto Area (www.targetkids.ca) (181). Healthy children during their scheduled health supervision visits are approached to participate by trained research assistants embedded in each practice. TARGet Kids! practices offer up to 10 scheduled health supervision visits between 2 months and 5 years of age. From June, 2008 to September 2013, a total of 13004 children (0-72 months) were found eligible to participate in the TARGet Kids! program and about 39% consented to participate (163).

Approval for data collection was received from the Hospital for Sick Children and St. Michael’s Hospital research ethics boards, and informed consent was received by parents of participating children. Data related to health, nutrition and socio-demographic characteristics of children were collected using a standardized parent-completed survey instrument based on the Canadian Community Health Survey (163, 170). Trained research assistants obtained height and weight of children using standardized instruments and also collected a sample of blood which was analyzed at Mount Sinai Services (MSS) laboratory. Blood analysis included serum ferritin and blood hemoglobin concentration. The laboratory results were sent back to the clinic sites in real-time. All data generated
from the TARGet Kids! research initiative were entered into a secure web-based data management system (Medidata Rave®). From this electronic data capture and repository, we were able to collect children’s hematological data and identify any iron related disorders, such as NAID and IDA.

At the clinics, in keeping with provincial standards, physicians once notified of the laboratory results were at liberty to provide management to children with NAID and document their activity in clinic charts (182). Using a standardized data collection form, we abstracted the following information from the clinic charts of children who were identified with NAID: documentation of any intervention and the types of interventions recommended by physicians to treat NAID; and documentation of any physician ordered follow-up laboratory test to re-evaluate iron status. A period of six months was selected as the follow-up duration, because this approximates the time for management and follow-up for children with IDA (33).

TARGel Kids! program offered parents the opportunity to have blood work for their children at their enrollment visit and also at any subsequent scheduled health supervision visits. Thus, we were able to longitudinally follow the hematological outcome of children. For the current study, the hematological outcome of children with NAID was identified either through the TARGel Kids! follow-up blood test results or from the physician prescribed follow-up blood tests results abstracted from the medical records of TARGel Kids! practices.

Iron status was measured using indicators suggested by the American Academy of Pediatrics (AAP) in their guideline for assessment of iron deficiency in young children (hemoglobin and serum ferritin with CRP (40). Values commonly considered low for serum ferritin are 10-12 µg/L (36, 39, 42). For our study NAID was defined as a serum ferritin level of ≤12 µg/L with CRP <10 mg/L and a normal hemoglobin level (≥110 g/L). IDA was defined as a serum ferritin level of ≤12 µg/L with CRP <10 mg/L and a low hemoglobin level (<110 g/L). Iron sufficiency (IS) was defined as having both a normal serum ferritin and hemoglobin level (serum ferritin >12 µg/L with CRP <10 mg/L and hemoglobin ≥110 g/L) (32, 36, 40, 183). Serum ferritin was measured using a Roche Modular platform, Roche Diagnostics and hemoglobin was measured using Sysmex platform, Sysmex Diagnostics.
Clinically important co-variates included child’s age (months), sex, BMI z-score, birth weight (lbs), total breastfeeding duration (in months), current bottle use (yes or no), volume of cow’s milk intake (cups/per day), maternal ethnicity and education (40, 41, 135). BMI was calculated as weight in kilograms divided by the height in meters squared \(^{(184,185)}\). BMI z scores were calculated using WHO growth standards. Maternal ethnicity was dichotomized to European and non-European groups (East Asian, South Asian, Southeast Asian, West Asian and North African, African, Caribbean, Latin American and others). Maternal education was dichotomized to college/university and high school/elementary school.

5.3.1 Statistical analysis

To assess the representativeness of our sample, we compared children who did and did not undergo screening laboratory tests, using independent t test or the chi-square test where applicable. Prevalence of NAID was described as percentage of children with NAID from the study sample during the specified study period. Descriptive statistics were used to describe the distribution of the clinically important variables in the NAID and iron sufficient (IS) groups. Characteristics were compared using either the independent t test for continuous variables or the chi-square test for categorical variables. The association between NAID (reference group) and all clinically important variables were examined using a multivariate logistic regression analysis where clinically important and not highly correlated variables [variance inflation factor (VIF) for all variables were < 2.5] were tested simultaneously \((186)\). Validity of the model was tested using influence diagnostics including Pearson residual, Leverage and DfBetas (plotted against all variables) \((186)\). The plots showed no significant influential points in the model and most cases were well accounted for by the model.

To describe physician practice patterns, we calculated the proportion of children with NAID for whom there was documentation of the following: a physician-prescribed intervention to treat NAID (dietary advice, oral iron, both, other); a physician-ordered follow-up laboratory test within 6 months. To describe the hematological outcomes of children with NAID at follow-up, we calculated the proportion of children with: resolution of NAID; persistence of NAID; and progression of NAID to IDA. Statistically significant results were those with values of \(p \leq 0.05\). All statistical analyses were performed by using SAS software version 9.1 (SAS Institute, Cary NC).
5.4 Results

During the study period, a total of 3814 children were enrolled in the TARGet Kids! research network and a total of 2276 children were screened for iron deficiency. Children, who did and did not undergo screening laboratory tests, were similar with respect to independent variables. Thus, the sample for this study appears representative of the entire TARGet Kids! sample (table 7).
Table 7: Comparison of children (12-60 months of age) with and without blood work (N= 3814)

<table>
<thead>
<tr>
<th>Variables</th>
<th>Blood work N = 2276</th>
<th>No blood work N=1538</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age of child (months) †</td>
<td>29.00 (14.71)</td>
<td>28.20 (14.17)</td>
<td>0.10</td>
</tr>
<tr>
<td>Sex of child ‡</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>1295 (52.60)</td>
<td>687 (50.81)</td>
<td>0.29</td>
</tr>
<tr>
<td>Female</td>
<td>1167 (47.40)</td>
<td>665 (49.19)</td>
<td></td>
</tr>
<tr>
<td>zBMI of child†</td>
<td>0.19 (1.08)</td>
<td>0.21(1.09)</td>
<td>0.56</td>
</tr>
<tr>
<td>Birth weight (lbs)†</td>
<td>6.53 (1.87)</td>
<td>6.55(1.74)</td>
<td>0.75</td>
</tr>
<tr>
<td>Maternal ethnicity ‡</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>European</td>
<td>1657 (70.09)</td>
<td>962 (73.27)</td>
<td>0.04</td>
</tr>
<tr>
<td>Non-European</td>
<td>707 (29.91)</td>
<td>351 (26.73)</td>
<td></td>
</tr>
<tr>
<td>Maternal education ‡</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>College/university</td>
<td>2143 (90.08)</td>
<td>1179 (90.48)</td>
<td>0.69</td>
</tr>
<tr>
<td>High and public school</td>
<td>236 (9.92)</td>
<td>124 (9.52)</td>
<td></td>
</tr>
<tr>
<td>Duration of breast feeding (months)†</td>
<td>10.89 (6.47)</td>
<td>10.78(6.27)</td>
<td>0.64</td>
</tr>
<tr>
<td>Currently use bottle ‡</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>871 (36.05)</td>
<td>453 (34.01)</td>
<td>0.21</td>
</tr>
<tr>
<td>No</td>
<td>1545 (63.95)</td>
<td>879 (65.99)</td>
<td></td>
</tr>
<tr>
<td>Volume of cow’s milk (cups/day) †</td>
<td>4.13 (2.19)</td>
<td>4.13(2.04)</td>
<td>0.97</td>
</tr>
</tbody>
</table>

† mean (SD); ‡ n (%)

According to the definition of NAID used for this study, 155 children aged 12-60 months were identified with NAID. Hence, the prevalence rate of NAID within the study period among children aged 12-60 months, enrolled in the TARGet Kids! research network was found to be 7% (95% CI, 6.0-8.1). Table 8 shows the distribution of the clinically important variables within NAID and IS (iron sufficient) groups.

Table 8: Comparison between NAID and IS children (N= 2276)

<table>
<thead>
<tr>
<th>Variables</th>
<th>NAID Group (N=155)</th>
<th>IS Group (N=2121)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age of child (months) †</td>
<td>24.70 (10.80)</td>
<td>34.88 (13.74)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Sex of child ‡</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>77 (57.89)</td>
<td>899 (52.79)</td>
<td>0.26</td>
</tr>
<tr>
<td>Female</td>
<td>56 (42.11)</td>
<td>804 (47.21)</td>
<td></td>
</tr>
<tr>
<td>zBMI of child †</td>
<td>0.39 (1.00)</td>
<td>0.24 (1.07)</td>
<td>0.09</td>
</tr>
<tr>
<td>Birth weight (lbs) †</td>
<td>6.62 (1.51)</td>
<td>6.53 (1.74)</td>
<td>0.56</td>
</tr>
<tr>
<td>Maternal ethnicity ‡</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>European</td>
<td>101 (78.91)</td>
<td>1118 (68.55)</td>
<td>0.01</td>
</tr>
<tr>
<td>Non-European</td>
<td>27 (21.09)</td>
<td>513 (31.45)</td>
<td></td>
</tr>
<tr>
<td>Maternal education ‡</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>College/university</td>
<td>123 (93.89)</td>
<td>1488 (89.96)</td>
<td>0.14</td>
</tr>
<tr>
<td>High and public school</td>
<td>8 (6.11)</td>
<td>166 (10.04)</td>
<td></td>
</tr>
<tr>
<td>Duration of breast feeding (mo) †</td>
<td>12.18 (6.11)</td>
<td>11.30 (6.92)</td>
<td>0.14</td>
</tr>
<tr>
<td>Currently use bottle ‡</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>43 (33.08)</td>
<td>391 (23.34)</td>
<td>0.01</td>
</tr>
<tr>
<td>No</td>
<td>87 (66.92)</td>
<td>1284 (76.66)</td>
<td></td>
</tr>
<tr>
<td>Volume of cow’s milk (cups/day) †</td>
<td>4.39 (2.14)</td>
<td>4.35 (1.96)</td>
<td>0.81</td>
</tr>
</tbody>
</table>

NAID: Non-Anemic Iron Deficiency; IS: Iron Sufficient; † mean (SD); ‡ n (%)
In the multivariate analysis, factors found to be significantly associated with NAID were: age (with each month decrease in age, children had 1.08 times greater odds of NAID); zBMI (with each unit increase in zBMI children had 1.22 times greater odds of NAID); duration of breastfeeding (with each month increase in breastfeeding duration children had 1.05 times greater odds of NAID); and volume of cow’s milk intake (with each cup/day increase in cow’s milk intake, children had 1.13 greater odds of NAID). See table 9 for 95% confidence interval of odds ratios and p-value.
Table 9: Multivariable analyses of factors associated with NAID in children aged 12-60 months

<table>
<thead>
<tr>
<th>Predictor variables</th>
<th>β coefficient</th>
<th>p-value</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age of child (months)†</td>
<td>0.0814</td>
<td>&lt;0.0001</td>
<td>1.08 (1.06, 1.11)</td>
</tr>
<tr>
<td>zBMI of child</td>
<td>0.2011</td>
<td>0.04</td>
<td>1.22 (1.01, 1.48)</td>
</tr>
<tr>
<td>Birth weight (lbs)</td>
<td>-0.0166</td>
<td>0.79</td>
<td>0.98 (0.87, 1.12)</td>
</tr>
<tr>
<td>Duration of breast feeding (months)</td>
<td>0.0475</td>
<td>0.01</td>
<td>1.05 (1.01, 1.08)</td>
</tr>
<tr>
<td>Volume of cow’s milk (cups/day)</td>
<td>0.1231</td>
<td>0.03</td>
<td>1.13 (1.01, 1.26)</td>
</tr>
<tr>
<td>Sex of child</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>0.3645</td>
<td>0.09</td>
<td>1.44 (0.94, 2.20)</td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Currently use bottle</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>-0.3539</td>
<td>0.17</td>
<td>0.70 (0.42, 1.17)</td>
</tr>
<tr>
<td>Yes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maternal ethnicity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>European</td>
<td>0.3064</td>
<td>0.23</td>
<td>1.36 (0.83, 2.23)</td>
</tr>
<tr>
<td>Non-European</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maternal education</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>College/university</td>
<td>0.1100</td>
<td>0.79</td>
<td>1.12 (0.49, 2.56)</td>
</tr>
<tr>
<td>High and elementary school</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

OR: Odds Ratio;
†Decreasing age was significantly associated with greater odds of having NAID

Clinic chart documentation showed, out of the 155 children with NAID, 57 (37%) were recommended an intervention. Table 10 shows the different types and proportion of interventions that were recommended by physicians to treat NAID. Furthermore, only 13 (8.4%) of the 155 children with
NAID had a physician prescribed follow-up laboratory test performed to re-evaluate their iron status. This was 23% of all those who were recommended a treatment (n = 57).

Table 10: Interventions recommended by physicians to treat NAID (N=57)

<table>
<thead>
<tr>
<th>Types of interventions</th>
<th>No.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral iron</td>
<td>25</td>
<td>43.9</td>
</tr>
<tr>
<td>Dietary advice</td>
<td>13</td>
<td>22.8</td>
</tr>
<tr>
<td>Oral iron + dietary advice</td>
<td>6</td>
<td>10.5</td>
</tr>
<tr>
<td>Dietary advice + others</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>Others‡</td>
<td>2</td>
<td>3.5</td>
</tr>
<tr>
<td>NR</td>
<td>7</td>
<td>12.3</td>
</tr>
</tbody>
</table>

NAID: Non-Anemic Iron Deficiency; NR: Not Recorded
‡Others: Multivitamins with iron

For hematological outcome of children identified with NAID, a total of 58 children had a follow-up (TARGet Kids! follow-up or physician prescribed) laboratory test to re-evaluate their iron status. The mean follow-up time was 1 year. Among them, 38/58 (65.5%) children had resolution of NAID, 15/58 (25.9%) children had persistence of NAID, and 2/58 (3.4%) children had progression of NAID to IDA (see figure 1). Of the children with documentation of a recommended intervention, 32 had a follow-up laboratory test with the following results: 22 had resolution of NAID, 7 had persistence of NAID, 1 child had progression from NAID to IDA and in 2 cases data was not available. Of children with no documentation of an intervention, 26 had a follow-up laboratory test with the following results: 16 had resolution of NAID, 8 had persistence of NAID, 1 child had progression from NAID to IDA and in 1 case data was missing (see figure 11).
5.5 Discussion

The study shows the prevalence of NAID to be 7% among healthy urban Canadian pre-school (12-60 months) children participating in the TARGet Kids! research network. Furthermore, we identified that children in the younger age group (12-36 months), the prevalence of NAID was 12.6%. Our data may be the first to identify the prevalence of NAID in a large sample of urban Canadian children in this age group. The Canadian Health Measures Survey (CHMS) has reported prevalence of anemia (hemoglobin <110 g/L, 0.5%) and low iron stores (serum ferritin <12 µg/L, 3.2%) separately for 487 children aged 3 to 5 years. (106, 107). The lower prevalence in the CHMS data as compared with our findings may be due to the older age of children in CHMS or the different methods of recruitment.
In our study, we used serum ferritin to screen for NAID in pre-school children in community practice setting; this appears feasible, especially in developed countries where the prevalence of IDA is low and sole use of hemoglobin becomes less effective in identifying iron deficiency in children. However, further research is necessary to evaluate and compare the diagnostic properties of serum ferritin with other indicators (such as transferrin receptor and reticulocyte hemoglobin) used to screen NAID in young children (40, 74). We have also identified risk factors significantly associated with NAID (younger age, higher zBMI, longer duration of breastfeeding and greater intake of cow’s milk per day). These modifiable risk factors being associated with the early stage of iron deficiency can be considered by primary care physicians in preventing the development of IDA in young children.

Our study findings also highlight substantial variation in clinical practice with respect to management and follow-up of children identified with NAID on screening. Thirty-seven percent of children identified with NAID on screening had documentation of a recommended intervention, and 8.4% of children had a physician-ordered follow-up laboratory test to re-evaluate their iron status. Also, five different types of interventions were used by clinicians to treat NAID. This level of practice variation suggests substantial physician uncertainty regarding the interventions and follow-up of children identified with NAID. In the absence of clinical guidelines for managing NAID in children, it is possible that physicians extrapolate from clinical guidelines for managing IDA (40, 42). However, our study shows that about one third of children who had a follow-up blood testing had persistent NAID (25.5%) or progressed to IDA (3.4%). The potential for incomplete resolution of NAID emphasize the need to develop clinical guidelines that not only target IDA but also NAID, the early latent stage of iron deficiency.

There are several limitations to this study. First, not all children enrolled in TARGet Kids! underwent laboratory testing. However, comparisons of children who did and did not undergo laboratory testing showed no difference between the two groups. Furthermore, the prevalence of NAID in our study is similar to those reported in other developed countries, supporting the generalizability of our findings (36, 42). Second, for the assessment of physician practice, we used clinic chart abstraction and necessarily relied upon the presence or absence of documented management as a proxy of actual management. Finally, although we identified 155 children with NAID following screening laboratory testing, only 58 had follow-up laboratory tests. This limits our interpretation of the natural history of
NAID and the potential effectiveness of intervention. Furthermore, it is possible that physicians laboratory test-ordering behavior was influenced by participating in TARGet Kids!, however we are unable to confirm this.

5.6 Conclusion

We assessed more than 2,000 young children (1-5 years), following laboratory screening for NAID and identified high prevalence, presence of modifiable risk factors, significant physician practice variation and poor resolution of hematological outcome in children with NAID. To strengthen the findings from our current study, a randomized controlled trial is currently being conducted through our research group to further evidence related to screening of NAID using iron specific indicators and the effect of NAID on the neurodevelopment of young children (187).
Chapter 6: Re-evaluation of serum ferritin cut-off values for the diagnosis of iron deficiency in infants aged 12 to 36 months.

This chapter describes the second of four original research works of the candidate. The purposes of this chapter are to:

1. Describe the evidence associated with SF cut-offs currently used to diagnose iron deficiency and their clinical relevance.
2. Identify SF cut-offs for diagnosing iron deficiency in children aged 12-36 months by examining its relationship with another iron status indicator, Hb concentration.
6.1 Abstract

Background: Hemoglobin (Hb) cut-off of <110 g/L is used to screen for iron deficiency anemia (IDA) in young children. Serum ferritin (SF) is a biomarker of iron status used to diagnose iron deficiency. Optimal cut-off values for SF in children age 12-36 months are not well delineated.

Objective: To identify clinically relevant SF cut-offs for the diagnosis of iron deficiency in children aged 12-36 months by examining its relationship with Hb concentration.

Methods: In this cross-sectional study the relationship between SF and Hb was examined using an adjusted restricted cubic spline (RCS) linear regression analysis. From the regression plot a plateau point where Hb concentration is maximized was estimated; the SF cut-off that predicted a mean Hb value of 110 g/L was calculated.

Results: Blood samples from 1257 children, 12-36 months of age, were analyzed. Mean ± standard deviation (SD) of age was 18.9 (5.9) months and 53.5% were male. Mean (±SD) of SF and Hb were 27.7 (19.7) µg/L and 119.3 (8.8) g/L respectively. The adjusted RCS model identified a plateau point at which the predicted Hb level is maximized and this point corresponded to a SF level of 17.9 µg/L. Furthermore, Hb with a predicted mean value of 110 g/L corresponds to a SF value of 4.6 µg/L.

Conclusion: Our study identified a cut-off for SF (17.9 µg/L) where the clinical impact of iron deficiency may not come into effect until values lower than this cut-off has been reached; and a SF cut-off (4.6 µg/L) that may have clinical impact on the neurodevelopment of children. Further research is needed to confirm their direct clinical impact and determine their diagnostic properties.
6.2 Introduction

Iron deficiency is the most common nutritional deficiency found in young children, peaks in prevalence between 1 and 3 years of age, and may lead to irreversible neurocognitive impairment (36, 40). The iron status of children can be assessed using hematological tests (i.e., hemoglobin concentration, hematocrit, mean cell volume, and red blood cell distribution width) and iron specific biomarkers (i.e., serum ferritin concentration, serum iron, erythrocyte protoporphyrin concentration, total iron-binding capacity, transferrin saturation, and serum transferrin receptors) (32, 33).

The American Academy of Pediatrics (AAP) recommends universal screening for anemia with determination of hemoglobin (Hb) concentration with a cut-off of <110 g/L for children aged 1-3 years (40). The concentration of the iron containing protein Hb reflects the amount of functional iron in the body (33). Thus, Hb concentration is regarded as an indicator of iron deficiency (36). Because changes in Hb concentration occur only at the late stage of iron deficiency, it is considered a late indicator of iron deficiency. The Hb concentration cut-off for anemia reflects a severity of iron deficiency that has been causally linked to delayed neuro-development in young children and necessitates clinical management (33, 40, 125). Hence, this cut-off (<110 g/L) has clinical importance for practitioners and iron deficiency researchers.

Serum ferritin (SF) is one of the most widely used and specific biomarker of iron status in young children (32, 33). It reflects the size of the iron store in the body (188). Currently recommended SF cut-off values for identifying iron deficiency in children range between <10-12 µg/L (32, 40). A review of the literature shows that these cut-offs (<10-12 µg/L) for children originated from a review by Dallman et al. published in 1980 and the reference for this recommended cut-off is a study by Siimes et al. published in 1974 (62, 68). In this study, children aged 0-15 years and had IDA (n=13) were identified to have a SF range of 1.5 - 9 ng/ml. Based on this range a SF cut-off <10 µg/L has been suggested for children of all age groups. More recently, two other studies evaluated the cut-off of SF in young children (0-5 years) using the fifth percentile of the distribution of SF as the cut-off for defining iron deficiency. One study identified the fifth percentile to correspond to a SF of <10 µg/L, while the other found this value to be 12 µg/L (72, 73).
Therefore, the evidence behind these thresholds is weak. Furthermore, they were not developed specifically for infants 12-36 months of age and were based on the distribution of SF in a very small number of children with IDA. The authors of a study targeting a younger age group of children (9-12 months) has also voiced concerns regarding currently used SF cut-offs (82). Thus, SF cut-offs for diagnosing iron deficiency in children needs to be re-evaluated.

It is important to understand the relationship between SF cut-offs and important child health outcomes such as neurodevelopment. Some evidence suggests that low SF alone may have significant impact on children’s neurodevelopment (118, 127, 161). However, this evidence is not conclusive and further research is needed to establish the clinical impact of low SF on children’s neurodevelopment (119). Examining the relationship between Hb (the functional form of iron in the body) and SF (the stored form of iron in the body) may provide an opportunity to identify clinically important SF cut-offs for diagnosing iron deficiency. Considering the long time course for development of chronic health outcomes that may be related to iron deficiency in children, identifying clinically important cut-offs for SF will enhance the laboratory diagnosis of iron deficiency in young children. The objective of this study was to identify clinically relevant SF cut-offs for the diagnosis of iron deficiency in children aged 12-36 months by examining its relationship with Hb concentration.

6.3 Methods

6.3.1 Study design and population

This was a cross-sectional study of healthy urban children living in Toronto, Canada (a high-income, developed country). Data was collected from May, 2010 to July, 2014. Study participants included children aged 12-36 months recruited during a scheduled health supervision visit with a physician participating in the TARGet Kids! primary care practice based research network (www.targetkids.ca) (189). Excluded children were those diagnosed with anemia other than iron deficiency, having C-reactive protein (CRP) ≥10 mg/L, previously diagnosed with a hematological disorder (thalassemia and other disorders of hemoglobin), developmental disorder, genetic, chromosomal or syndromic condition and chronic medical conditions (except asthma and allergy).
6.3.2 Data collection

Data was collected prospectively on children’s health, nutrition and socio-demographic characteristics using a standardized parent-completed survey instrument. A sample of blood was also collected and analyzed to determine children’s iron status using iron specific indicators – hemoglobin (Hb), serum ferritin (SF) and C-reactive protein (CRP). SF was measured using a Roche Modular platform, Roche Diagnostics and Hb was measured using Sysmex platform, Sysmex Diagnostics (190, 191). All diagnostic assessments were performed at the Mount Sinai Services Laboratory (www.mountsinaiservices.com).

Approval for data collection was received from the Hospital for Sick Children and St. Michael’s Hospital research ethics boards, and informed consent was received from parents of participating children.

6.3.3 Statistical analysis:

Descriptive statistics were used to describe the distribution of the variables: child age and sex, Hb and SF. Non-linearity assumptions were tested by visual inspection and by performing a likelihood ratio (LR) test to compare the nested univariate models of linear predictor versus model with restricted cubic spline (186).

To examine the relationship between SF (independent variable) and Hb (dependent variable), an adjusted restricted cubic spline (RCS) regression model with 5 knots was constructed (186). For a large sample (N >100), 5 knots has been recommended as a good choice. It provides enough flexibility for a reasonable loss of precision caused by overfitting the data (186, 192). Children’s age and sex have been shown to be significantly associated with iron status indicators (Hb and SF) (73, 193-195). Hence, both these variables were adjusted for in the model, however, the spline function was performed only for the association between Hb and SF.
Using the RCS regression model we identified two SF cut-off levels. First, the predicted value of Hb that corresponded to a maximum plateau point was calculated. The plateau point was estimated by finding the root of the derivative of the predicted mean Hb as a function of SF.

Second, using the same model, a SF value that predicted a Hb value of 110 g/L was calculated. Since an Hb cut-off of 110 g/L has significant clinical importance, we intended to find a SF cut-off that predicted an Hb of 110 g/L from the RCS regression model.

Data analyses were performed using SAS version 9.1 (SAS institute Inc., Cary, NC, USA). The R version 3.0.1 was used for restricted cubic spline analysis. Alpha (two-tailed) was set at values less than 0.05.

6.4 Results

Blood samples of 1257 children (12-36 months of age) were analyzed for Hb and SF. The mean (±standard deviation) age of children was 18.9 (5.9) months and 53.5% were male. Mean (±SD) of SF and Hb were 27.7 (19.7) µg/L and 119.3 (8.8) g/L respectively.

The adjusted RCS regression model identified a non-linear relationship between SF and Hb. When compared to nested univariate models of linear predictors, the model with RCS showed a LR chi-square value of 91.07 (p < 0.001), indicating that a non-linear relationship (between SF and Hb) had a better fit to the data.

RCS regression showed Hb to be significantly associated with SF (p < 0.0001). The plateau point at which predicted Hb level was maximized (120.6 g/L) corresponded to a SF level of 17.9 µg/L (see figure 12). Furthermore, Hb with a predicted value of 110 g/L corresponded to a SF value of 4.6 µg/L (mean age 18.9 months and sex = male). Age was statistically significant (p=0.003) while sex was not (p=0.53).
6.5 Discussion

Based on restricted cubic spline modeling, our study identified that the predicted value of Hb increased until reaching a SF value of 17.9 µg/L, and plateaued thereafter. This SF level (17.9 µg/L) represents the point where Hb level is maximized (120.6 g/L). Hb represents the functional form of iron in the body (8). Thus, in healthy children a cut-off of SF at which Hb concentration is maximized, may have important clinical implication. Since SF is the storage form of iron in the body, this cut-off may represent the optimal amount of storage iron required to carry out the functional processes associated with iron metabolism (8). Hence, SF values below this threshold may negatively affect functional processes that may have clinical implications.
Another finding from our study was the identification of a SF cut-off (4.6 µg/L) that predicted a Hb concentration of 110 g/L. In children with iron deficiency, this Hb cut-off has been used to define anemia, and has been found to be associated with adverse neurodevelopmental outcomes in young children (127, 140). Hence when children are diagnosed with iron deficiency anemia based on Hb level of <110 g/L, they may have a SF as a low as 4.6 µg/L.

This study may be the first to assess the relationship between two iron status indicators (Hb and SF) using restricted cubic spline functions in a large sample of pre-school children (N = 1257). From our study, we identified SF cut-offs (17.9 µg/L and 4.6 µg/L) that signify different stages in the pathway of iron metabolism. A value of 17.9 µg/L may indicate that the functional/clinical impact of iron deficiency does not come into effect until values lower than this cut-off has been reached (196). On the other hand, the 4.6 µg/L value may correspond to a cut-off that has definite clinical impact through its association with a clinically important Hb cut-off for anemia (125). Intermediate values mandate further investigation.

Despite the benefits associated with assessing SF in diagnosing iron deficiency, it is also subject to certain limitations. Because SF is an acute-phase reactant, concentration of SF may be elevated in the presence of chronic inflammation, infection, malignancy or liver disease (40). Thus, different cut-off levels of SF have been recommended for populations where the prevalence of infectious and inflammatory disorders is high (38). Additionally, combining SF concentration with determination of another acute-phase reactant such as C-reactive protein (CRP) or alpha 1-acid glycoprotein has been suggested and applied in our study (40, 75).

Based on the methodology we used, these SF cut-off values are statistical markers that may be associated with clinical relevance. Considering this as a potential limitation of this analytic approach, these cut-off values may not represent optimal thresholds for diagnosing iron deficiency. However, the results of this study are in agreement with another study that had attempted to identify SF cut-offs in the same pediatric age group using a different statistical analysis. They reported a SF cut-off that corresponded to an Hb level of <110 g/L was ≤ 8.7 µg/L (95% CI: 6.8, 11.1 µg/L) (39). Despite similar findings, it is crucial to evaluate the direct impact of SF cut-offs identified from this study on important clinical outcomes such as children’s neurodevelopment. Furthermore, the diagnostic accuracy of these
cut-offs need to be determined using epidemiological methods such as Receiver Operating Characteristics (ROC) analysis and likelihood ratios (64, 74). Assessment of the direct clinical impact and diagnostic properties will advance our understanding of the optimal cut-off of SF for laboratory diagnosis of iron deficiency in this age group. This study may be considered the initial step towards finding SF cut-offs that signify clinical relevance rather than being merely distribution-based.

6.6 Conclusion

An ongoing challenge has been determining SF cut-offs that have clinical importance in the diagnosis of iron deficiency in young children. The current study identified two SF cut points associated with clinically important cut-offs of Hb for the diagnosis of iron deficiency in young children. Knowledge of clinically relevant SF cut-offs may greatly influence the prevalence and iron deficiency prevention strategy in young children.
Chapter 7: Optimizing Early Child development for young children with non-anemic iron deficiency in the primary care practice setting (OptEC): study protocol for a randomized controlled trial

This chapter describes the third of four original research works of the candidate. The purposes of this chapter are to:

1. Describe the protocol of a randomized trial (OptEC) that aimed to evaluate the effectiveness of oral iron treatment plus dietary advice versus placebo plus dietary advice in children with NAID.
2. Describe the rationale and aim of an internal pilot study conducted within the study design of the OptEC trial.
3. Describe the sample size and methods for conducting an internal pilot study

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One of the research priorities of the TARGet Kids! research network is to build a platform for conducting pragmatic randomized trials of interventions for the prevention of common pediatric nutritional disorders using the primary health care setting. With this goal a randomized controlled trial, called OptEC: Optimizing Early Child Development in the Primary Care Practice Setting, to compare the effect of iron treatment plus dietary counseling versus placebo plus dietary counseling in improving developmental and laboratory outcomes in young children with non-anemic iron deficiency, is currently being conducted using the TARGet Kids! platform. The third study of this thesis is the REB approved study protocol of this trial.

7.1 Background and rationale

Child development, specifically early child development (ECD) has been recognized as the most important indicator of subsequent healthy developmental trajectories in children (197, 198). Experience-based brain and biological development in early years can set trajectories that affect the competence, health and well-being in individuals throughout life (199, 200). In recognition and support of the ground-breaking research that had introduced these concepts, national and international strategies/recommendations to enhance early child development have been suggested (197). In Canada, programs to enhance early child development were introduced using the primary health care setting and efforts are currently underway to establish a universal integrated ECD program (201, 202).

The consequences associated with iron deficiency in early childhood have great relevance and impact on children’s healthy developmental trajectory. Evidence indicating iron deficiency to be most prevalent during the early years of childhood (see section 1.2.5) and the evidence of its detrimental effect on child development (chapter 2 and 3), emphasizes the need to treat and prevent this disorder as early as possible in the life trajectory.

Three decades of research suggests that prevention of iron deficiency in the primary care practice setting may be an unrealized and unique opportunity to prevent poor developmental outcomes in children. This body of literature has largely focused on the most severe and late stage of iron deficiency with anemia (IDA) (see section 2.4 and 2.5); however there is remarkably little research regarding the early stage, non-anemic iron deficiency (NAID) which too may be associated with
delaying the development of young children (see section 3.5.1). This provides a critical opportunity for early identification and prevention of NAID and fulfils several of the World Health Organization guiding principles for screening (88).

Current Canadian guidelines do not recommend screening all children for iron deficiency; because there is not enough good quality research to prove that screening is effective (see section 3.5.5). Therefore, there is an urgent need to evaluate and compare the developmental outcomes of young children with NAID who undergo screening followed by treatment or no treatment with iron specific interventions. This question is highly relevant to child health, is pragmatic and responsive to physician care in primary care practice settings, and is feasible and efficient to study within the practice based research platform called TARGGet Kids! (chapter 4).

7.2 Objectives and hypotheses

The primary objective of this trial is to assess the effectiveness of four months of oral iron plus dietary advice versus placebo plus dietary advice, in children with NAID aged 12 to 40 months, to improve their developmental outcomes. We hypothesize that children receiving four months of oral iron plus dietary advice will have better developmental outcomes than those who receive placebo plus dietary advice.

Secondary objectives include comparing four months of oral iron treatment plus dietary advice versus placebo plus dietary advice, for the following secondary outcomes: laboratory measures of iron indicators (serum ferritin and hemoglobin), and behavioral outcomes such as temperament, in children with NAID.
7.3 Methods

7.3.1 Study design, setting, participants, interventions and control, outcomes and measures and participant timeline

Study design

The ‘Optimizing Early Child Development for Young Children with Non-Anemic Iron Deficiency in the Primary Care Practice Setting’ (OptEC) study is designed as a multi-site, pragmatic, placebo controlled, superiority randomized trial. From a screened cohort, children identified with NAID are randomly allocated in a 1:1 ratio to each treatment group. This trial has been designed along the pragmatic end of the pragmatic-explanatory continuum, since it was designed to primarily inform decision making (203). Specifically, eligibility criteria, participant compliance, intensity of follow-up, and primary analysis follow pragmatic approaches, while practitioner expertise and adherence, intervention, and follow-up of outcomes follow approaches midway along the pragmatic-explanatory continuum. This protocol was designed following the Standard Protocol Items: Recommendations for Interventional Trials (SPIRIT) guidelines, and results will be reported according to the 2008 Consolidated Standards for Reporting Trials (CONSORT) guidelines for pragmatic trials (204, 205).

Setting

This multi-site study is being conducted in the offices of primary care practices participating in the TARGet Kids! practice-based research network. The “Optimizing Early Child Development in the Primary Care Practice Setting: Pragmatic Randomized Trial of Iron Treatment for Young Children with Non-anemic Iron Deficiency (OptEC)” trial is embedded in the TARGet Kids! cohort. The screening cohort includes all eligible children attending their well-child visit with their primary care physician. To date, TARGet Kids! practice sites are located in Toronto, Ontario. There are currently seven pediatric practices and three family medicine practices involved in patient recruitment. Each practice has between three and 10 practicing physicians.

Participants

Children aged 12 to 40 months, whose parents consent to participate in the TARGet Kids! study, constitute the screening cohort of this trial. These parents are given a letter containing a short
description of the OptEC trial. This letter states that if their child is found eligible, the parents will be contacted by phone to invite them to participate in the OptEC trial.

Eligibility for randomization

All participants in the screening cohort undergo laboratory screening for iron deficiency, and children are assigned to one of three categories based upon the results of their hemoglobin, serum ferritin, and C-reactive protein (CRP):

1. NAID, determined by hemoglobin ≥110 g/L, serum ferritin <14 μg/L, and CRP <10 mg/L.
2. IDA, determined by hemoglobin <110 g/L, serum ferritin <14 μg/L, and CRP <10 mg/L.
3. IS, determined by hemoglobin ≥110 g/L, serum ferritin ≥14 μg/L, and CRP <10 mg/L.

In this trial, only children diagnosed with NAID are randomized to the intervention and control groups. The other two groups (IDA and IS) are non-randomized comparators (Figure 13: schematic of study plan). However, for all three groups, we exclude children with any of the following: a CRP level ≥10 mg/L; a previously diagnosed developmental disorder; a genetic, chromosomal or syndromic condition; chronic medical condition (with the exception of asthma and allergies), including chronic anemia, iron deficiency, or recent oral iron supplementation or treatment; prematurity, with a gestational age of less than 35 weeks; low birth weight less than 2,500 g; attending the office for an acute illness, such as a viral illness, or other health concern other than for a well-child assessment; any contraindications to receiving elemental iron; the use of any natural health product containing the same medicinal ingredient(s) as the investigational product; or if English is not spoken to the child in the home or in a child care setting.
Figure 13: Schematic of the “Optimizing early child development in the primary care physician practice setting: Pragmatic randomized trial of iron treatment for young children with non-anemic iron deficiency (OptEC) trial.

**Baseline Measurements:**
- **TARGET Kids! Child and Family Questionnaire**
- Laboratory measurements: Hemoglobin, serum ferritin, mean corpuscular volume (MCV), C-reactive protein (CRP)
- Individual child behavior characteristics/ temperament assessed using the IBQ/ECBQ/CBQ
- Physical measurements: Child height, weight and waist circumference and parent weight and height

**Baseline Developmental Assessment:**
- Mullen Scales of Early Learning (MSEL)

**SCREENING COHORT**
- N = 1,500
- Baseline Measurements:
  - Non-Anemic Iron Deficiency (NAID) hemoglobin >110 g/L and serum ferritin <14 μg/L
  - Iron Deficiency Anemia (IDA) hemoglobin <110 g/L and serum ferritin <14 μg/L
  - Iron sufficient (IS) hemoglobin >110 g/L and serum ferritin ≥14 μg/L

**CHILDREN ELIGIBLE FOR THE RANDOMIZED CONTROLLED TRIAL**
- N = 150
- Baseline Developmental Assessment: Mullen Scales of Early Learning (MSEL)
- Randomize
  - N = 75 Oral iron treatment x 4 months
  - N = 75 Oral placebo x 4 months Plus dietary advice

**CHILDREN NOT ELIGIBLE FOR THE RANDOMIZED CONTROLLED TRIAL**
- N = 25
- Baseline Developmental Assessment: Mullen Scales of Early Learning (MSEL)
- Oral iron x 4 months Plus dietary advice
  - No treatment

**OUTCOMES**
- **Primary outcomes at 4 months:**
  - Child development assessed with the Mullen Scales of Early Learning (MSEL)
- **Secondary outcomes at 12 months:**
  - Laboratory measures of iron status: Hemoglobin and Serum Ferritin

**SCREENING COHORT**
- Baseline Developmental Assessment: Mullen Scales of Early Learning (MSEL)

**CHILDREN ELIGIBLE FOR THE RANDOMIZED CONTROLLED TRIAL**
- Baseline Developmental Assessment: Mullen Scales of Early Learning (MSEL)
- Randomize
  - N = 75 Oral iron treatment x 4 months
  - N = 75 Oral placebo x 4 months Plus dietary advice

**OUTCOMES**
- **Primary outcomes at 4 months:**
  - Child development assessed with the Mullen Scales of Early Learning (MSEL)
- **Secondary outcomes at 12 months:**
  - Laboratory measures of iron status: Hemoglobin and Serum Ferritin

Intervention and control

Children with NAID are randomized to receive either oral iron treatment (6 mg elemental iron/kg/day) or placebo (equivalent volume) in two divided doses for four months (206). A drop-based formulation containing ferrous sulfate (Fer-In-Sol™, Mead Johnson Nutrition, Evansville, Indiana, USA)) was chosen as the active agent to facilitate ease of administration to young children. The placebo is developed by the Hospital for Sick Children compounding pharmacy, and similar in color and taste to the active agent.

Children in both the oral iron and placebo group are also given dietary advice to improve iron intake. A guideline for improving iron intake was developed to serve this purpose (see appendix 4: iron intake guideline). It is based on the recommendations in Canada’s Food Guide and the Hospital for Sick Children’s online guideline to improve iron intake in children (112, 207). Dietary advice includes recommendations on the varied sources of foods containing high amounts of iron, foods that increase and inhibit iron absorption, and dietary habits that may prevent iron deficiency (such as maximum daily cow’s milk intake and limiting the intake of juice).

Concomitant interventions permitted include over the counter multivitamins which do not contain iron; those prohibited include additional over the counter iron and prescription iron. To monitor adherence at the end of the trial, parents are asked to return bottles, and the amount of iron administered is calculated based on the volume of solution remaining. Parents in both groups are advised of possible adverse effects of oral iron (constipation and black stools), which are reversible and non-harmful, and are encouraged to remain compliant if these develop. A participant information sheet that contains information on the study drug is provided to parents who agree to participate in the trial. No specific criteria are being used for discontinuation or modification of the interventions, as the dose of iron is within the safe and recommended dosages for children (206).

Assignment of the interventions to the treatment groups is randomized and the randomization is stratified by clinic site. Block randomization is generated with blocks of variable sizes to ensure that group sizes are similar at the end of each block (208). The randomization sequence for each clinic site is generated using computer-generated random numbers by a biostatistician. Allocation concealment is achieved by having the pharmacy department at the Hospital for Sick Children prepares the treatment and placebo in sealed, serially numbered bottles of similar appearance and weight, according to the
allocation sequence. Parents, attending physicians, laboratory personnel, and study personnel conducting the outcome assessments, and data analysts and investigators are blind to the group allocation. Study medication and placebo are supplied in bottles that look identical, and the appearance, consistency, and taste of the liquid are similar. Group allocation will remain concealed until the final data analysis is performed.

If a subject in the randomized study deteriorates or has persistent, severe, bothersome side effects then unblinding may be necessary. Emergency unblinding will only be done when the clinical treatment of the patient will be different by knowing which arm of the study the patient was on. The physician caring for the subject will contact the principal investigator or co-investigator first to discuss the unblinding procedure. The study investigators should remain blinded if possible.

Non-randomized children

Children who are identified with IDA and IS constitute the non-randomized part of the OptEC trial (Figure 1: schematic of study plan). Children with IDA receive oral iron treatment, 6 mg elemental iron/kg/day, in two divided doses for four months plus dietary advice, which is considered standard of care. Children with IS do not receive any intervention.

Outcomes and measures

The primary outcome for the OptEC trial is the Early Learning Composite (ELC), assessed using the Mullen Scales of Early Learning (MSEL). The MSEL measures five distinct developmental skills: gross motor and four ‘cognitive’ skills (fine motor, visual reception, receptive language, and expressive language). The four cognitive skills are summarized and converted into age-adjusted normalized ELC scores, which has a mean of 100 and a standard deviation of 15 (209). Secondary outcomes include two laboratory measures of iron status (hemoglobin and serum ferritin levels) and measurement of individual child behavior characteristics (known as child temperament), which is assessed using age-appropriate parent reported questionnaires: the Infant Behavior Questionnaire (IBQ), the Early Childhood Behavior Questionnaire (ECBQ), or the Children’s Behavior Questionnaire (CBQ).

Rationale for selection of outcome measures for this study
Assessment of cognitive and motor function

The MSEL is an individually administered scale for assessment of development that may be applied to young children from birth to 68 months (209). The standardization sample mainly represented Caucasian American children from urban communities with middle class socioeconomic status. All scales of this tool have adequate test floors: that is, a child of any age can score at least two standard deviations below their respective means. Administration of the MSEL requires approximately 30 to 40 minutes. The psychometric properties of the MSEL have been shown to be adequate (210). The MSEL has been used to assess development in several pediatric conditions, including autism spectrum disorders, profound hearing loss, genetic conditions, biliary atresia, language delay, and congenital hypothyroidism (211-214). Although the Bayley Scales of Infant Development (BSID) is the most common development assessment tool used in previous studies of iron deficiency, a recent study has demonstrated that the BSID may underestimate developmental delay in children (125, 215). For the current study, considering its strong psychometric properties, similarity of the population used in this trial with the standardization population, and its extensive use in the pediatric population, the MSEL has been selected as the scale to assess cognition and motor development in children.

Assessment of children’s social and emotional behavior

There is a reported association between iron deficiency and altered infant social-emotional behavior, including shyness, frustration, poor engagement, sootheability, and affect, as measured in laboratory settings (216-218). We have selected three age-appropriate validated parent-completed questionnaires for the assessment of individual characteristics of child behavior, specifically known as child temperament: the IBQ for infants aged 12 to 17 months old, the ECBQ for toddlers aged 18 to 36 months, and the CBQ for preschoolers aged 37 to 72 months (219, 220). Three dimensions of temperament are measured: negative affectivity, surgency or extraversion, and effortful control. Our research team has recently studied pre-school children’s temperament using the CBQ and found children who scored highly on the negative affectivity scale had significant association with higher nutrition risk, suggesting that child temperament may be associated with nutritional disorders such as obesity and iron deficiency (221).

Laboratory measures of iron status (hemoglobin and serum ferritin)
The hemoglobin cut-off level of >110 g/L distinguishes anemia from non-anemia in children under five years of age (41, 53, 72). In adults, serum ferritin has been found to be the most appropriate test for diagnosis of IDA, with a cut-off level of ≤15 μg/L, and a recommended cut-off level of <10 or <12 μg/L for children (39, 40, 64). Our laboratory measures serum ferritin using a Roche modular platform (Roche Diagnostics Limited, Rotkreuz, Switzerland) and hemoglobin is measured using the Sysmex platform (Sysmex Canada, Mississauga, ON, Canada) (190, 191). The Roche modular platform uses a corrective method to analyze serum ferritin (222). This correction increases the cut-off level of serum ferritin for children to <14 μg/L. Hence, for operational purposes, our trial uses this corrective value of <14 μg/L of serum ferritin to distinguish between iron deficiency and iron sufficiency. Other population-based research, such as the National Health and Nutrition Examination Survey (NHANES), uses similar serum ferritin levels to identify iron deficiency in children (222).

Because serum ferritin is an acute phase reactant, concurrent measurement of CRP has been recommended (40). An elevated level of CRP suggests that the ferritin level may be falsely elevated (9). Hence, we excluded these children from our sample.

Participant timeline
Participants (NAID, IDA, and IS) are assessed at three time points: baseline, four, and 12 months after the baseline visit. At the baseline assessment children are first screened for serum ferritin, hemoglobin, and CRP to identify their iron status. A venous blood sample (3 mL) is used to measure the baseline iron status. According to the results of the screening laboratory test, those who are eligible and agree to participate in the OptEC trial are asked to come back to the physician’s office to complete their baseline developmental testing (MSEL). Other baseline data include a parent-completed, standardized data collection form based on questions used in the Canadian Community Health Survey (170). The following data is collected: child and family characteristics (including demographic data, socioeconomic status, ethnicity, family structure, child care, and familial illnesses), child diet, physical activity, and health. Individual child behavior characteristics known as child temperament are also assessed using the IBQ, ECBQ, or CBQ. Measurement of height and length, weight, and waist circumference of participants and their accompanying parent is performed using standardized anthropometric protocols (223).
After the baseline assessment, intervention is provided for four months. The four-month follow-up visit is considered the time of the primary outcome assessment, and includes measurement of: development (MSEL), temperament (IBQ, ECBQ, or CBQ), and anthropometric and laboratory tests (serum ferritin, hemoglobin, and CRP). Parents are asked to complete a follow-up questionnaire (see appendix 5: four-month follow-up form) that collects data related to administration of the study drug, causes of non-adherence, adverse effects, and an approximate per week rate of missed doses of the study drug. The questionnaire also collects data on children’s frequency of illness during the 4 month period.

The 12-month follow-up visit is a non-intervention longitudinal follow-up visit for children in all three iron groups (Table 11: Schedule of procedures, assessments, and interventions). At the 12-month follow-up visit developmental, temperament, anthropometric, and laboratory (serum ferritin, hemoglobin, and CRP) testing is performed.
Table 11: Schedule of procedures, assessments, and interventions

<table>
<thead>
<tr>
<th>TIME POINT</th>
<th>1 weeks</th>
<th>0</th>
<th>4 months</th>
<th>4 months</th>
<th>12 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>STUDY PERIOD</td>
<td>Pre-randomization</td>
<td>Intervention</td>
<td>Post-intervention follow-up</td>
<td>Post-intervention follow-up</td>
<td></td>
</tr>
<tr>
<td>SUBJECTS</td>
<td>Screening Cohort N=1500</td>
<td>RCT subjects n=150</td>
<td>RCT subjects n=150</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**INTERVENTIONS:**

- Intervention
  - X
  - X
- Control
  - X
  - X

**PROCEDURES:**

- Informed consent
  - X
- Eligibility screen
  - X
- Randomization
  - X

**ASSESSMENTS:**

- **TARGet Kids! Child and Family Questionnaire**
  - X
- Laboratory measurements:
  - Hemoglobin, serum ferritin, mean corpuscular volume (MCV), C-reactive protein (CRP)
  - X
  - X
  - X
- IBQ (12-17 months infants) / ECBQ (18-36 months children) / CBQ (37-72 months children)*
  - X
  - X
  - X
- Physical measurements: height and weight, waist circumference
  - X
  - X
  - X
- Mullen Scales of Early Learning (MSEL)
  - X
  - X
  - X
- 4 month follow-up questionnaire
  - X

* IBQ: Infant Behavior Questionnaire; ECBQ: Early Childhood Behavior Questionnaire; CBQ: Children’s Behavior Questionnaire
7.3.2 Recruitment, retention and data collection methods

Recruitment and retention
The TARGet Kids! consent form informs parents that their child, once enrolled in the cohort, may be eligible for a trial (such as the OptEC trial). Based on the laboratory results of the screening cohort, research assistants (RA) are instructed to contact the parents of potential participants by phone and request that they return to the physician's office if they agree to participate. The RAs are provided with a scripted telephone dialogue in order to standardize the patient recruitment process. At the physician’s office, the RA reviews the consent form with the parent, and the clinic nurse reviews the iron status of the child with the parent and obtains informed written consent. The family has the opportunity to ask the RA, the pediatrician, and/or clinic nurse any questions at any time. For families who provide consent, RAs conduct follow-up phone calls to ensure that the child is taking the study drug and address any questions parents may have. Since the study drug is given for four months, after the four-month follow-up visit no further contact with the participant is conducted before the 12-month follow-up visit. Two weeks prior to the 12-month follow-up visit the RA contacts the participant via phone and schedules the visit.

Data collection methods
Questionnaire data and physical measurements are collected by the RAs embedded in the practices. The RAs have been trained to ensure accuracy of data collection and the questionnaires have been extensively pilot tested. Blood work is obtained by trained personnel according to the arrangements established at each of the practice sites. The RA is responsible for ensuring the blood is delivered to Mount Sinai Services ((MSS) Toronto, Canada) for laboratory testing. MSS provides customized laboratory and research services to pharmaceutical and biotech companies, and researchers. Laboratory results are sent electronically to the data management center, as well as faxed to the practicing physicians offices. Children eligible for the randomized trial return to the physician’s office where developmental testing is completed using the MSEL, administered by a trained psychometrist under the supervision of a registered psychologist. At the four and 12-month follow-up visits, the RA is responsible for ensuring that the questionnaires and physical and laboratory measures are completed. The psychometrist completes the MSEL.
7.3.3 Data management, trial monitoring and adverse event reporting

Data management
The Applied Health Research Centre (AHRC) of the Keenan Research Centre, Li Ka Shing Knowledge Institute of St Michael’s Hospital, University of Toronto, serves as the data management centre for this trial. AHRC employs state-of-the-art web-based data management software RAVE™ (version 5.6.3, Medidata Solutions Inc., New York, USA), which uses secure encrypted web-based data capture technology and is the repository for data collected during this study. It has user configurable workflows, sophisticated case report form (CRF) design, complex edit checking, and customized security parameters. Our RAs enter data remotely in real time to the central database from any of the practice sites. RAVE has extensive built-in reporting capabilities, and data can be exported to standard formats for data analysis [for example, to SAS (Statistical Analysis System) software]. Laboratory tests are directly uploaded to RAVE through a secure web portal.

Trial monitoring
A Data Monitoring Committee was not deemed necessary, as the experimental intervention (oral iron treatment with 6 mg elemental iron/kg/day given once daily or in two or three divided doses daily for four months plus dietary counseling) is the standard of care for children in the same age group with IDA, and the side effect profile is well known. In the current study of children with NAID, similar side effects are expected and are collected.

Adverse event reporting
All adverse events will be reported to the Hospital for Sick Children Research Ethics Board, according to the Hospital for Sick Children’s adverse event reporting requirements. All adverse drug reactions to the study medication will be reported to Health Canada within 15 calendar days or, for death or life-threatening events, within seven calendar days. In the latter case, a follow-up report must be filed within eight calendar days. Adverse reactions will be managed according to the Hospital for Sick Children’s standard clinical management practices.

7.3.4 Statistical analysis and power calculation

Statistical analysis
From the screening cohort, the baseline characteristics of the three groups (NAID, IDA, and IS) will be compared with descriptive statistics and significance testing. Categorical variables will be compared with a chi-square test, and continuous variables will be compared with an analysis of variance (ANOVA), or non-parametric equivalent. For participants with NAID randomized to treatment or placebo groups, no significance testing will be performed on the baseline characteristics; however, we will note any imbalances that have arisen by chance which may be clinically meaningful. All children with NAID randomized to treatment or placebo will be analyzed in the group to which they were randomized, following the intention-to-treat principle. In the primary analysis, the difference in developmental and hematologic measures in children with NAID randomized to treatment versus placebo will be assessed using linear regression, with the initial baseline measures included as the adjusting variable [analysis of covariance (ANCOVA) method] (224). In a secondary analysis, additional covariates of clinical or statistical significance (including parent education and family income) will be included in the model. The primary analysis will be a fixed effects model, ignoring stratification by clinic site. A secondary analysis will include a confirmatory analysis using a mixed effects model, with interventions and MSEL scores as the fixed effect and clinic site as the random effect. The non-randomized groups (IS and IDA) will be compared with the NAID groups for difference in their follow-up developmental and hematological outcome using ANOVA. Although efforts to ensure complete data collection and participant follow-up will be maximized, analytic strategies to handle missing data will include imputation techniques, if appropriate. If more than 20% of participants are lost to follow-up, a per-protocol analysis will be carried out in addition to the intention-to-treat analysis.

Power calculation

Clinically meaningful difference in tests of cognition
The minimal clinically important difference (MCID) for the primary outcome of interest (child development as measured by MSEL) has been carefully considered by our research team (225). In a landmark longitudinal study of infants with IDA compared with IS infants followed from infancy (12 to 23 months) through to 19 years of age, infants with IDA were found to have an eight to nine point cognitive disadvantage in infancy. In a subset of low-income infants the gap widened from 10 points in infancy to 25 points by 19 years (134, 137-139). Another pivotal study by Walter et al. has suggested
that the MCID for cognitive difference in children may be as low as six points (226). Studies in older children have shown that a 15 point cognitive disadvantage at age 11 years conferred a relative risk of 0.79 of being alive 65 years later, and a 30 point disadvantage reduced this to 0.63 (227). From these studies (and a larger body of literature identifying the association between cognition and education, employment, and health) 15 points or greater is clearly clinically meaningful. However, it is important for a trial to have the power to identify the minimally important difference, which might be as low as a six to eight point difference.

Sample size calculation for the randomized part of the OptEC trial (see Figure 13: schematic of study plan) is based primarily on a presumptive effect estimate of the ELC score, which we considered as an MCID. ELC being a summarized indicator of child cognition, we arrived at a sample size estimate through a sensitivity analysis considering an array of possible MCIDs (six to eight point difference) for children’s cognitive development.

From previous research, it is anticipated that the mean ECL score for children with NAID is 90, and the standard deviation is ± 15 (127, 128, 161). To detect a six to eight point difference in post-treatment ELC score, with a power of 80% and a significance level of 5%, a total sample size ranging from 112 to 198 (56 to 99 per group) is required. We targeted an approximate sample of 150 (75 per group). Sample size calculation was performed using the t-test formula (224). With an estimated prevalence of NAID of 10%, it is anticipated that screening approximately 1,500 children will identify 150 children with NAID to be randomized over a four-year period (41, 222, 228). Expecting potential drop-outs and withdrawals to be between 0 and 20%, a total of 180 NAID children will be randomized.

For the non-randomized children (IDA and IS), it is anticipated that the mean ELC score for children with IDA is 85, and the mean developmental score for IS children is 100, and both have an ELC standard deviation of ± 15 (127, 128, 161). From the screening cohort of 1,500, it is anticipated that 1 to 2% will have IDA (n = 25), and an equal number of randomly selected children with IS (n = 25) will be sampled for comparison (222, 228). To randomly select children with IS, once a child with IDA is identified, the immediate next child identified with IS who agrees to participate is enrolled in the trial.

7.3.5 Ethics and dissemination
Ethical conduct of the OptEC trial

The OptEC trial was granted ethics approval by The Hospital for Sick Children Research Ethics Board [(REB) file number: 1000027782] on 10 May 2012 and approval is renewed annually by the REB. This study has been registered as a clinical trial (Clinicaltrials.gov identifier: NCT01481766). Written informed consent is obtained from parents of all child participants prior to any data collection. The OptEC trial has different consent forms for the three groups of children (NAID, IDA, and IS) based on their iron status and provision of intervention. All data collection forms and supporting documents (iron intake guideline, participant information sheet, and telephone script) were approved by the Hospital for Sick Children REB. Blood results are provided to the child’s physician within 24 to 48 hours. Detailed reports of the MSEL are available in approximately four weeks. Parents and physicians may perceive the opportunity for a cognitive assessment and laboratory testing to be a direct benefit of participation in this study. The investigators considered the inclusion of dietary advice and assessment of outcomes at four months in all randomized children to be consistent with good clinical practice. If at four months children in either the intervention or control group have persistent NAID or have progressed to IDA, they are treated and monitored accordingly by their primary physician.

7.4 Knowledge translation / Dissemination

Findings from this research will be disseminated directly to the physician participants and to their patients. An annual meeting of all the TARGet Kids! Practice staff (physicians, nurses, and office staff), research team (investigators, research assistants, and students), and policy leaders (representatives from Section of Community Pediatrics, Department of Family and Community Medicine, and parent representatives) will occur. Parents of participants will receive the summary of their child’s developmental assessment, anthropometric measures, and laboratory measures, leading to a direct benefit for individual participants. Further downstream dissemination to primary care physicians will occur through formal and informal venues at local levels, such as educational rounds (for example City Wide Pediatric Rounds and SickKids Annual Pediatric Update) and held by local physician groups. End of grant knowledge will be shared with the academic community through publication in relevant journals and presentations at national and international conferences (Annual Meetings of the Pediatric Academic Societies, and the Canadian Paediatric Society), and locally
through hospital rounds and presentations, and through our TARGet Kids! website (189). Messages will be relevant to professionals working in the fields of pediatrics, family medicine, developmental pediatrics, nutrition, nursing, dietetics, and public health. We will also share our findings with colleagues at the Canadian Paediatric Society and the American Academy of Pediatrics. The principal applicant is a member of the Canadian Task Force for Preventive Health Care and will participate in the upcoming guideline development for developmental screening and screening for IDA. Opportunities for coverage in lay publications and media will be sought using an experienced knowledge broker at SickKids Department of Public Relations.

7.5 Discussion: An internal pilot study to guide the OptEC trial

The estimates used for the calculation of the sample size for the OptEC trial were derived from previous trials which differed from the trial currently being designed; for example, different patient population, small numbers of centers, and different treatment duration (229, 230). Application of prior estimates for power calculation of the current trial may lead to an unnecessarily large trial, or the trial may not be large enough to have sufficient power for detection of a clinically relevant treatment effect (229, 231). Therefore, an internal pilot study was initiated.

7.5.1 Rationale for internal pilot study

An internal pilot is incorporated into the main study design of a randomized controlled trial to obtain important parameter estimates. It forms an integral part of the trial itself and is not a separate study. The protocol of the randomized controlled trial designates the first phase of the trial as a ‘pilot’ phase. These estimated parameters are then used to recalculate the sample size or improve the design and conduct of the clinical trial (229, 230). At the end of the trial, data analyses incorporate those collected during the internal pilot, as well as those collected subsequently. Building an internal pilot study into a clinical trial has very small adverse effect on the significance level (229, 232). Other possible uses of internal pilot data include checking the assumptions regarding adherence of the participants to the study intervention (229, 233). Non-adherence may decrease the probability of detecting treatment differences and affect the interpretation of observed differences. Poor adherence can also jeopardize the outcome of clinical trials by reducing their power. Using data from an internal pilot can provide the
anticipated adherence level of participants of a larger clinical trial and can be used to implement strategies to enhance compliance (234, 235).

7.5.2 Aim of the OptEC trial internal pilot study

The objectives of the internal pilot study are: to obtain a reliable estimate of the standard deviation ($S_2$) of the primary outcome of the OptEC trial; to obtain the correlation between the baseline and follow-up measurement of the primary outcome; to recalculate the sample size of the OptEC trial using the estimates generated from the internal pilot; and to assess the adherence rate and causes of non-compliance in children enrolled in the pilot study.

7.5.3 Sample size for the internal pilot study

Several authors have considered approaches to pre-selecting the sample size for internal pilot studies. One approach has shown through simulation that to receive a reliable estimate of the true population parameter, the minimum size for an internal pilot should be at least 10 subjects per treatment group for a two-group randomized study (229). The sample size for the OptEC trial is approximately 150 (75 per group) NAID subjects to be randomized over a period of four years. Thus, we planned an internal pilot using the first 30 NAID (15 per group) subjects randomized to the two treatments groups.

7.5.4 Methods

When the trial has assessed the endpoints for the 30 participants in the internal pilot, we will calculate the observed standard deviation within each group and pool them to obtain an estimated standard deviation ($S_2$). If $S_2 \leq 15$, the trial will continue as planned, so that the total sample size remains between 112 and 198. However, if $S_2 > 15$, we will recalculate the sample size using the new estimate of the standard deviation ($S_2$) (229). Recalculation of the sample will be performed using the ANCOVA method. This method uses the correlation between the baseline and follow-up measurement of the primary outcome in sample size calculation (224).
For assessment of adherence, an adherence rate will be calculated using data from the internal pilot study (235, 236). The four-month follow-up form of the OptEC trial collects data on causes of non-adherence, adverse effects, and an approximate per week rate of missed doses of the study drug. This self-reported measure of missed doses will be used to assess the rate of compliance using a method proposed by Klerk et al. (237). In this method, summaries such as the total number of days in which no doses were taken, the length of the monitored interval, and the overall percentage of prescribed doses taken is used to calculate a rate of adherence (236, 237). Adherence rate will be described by placing participants into broad bands, with the percentage of patients in each band. The causes of non-adherence will be identified and summarized as percentage.
Chapter 8: An internal pilot study for a randomized trial aimed at evaluating the effectiveness of iron interventions in children with non-anemic iron deficiency: the OptEC trial

This chapter describes the last of the four original research works of the candidate. The purposes of this chapter are to:

1. Describe the rationale for conducting an internal pilot study for the OptEC trial
2. Describe the objectives, methods, results and implication of the internal pilot study

Published in manuscript form:

This manuscript has been peer reviewed. See responses to peer review in appendix 6

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8.1 Abstract

Background: The OptEC trial aims to evaluate the effectiveness of oral iron in young children with non-anemic iron deficiency (NAID). The initial sample size calculated for the OptEC trial ranged from 112-198 subjects. Given the uncertainty regarding the parameters used to calculate the sample, an internal pilot study was conducted.

Objectives: To obtain reliable estimate of parameters (standard deviation and design factor) to recalculate the sample size and to assess the adherence rate and reasons for non-adherence in children enrolled in the pilot study.

Methods: The first 30 subjects enrolled into the OptEC trial constituted the internal pilot study. The primary outcome of the OptEC trial is the Early Learning Composite (ELC). For estimation of the SD of the ELC, descriptive statistics of the 4 month follow-up ELC scores were assessed within each intervention group. The observed SD within each group was then pooled to obtain an estimated SD ($S_2$) of the ELC. Correlation ($\rho$) between the ELC measured at baseline and follow-up was assessed. Recalculation of the sample size was performed using analysis of covariance (ANCOVA) method which uses the design factor (1 - $\rho^2$). Adherence rate was calculated using a parent reported rate of missed doses of the study intervention.

Results: The new estimate of the SD of the ELC was found to be 17.40 ($S_2$). The design factor was, (1 - $\rho^2$) = 0.21. Using a significance level of 5%, power of 80%, $S_2$ = 17.40 and effect estimate ($\Delta$) ranging from 6-8 points, the new sample size based on ANCOVA method ranged from 32-56 subjects (16-28 per group). Adherence ranged between 14% and 100% with 44% of the children having an adherence rate $\geq$86%.

Conclusion: Information generated from our internal pilot study was used to update the design of the full and definitive trial (recalculation of sample size, determine the adequacy of adherence and application of strategies to improve adherence).
8.2 Introduction

This update reports the findings from an internal pilot study that aimed to obtain parameter estimates for recalculation of the sample size of the OptEC trial (Optimizing Early Child Development in the Primary Care Practice Setting) and also assess the adherence rate of the participants in the internal pilot study.

The OptEC trial aims to evaluate the effectiveness of oral iron plus nutritional guidance over placebo plus nutritional guidance in children with non-anemic iron deficiency (NAID) in improving their developmental, hematological and behavioral outcomes. Sample size for the OptEC trial ranged from 112-198 (N_a) participants (using a standard deviation, S_1 = 15 and a range of effect estimates, Δ of 6-8 points). The sample size was calculated using the t-test. A detailed description of the OptEC trial has previously been published (187).

The design of the OptEC trial includes an internal pilot study (238). The rationale for conducting an internal pilot for the OptEC trial was three fold. First, there was uncertainty regarding the parameters used to calculate the sample size for the OptEC trial (230). The estimates used were obtained from previous trials which had different study conditions, for example, different population, small numbers of centers and different treatment duration. Thus, prior estimates may not be representative of the current trial. Inaccurate estimates may lead to an unnecessarily large trial or a trial not large enough to have sufficient power for detection of a clinically relevant treatment effect. Data generated from internal pilots are used to obtain more reliable estimates of parameters for recalculation of sample size of clinical trials (229-232).

Second, we aimed to recalculate the sample size for the OptEC trial using the method known as the ANCOVA (analysis of covariance). One advantage of the ANCOVA method is that it accounts for the correlation between the baseline and follow-up assessment of the primary outcome, in the calculation of the sample size (224). Thus the sample size calculated using this method will have the same power as the t-test but will require fewer subjects (224). In order to use the ANCOVA method, we need to know the correlation between the baseline and follow-up assessment of the primary outcome of the OptEC trial which we intend to calculate using our internal pilot data.
Third, evidence suggests low level of adherence to interventions in clinical trials investigating the efficacy of oral iron interventions (156). A review of adherence in primary school children showed the adherence rate to oral iron to range between 50%-90% (141). Lack of adherence may decrease the probability of detecting treatment differences and affect the interpretation of observed differences. However, partial adherence is usually sufficient to evaluate the effectiveness of an intervention. According to previous research, if 40% of the participants in a randomized trial have at least 90% of adherence then the effectiveness assessment process remains unimpaired (239). Data from an internal pilot study can be used to check the level of adherence in participants of clinical trials (234-236). If adherence is found to be less than desired, then strategies can be implemented to improve adherence.

The overall aim of the internal pilot was to inform the design of the full and definitive trial. Thus, the objectives of the internal pilot study were (1) to obtain a reliable estimate of the standard deviation ($S^2$) of the primary outcome of the OptEC trial; (2) to obtain the correlation between the baseline and follow-up measurement of the primary outcome; (3) to recalculate the sample size ($N_r$) of the OptEC trial using the estimates generated from the internal pilot; and (4) to assess the adherence rate and causes of non-adherence in children enrolled in the pilot study.

At the end of the pilot study, if necessary the sample size will be recalculated and compliance measures may need to be enhanced. Otherwise, the OptEC trial will continue following the protocol as previously reported (187). During the collection and analysis of data for the internal pilot study the recruitment of the trial continued.

### 8.3 Methods

The internal pilot study is an integral part of the OptEC trial, which consisted of the first few participants enrolled in the trial. Hence, it follows the same design and conduct of the main trial. In the following sections we describe methods that are particularly relevant to the internal pilot study as recommended by Thabane et al. for reporting of pilot study results (240).

8.3.1 Design, participants, intervention and control, primary outcome and measures
Design
The OptEC trial, hence the internal pilot study was a multi-site, pragmatic, placebo controlled, superiority randomized trial (187).

Participants
Eligibility criteria for participation in the internal pilot were the same as those for the OptEC trial. Inclusion criteria: children with NAID [hemoglobin >110 g/L, serum ferritin < 14 µg/L and C-reactive protein (CRP) <10 mg/L]; and age 12 to 40 months. Exclusion criteria: CRP level >10 mg/L, previously diagnosed developmental disorder, genetic, chromosomal or syndromic condition, chronic medical conditions (except asthma and allergies), including chronic anemia, recent oral iron supplementation or treatment, gestational age less than 35 weeks, low birth weight less than 2500 grams, attending the office for an acute illness, any contraindications to receiving elemental iron, the use of any natural health product containing the same medicinal ingredient(s) as the investigational product, English not spoken to the child in the home or in a child care setting.

Intervention and control
Children are randomized to receive either oral iron treatment (6 mg elemental iron/kg/day) or placebo (equivalent volume) twice daily for four months. Children in both the oral iron and placebo groups are also given nutritional guidance to improve iron intake which includes recommendations on the varied sources of high iron containing foods, foods that increase and inhibit iron absorption, and dietary habits that may prevent iron deficiency (such as - maximum daily cow’s milk intake, limiting the intake of juice). Concomitant interventions permitted include over the counter multivitamins which do not contain iron; those prohibited include additional over the counter iron and prescription iron.

Primary outcome and measures
The primary outcome for the OptEC trial is the Early Learning Composite (ELC) assessed using the Mullen Scales of Early Learning (MSEL) (209). The MSEL measures five distinct developmental skills, gross motor and four “cognitive” skills - fine motor, visual reception, receptive language, and expressive language. The four cognitive skills are summarized and converted into age adjusted normalized ELC, which has a mean of 100 and a standard deviation of 15. Developmental assessment using the MSEL is performed at baseline and after 4 months of intervention by a trained psychometrist.
under the supervision of a registered psychologist. All individuals involved with data collection, entry and analysis are blind to the group assignment.

8.3.2 Collection of other variables

Baseline data collection for the internal pilot included age and sex of child, birth weight, weight, length/height, maternal ethnicity and education, family income and some nutritional behavior characteristics (total duration of breastfeeding, volume of cow’s milk intake and current bottle feeding). These data were collected using a parent-completed, standardized data collection form based on questions used in the Canadian Community Health Survey. We dichotomized family income based on median income of families in the city of Toronto (241).

Adherence related data were collected during the 4 month follow-up visit which included reasons for non-adherence, adverse effects and a parent reported weekly rate of days the study intervention was not taken.

8.3.3 Sample size for the internal pilot study

The minimum size for an internal pilot study should be at least 10 subjects per treatment group, for a two group randomized trial (229). The pre-planned sample size for the OptEC trial ranged from 112-198 (approximately 150 subjects) (187). The first 15 participants per treatment group enrolled in the OptEC trial were considered as the internal pilot study (total n=30).

8.3.4 Statistical methods for the internal pilot study

For estimation of parameters for sample size recalculation, descriptive statistics of the 4 month follow-up developmental data were assessed within each treatment group. The observed standard deviation of the ELC score within each treatment group was pooled to obtain an estimated standard deviation ($S_2$) of the ELC score (appendix 7 shows the formula for calculating the pooled standard deviation) (230, 242).
Recalculation of the sample size was performed using the analysis of covariance (ANCOVA) method which uses the design factor (or variance deflation factor) to calculate the sample size (224). The design factor is \((1 - \rho^2)\), where \(\rho\) is the correlation between the baseline and follow-up measurement of the primary outcome. The ANCOVA uses a two-step method to calculate sample size (224). Step 1: First, a sample size is determined using the two sample independent t-test and the new estimate of the SD \((S_2)\). Step 2: Then, the correlation \((\rho)\) between the baseline and 4 month follow-up measure of the ELC is calculated using Pearson’s correlation. This value is used to determine the design factor \((1 - \rho^2)\). The value of the sample sizes calculated using the t-test is then multiplied by the design factor \((1 - \rho^2)\) to produce the number of participants \((N_t)\) required by the ANCOVA method.

The reasons for non-adherence were identified and summarized. Adherence rate was calculated using a method proposed by Klerk et al (237). In this method, summaries such as - the total number of days per week the child received the study intervention, the length of the monitored interval and the over-all percentage of the study intervention taken during the monitored interval was used to calculate a rate of adherence. Furthermore, adherence rate was described by placing participants into broad bands of adherence. The adherence bands corresponded to the number of days per week the participants received the intervention, such as, \(\geq 6\) days correspond to 86% - 100% adherence; 4 - 5 days to 57% - 85%; 2 - 3 days to 28% - 56%; and \(\leq 1\) day to 0 – 27%. The percentage of children in each band was also determined.

For the purpose of the internal pilot study, the two treatment groups were identified as group A and group B by a third party, so as to keep group assignment blinded to all persons associated with the internal pilot study. Statistical analyses were performed using SAS software version 9.1 (SAS Institute, Cary NC) and sample size calculation was performed using the Vanderbilt University, Department of Biostatistics, power and sample size calculator (243).

Ethics approval
The OptEC trial was granted ethics approval by The Hospital for Sick Children Research Ethics Board (REB File No.: 1000027782) on May 10, 2012 and approval is renewed annually by the REB. Written informed consent is obtained from parents of all child participants (including the internal pilot study) prior to any data collection.
8.4 Criteria for success of the internal pilot study

Pre-specified criteria for success (240) for recalculation of the sample size were as follows: if the estimated SD $S^2 \leq 15$, the trial would continue as planned, that is the initial sample size ($N_a = 112-198$) will not change. However, if the estimated SD, $S^2 > 15$, then the sample size will be recalculated (230). For adherence related data, we projected that 40% of the participants in the internal pilot study will have 86% - 100% of adherence (the highest band of adherence). There was no stopping rule for the internal pilot study that would halt the OptEC trial.

8.5 Results

*Participant flow and baseline characteristics*

A total of 107 children with NAID were identified between June, 2012 and June, 2014. Of these children ultimately a total of 30 were randomized to the two intervention groups (see figure 14: participant flow diagram). Table 12 depicts the baseline characteristics of the participants in the internal pilot of the OptEC trial.
Figure 14: Participant flow diagram for the internal pilot study

Identified with NAID (n=107)

- Refused to participate (n = 24)
- Could not be contacted (n= 12)
- Did not meet eligibility criteria (n= 27)
- Participating in another trial (n= 14)

Randomized (n=30)

Participant left the study after randomization (n = 1)

Allocated to Group A intervention (n= 15)

- Lost to follow-up (n= 1)

Allocated to Group B intervention (n = 14)

Discontinuation of study drug and lost to follow-up (n = 1)

Analyzed (n= 14)

Analyzed (n= 13)
Table 12: Baseline characteristics of the children in the internal pilot study (N=30)

<table>
<thead>
<tr>
<th>Variables names</th>
<th>Descriptive data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age of child (months)</td>
<td>23.5 (6.95) †</td>
</tr>
<tr>
<td>Sex of child</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>15 (50) ‡</td>
</tr>
<tr>
<td>Female</td>
<td>15 (50) ‡</td>
</tr>
<tr>
<td>zBMI of child</td>
<td>0.49(0.93) †</td>
</tr>
<tr>
<td>Birth weight (kg)</td>
<td>3.3 (0.43) †</td>
</tr>
<tr>
<td>Maternal ethnicity</td>
<td></td>
</tr>
<tr>
<td>European</td>
<td>20 (74.07) ‡</td>
</tr>
<tr>
<td>Non-European</td>
<td>7 (25.93) ‡</td>
</tr>
<tr>
<td>Maternal education</td>
<td></td>
</tr>
<tr>
<td>University/Diploma</td>
<td>19 (70.37) ‡</td>
</tr>
<tr>
<td>College/non-university Diploma</td>
<td>5 (18.52) ‡</td>
</tr>
<tr>
<td>High School</td>
<td>2 (7.41) ‡</td>
</tr>
<tr>
<td>Family income*</td>
<td></td>
</tr>
<tr>
<td>Above median income</td>
<td>22 (81.48) ‡</td>
</tr>
<tr>
<td>Below median income</td>
<td>5 (18.52) ‡</td>
</tr>
<tr>
<td>Duration of breastfeeding (months)</td>
<td>14.56 (4.97) †</td>
</tr>
<tr>
<td>Volume of cow’s milk (cups/day)</td>
<td>2.46 (1.20) †</td>
</tr>
<tr>
<td>Currently use bottle</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>11 (42.31) ‡</td>
</tr>
<tr>
<td>No</td>
<td>15 (57.69) ‡</td>
</tr>
<tr>
<td>Serum ferritin (µg/L)</td>
<td>8.90 (2.47) †</td>
</tr>
<tr>
<td>Hemoglobin (g/L)</td>
<td>120.48 (9.34) †</td>
</tr>
</tbody>
</table>

† mean (SD)
‡ n (%)
*Income based on median income of families in the city of Toronto (11)
Estimation of the standard deviation ($S_2$) and recalculation of the sample size

Table 13 shows the SD of the follow-up developmental data. The SD of the ELC in group A and group B was 21.17 and 12.05, respectively. These two values were pooled to calculate an estimated SD, $S_2 = 17.4$. However, we observed a large difference between the SDs of the two treatment groups. An F-test was performed, where we were unable to reject the null hypothesis that the variances were equal ($F \text{ value} = 3.09$ and $p= 0.06$) (242). Therefore, the pooled estimate of the SD ($S_2$) was used to recalculate the sample size.

Table 13: Follow-up developmental data of the participants in the internal pilot study

<table>
<thead>
<tr>
<th>Developmental score</th>
<th>Group A n=14</th>
<th>Group B n=13</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SD</td>
<td>SD</td>
</tr>
<tr>
<td>Early Learning Composite (ELC)</td>
<td>21.17</td>
<td>12.05</td>
</tr>
</tbody>
</table>

SD: Standard Deviation

Using a significance level of 5%, a power of 80%, SD value of $S_2 = 17.4$ and clinically meaningful effect estimate ranging from 6-8 points, a range of sample sizes were calculated first using the t-test method (see table 14). Correlation ($\rho$) between the ELC measured at baseline and 4 month follow-up was 0.89. Hence, the design factor for assessment of the ELC was, $(1 - \rho^2) = 0.21$. Then, following the ANCOVA method for calculating sample size, the design factor was multiplied to the values of the sample size calculated using the t-test and a range of sample sizes for the OptEC trial was recalculated ($N_r = 32-56$) (see table 14).
Table 14: Range of sample sizes recalculated using internal pilot data

<table>
<thead>
<tr>
<th>Calculated sample size</th>
<th>( \alpha = 5%; 1-\beta = 80%; \text{ and } S_2 = 17.4 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \Delta = 8 )</td>
<td>( \Delta = 7 )</td>
</tr>
<tr>
<td>Using t-test</td>
<td>150</td>
</tr>
<tr>
<td></td>
<td>Multiplication by the design factor ( (1 - \rho^2) = 0.21 )</td>
</tr>
<tr>
<td>Using ANCOVA</td>
<td>32</td>
</tr>
</tbody>
</table>

\( \alpha \): significance level; 1-\( \beta \): power; \( S_2 \): SD and \( \Delta \): effect estimate

Assessment of adherence rate and causes of non-adherence

The main reasons for non-adherence among the internal pilot sample were - the study drug takes too long to administer, is too messy, child did not like it, too difficult to administer and forgot to give the study drug. Adverse effects reported by the parents included vomiting (19%), staining of teeth (34%), constipation (38%), loose stool (35%) and passage of black stool (46%). We found adherence to the study intervention to range between 14% and 100%. We then identified the number and proportion of children in each adherence band (see table 15). The highest adherence band (86 % - 100%) had a total of 12 (44%) children.

Table 15: Adherence rates of participants in the internal pilot study (N=27)

<table>
<thead>
<tr>
<th>Bands of adherence rate (%)</th>
<th>Number of participants in each band (n)</th>
<th>Percentage of participants in each band (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>86 - 100</td>
<td>12</td>
<td>44.44</td>
</tr>
<tr>
<td>57 - 85</td>
<td>6</td>
<td>22.22</td>
</tr>
<tr>
<td>28 - 56</td>
<td>2</td>
<td>7.41</td>
</tr>
<tr>
<td>0 - 27</td>
<td>7</td>
<td>25.93</td>
</tr>
</tbody>
</table>
8.6 Discussion

This internal pilot study allowed a data-driven recalculation of the sample size for a randomized controlled trial. If knowledge about the standard deviation of the primary endpoint is weak, this type of approach is superior to an ordinary fixed sample design, because the initial sample size can be appropriately adjusted.

According to our first objective, an estimate of the SD ($S_2 = 17.4$) of the primary endpoint was determined by pooling the observed SD in the two treatment groups (table 13). This estimate was found to be larger than the SD ($S_1 = 15$) we used to calculate the initial sample of the OptEC trial. Hence, as stated in our criteria of success, the new estimate of the SD was used to recalculate the sample size.

Our second objective, recalculation of the sample size involved the application of the ANCOVA method (224). This method requires the estimation of the design factor that accounts for the correlation between the baseline and follow-up assessment of the primary outcome, our third objective. We determined the design factor $[(1 - \rho^2) = 0.21]$ using data from the internal pilot study. The sample sizes calculated using the ANCOVA method ranged between 32-56 subjects which will have the same power as the sample sizes calculated using the t-test (150-266), despite being considerably smaller (see table 14) (224). In situations where recruitment of participants is a challenge, application of the ANCOVA method is a valid approach to minimize the sample size in clinical trials without affecting the power of the trial. In our OptEC trial, we aim to enroll a total of 56 participants (28/group), in order to detect a treatment difference of 6 points in the ELC score.

Our fourth objective was to determine the adherence rate in our internal pilot study. According to previous evidence $\geq90\%$ of adherence in 40\% of participants was shown to be ideal for effectiveness assessment (239). Since our adherence assessment was based on the number of days per week the child received the study intervention, our study team modified this criteria for success to $\geq86\%$ adherence, as this corresponds to $\geq6$ days in a week. In our study we found 44\% of participants having an adherence rate $\geq86\%$ (see table 15). We believe this level of adherence will be sufficient to meet the objective of the OptEC trial to assess the effectiveness of the study intervention.
Because some children had adherence rate as low as 14%, we have implemented strategies to improve adherence, such as - informing parents on possible adverse effects, decreasing the dose of the study intervention when children experience adverse effects like vomiting and diarrhea, counseling parents on minimizing the difficulty of giving the study intervention and motivating parents on continuing the study intervention when children experience non-harmful and reversible adverse effects like passage of black stool and staining of teeth. This information has been incorporated into a participant information handout which is given to parents when they agree to enroll their child in the OptEC trial.

At the end of the trial, participants in the internal pilot study will contribute to the overall sample and outcome analysis of the full trial. According to internal pilot methodology, this inclusion will have minimal impact on the significance level of the test of treatment effect (229). The adherence rate reported in this study may be an overestimate of the actual adherence, due to the fact that it was based on parent reported measures.

**8.7 Conclusion**

Information generated from our internal pilot study was used to update the currently ongoing OptEC trial. It not only provided us with a more reliable and representative estimate of the SD of the primary outcome, but also provided us with the necessary parameters needed to calculate our sample size using the ANCOVA method. Furthermore, our study depicts other valid uses of internal pilot data such as the estimation of adherence rate. The internal pilot design is one of the methods suggested by the StaR (Standards for Research in Child Health) standard development groups to overcome challenges faced when attempting to derive sample size estimates in pediatric research (244). The data generated from our internal pilot can inform future trials to be performed in similar population, particularly those in developed country settings.
Chapter 9 : A synopsis of the four original studies included in this thesis

In this thesis the findings from four original research studies are reported in chapter 5, 6, 7 and 8. The purposes of this final chapter are to:

1. Summarize the findings from these four studies
2. Discuss the strengths and weaknesses
3. Discuss the implication of the findings for clinicians and policy-makers
4. Provide a brief discussion on unanswered questions and plans for future research
9.1 Summary of research

Risk factors, practice variation and hematological outcomes of children identified with non-anemic iron deficiency, following screening in primary care setting.

This study had an epidemiological context where we identified the prevalence, risk factors and hematological outcomes associated with NAID in an urban Canadian pediatric population. The study found NAID to be a common disorder in early childhood having a prevalence rate of 7% (95% CI, 5.95%-8.05%). We also identified risk factors significantly associated with NAID. These included younger age (with each month decrease in age, children had 1.08 times greater odds of NAID; 95% CI: 1.06, 1.11), higher zBMI (with each unit increase in zBMI children had 1.22 times greater odds of NAID; 95% CI: 1.01, 1.48), longer duration of breastfeeding (with each month increase in breastfeeding duration children had 1.05 times greater odds of NAID; 95% CI: 1.01, 1.08) and volume of cow’s milk intake (with each cup/day increase in cow’s milk intake, children had 1.13 greater odds of NAID; 95% CI: 1.01, 1.26). The study also depicted practice patterns associated with the management and follow-up of children identified with NAID by community physicians. The results demonstrated substantial variation where 37% of children with NAID received an intervention and only 8.4% of children had a physician-ordered follow-up laboratory test. Based on the results of the follow-up laboratory tests, among children identified with NAID, 65.5% had resolution of NAID, 25.9% had persistence of NAID, and 3.4% had progression of NAID to IDA.

Re-evaluation of serum ferritin cut-off values for the diagnosis of iron deficiency in infants aged 12 to 36 months.

The second original study of this thesis is an exploration of the relationship between two important iron status indicators. Using a restricted cubic spline (RCS) regression analysis we constructed a spline curve showing the association between Hb and SF in children aged 12-36 months (N=1257). The spline curve showed an Hb plateau point at a SF level of 17.9 µg/L. That is, the predicted Hb level was maximized at this point and plateaued thereafter. Furthermore, using the RCS regression model the SF level that predicted a mean Hb value of 110 g/L was found to be 4.6 µg/L.
From this study it was suggested that these SF values (17.9 µg/L and 4.6 µg/L) may be associated with clinical relevance through their association with important Hb cut-offs. In healthy children a SF value at which Hb concentration is maximized, may indicate the beginning of functional impairment, such as hematopoiesis (196). The other SF cut-off (4.6 µg/L) identified in this study was shown to be associated with an Hb cutoff that is used to identify anemia in young children. Hence, when children are diagnosed with IDA based on Hb level of <110 g/L, they may have a SF as a low as 4.6 µg/L. This cut-off of Hb has been causally linked to delayed neuro-development in young children (125). Hence a SF cut-off of 4.6 µg/L may also be associated with delayed neurodevelopment.

However, these are statistical markers of SF thresholds that have been shown to have indirect clinical relevance. Their direct impact on important clinical outcomes such as: children’s neuro-development need to be established. Only then can we understand their true clinical significance and whether they can be used to diagnose iron deficiency in children aged between 12-36 months of age.

*Optimizing early child development for young children with non-anemic iron deficiency in the primary care practice setting (OptEC): study protocol for a randomized controlled trial.*

This study reports the protocol of a multi-site, pragmatic, placebo controlled, superiority randomized trial (OptEC: Optimizing Early Child development for young children with non-anemic iron deficiency in the primary care practice setting). It aims to assess the effectiveness of four months of oral iron plus dietary advice versus placebo plus dietary advice, in children with NAID aged 12 to 40 months, to improve their hematological and developmental outcomes. A protocol is defined as a document that provides sufficient detail to enable the understanding of the background, rationale, hypothesis, objectives, study population, interventions, methods, statistical analyses, ethical considerations and knowledge translation plans to be used in the trial, before it begins (204, 245). In this protocol in addition to describing the above elements, the rationale for selection of the outcome measures; the process of selecting a clinically meaningful difference in tests of cognition in order to perform a power calculation; and the methods of an internal pilot study performed to improve the design and conduct of this clinical trial are discussed.
An internal pilot study for a randomized trial aimed at evaluating the effectiveness of iron interventions in children with non-anemic iron deficiency: the OptEC trial.

The final original study in this thesis is an internal pilot that was performed to recalculate the sample size and refine the conduct of a randomized clinical trial aimed at evaluating the effectiveness of iron interventions to improve the developmental and hematological outcomes in children with NAID (OptEC trial) (187). Using internal pilot data a new estimate of the standard deviation (SD) of the primary outcome was determined ($S_2 = 17.40$). Other parameters such as the correlation between the baseline and follow-up outcome assessment ($\rho = 0.89$) were also determined. Using these parameters and the ANCOVA method, a range of sample size was re-calculated for the OptEC trial. The final sample sizes ranged between 32-56 subjects, which were 70% less than the previously projected sample sizes (112-198 subjects).

Since adherence of participants in the internal pilot can provide assumptions on the adherence of participants in the main clinical trial, additional data collected from our internal pilot study included the adherence rate of the participants to the study intervention. We identified the adherence to range between 14% -100% and 44% of the children had an adherence $\geq 86\%$. Furthermore, the main reasons for non-adherence were identified to be: the study drug takes too long to administer, is too messy, the child did not like it, too difficult to administer and forgot to give the study drug. Adverse effects reported by the parents included vomiting (19%), staining of teeth (34%), constipation (38%), loose stool (35%) and passage of black stool (46%).

Based on these findings specific strategies were implemented to improve adherence in main trial, such as - informing parents on possible adverse effects, decreasing the dose of the study intervention when children experience adverse effects such as vomiting and diarrhea, counseling parents on minimizing the difficulty of giving the study intervention and motivating parents on continuing the study intervention when children experience non-harmful and reversible adverse effects like passage of black stool and staining of teeth. This information was incorporated into a participant information handout which is given to parents when they agree to enroll their child in the OptEC trial.
9.2 Strengths and weaknesses of the studies

Risk factors, practice variation and hematological outcomes of children identified with non-anemic iron deficiency, following screening in primary care setting.

NAID signifies a particular stage in the development of iron deficiency which can only be diagnosed using iron specific indicators. Lack of screening for iron deficiency using iron specific indicators, currently prevents us from understanding the true extent of NAID in Canadian pediatric population. Our study may be the first to identify the prevalence of NAID in a large sample of urban Canadian children (N=2276). However, we need to understand the generalizability of our findings.

Most epidemiological studies are based on a limited population selected either for convenience or on voluntary basis. Extrapolation and generalization from studies in any single selected population must consider specific demographic or socioeconomic variables relevant to the study question (246). Our population was selected on voluntary participation of children in a community practice based research initiative (see chapter 4 for TARGet Kids! recruitment method). It included children aged 1 to 5 years, coming from families predominantly of European ethnicity and having higher socio-economic status as measured by maternal education (see table 9). We also identified risk factors significantly associated with the development of NAID which included children with younger age, higher zBMI, longer duration of breastfeeding and greater intake of cow’s milk per day.

Most prominent epidemiological data on iron deficiency have been reported from the US population, specifically the 1999-2000 National Health and Nutrition Examination Survey (NHANES 1999-2000) data (36). The NHANES survey samples US civilians, non-institutionalized population and collects data through household interviews and physical examinations. This survey collects blood from all persons aged ≥1 year. A total of 682 children aged between 1 to 5 years were included in the 1999-2000 survey. These children did not have a dominant ethnic or socio-economic disposition. Similar to our study, NHANES used serum ferritin, hemoglobin and as an ancillary indicator of iron status, C-reactive protein (CRP) to diagnose iron deficiency. Risk factors of iron deficiency identified from this this populations were very similar to those observed in our study (40, 41).
Despite differences in the ethnic and socio-demographic characteristics of the two samples, the prevalence of iron deficiency (age 1 to 5 years) reported from the NHANES survey is similar to that identified in our study [7% (Canadian) vs. 9.2% (US)] (36, 40). The prevalence reported in the NHANES (1999-2000) survey is expected to be higher than that identified from our study, because by case definition, it reported the prevalence of iron deficiency that included NAID plus IDA. Whereas, the prevalence reported in our study was strictly indicative of the early stage known as NAID. Our data is also consistent with the prevalence of iron deficiency and risk factors reported in other developed European countries (42). The similarity found between our population and those of other developed countries (US and European countries) and the consistency of the risk factors identified from the different populations, supports the generalizability of our data.

Importantly, the findings from the first study highlight substantial variation in clinical practice with respect to management and follow-up of children identified with NAID on screening. The longitudinal design of our study enabled us to identify poor resolution of hematological outcomes over time in children screened with NAID (25.5% had persistent NAID and 3.4% of children progressed to IDA). These findings emphasize the need for appropriate guidelines to detect and manage NAID in young children and stress the importance of developing surveillance programs for pediatric population (40). For surveillance purposes, the sampled population should be representative of those populations targeted for a universal or specific intervention program (32). Since screening for iron deficiency is not the standard of care, children who do not volunteer to participate in the TARGet Kids! research initiative can be considered the general pediatric population and our target population (71). Our study also compared children under-going screening for iron deficiency and those who were not and found no difference between the two groups in terms of their age, sex, ethnicity, socio-economic status and dietary habits (see table 7). Hence, the sample we used can be considered a representative sample of an urban Canadian pediatric population.

Not all children enrolled in TARGet Kids! undergo laboratory testing to screen for iron deficiency. About 60% of children enrolled in TARget Kids! during our study period underwent screening for iron deficiency. Incomplete participation may introduce bias in the results of our study. Children declining laboratory testing may be systematically different from those who undergo laboratory testing.
However, comparisons of children who did and did not undergo laboratory testing showed no difference between the two groups (see table 7).

For assessment of physician practice patterns, we abstracted data from clinic charts and necessarily relied upon the presence or absence of documented management as a proxy of actual management. Review of charts carries limitation associated with documentation, such as varying level of detail, legibility and potential for missing data. Hence, the results of our study were also affected by these drawbacks related to chart review. Finally, in our study not all children identified with NAID had a follow-up blood sample (8.4%). This limits our interpretation of the natural history of NAID and the potential effectiveness of interventions that were used by clinicians to treat NAID in children. Furthermore, the low rate of follow-up is an indication of the challenges associated with screening. This finding is consistent with previous evidence indicating challenges related to screening for iron deficiency in young children (86).

*Re-evaluation of serum ferritin cut-off values for the diagnosis of iron deficiency in infants aged 12 to 36 months.*

This study may be the first to assess the relationship between two iron status indicators (Hb and SF) using restricted cubic spline functions in a large sample of pre-school children (N = 1257). An ongoing challenge in the diagnosis of iron deficiency in young children has been determining SF cut-offs that have clinical importance. Evidence shows that currently recommended SF cut-off values (<10-12 µg/L) for identifying iron deficiency are based on either one of two approaches (80). In the first approach subjects are identified as being iron deficient (using the criteria of another indicator) and then thresholds were established from the range of SF values found in this iron deficient sample. The second approach is to measure the concentration of SF in healthy subjects not likely to be iron deficient and calculate appropriate threshold values based on either 95% confidence intervals or values below the 5th centile (80). Thus, the clinical importance of these cut-offs have not been appropriately investigated. Evidence has shown that when the severity of iron deficiency reaches the point of anemia, children’s neuro-development is affected (126, 132). Thus, a Hb level indicating anemia (<110 g/L) has significant clinical importance both in clinical practice and research (40, 42). However, the
degree of SF that can have negative clinical impact on children’s development is still inconclusive (119). The current study aimed to identify clinically important SF cut-offs, through its association with another iron status indicator Hb.

SF cut-offs (17.9 µg/L and 4.6 µg/L) identified from this study have indirect clinical significance through its association with clinically important Hb cut-offs. Findings from other studies that have attempted to identify SF cut-offs based on its relationship with Hb, have also reported values different than those currently recommended (<10-12 µg/L). In a study by Schneider et al (2005), the researchers attempted to identify abnormal values for SF by regressing Hb on SF values using a 2-phase segmented linear regression model (39). Segmented regression analysis determined that the breakpoint for SF that corresponded to an Hb level of <110 g/L was ≤ 8.7 µg/L. Hence, our findings are in agreement with other research that has aimed to determine SF cut-offs through its relationship with Hb.

Since the SF cut-off values identified in the current study were established using a statistical technique (restricted cubic spline), these values may not represent optimal SF for diagnosing iron deficiency in young children. Considering this as a potential limitation of this analytic approach, the direct impact of these cut-offs on important child health and developmental outcomes need to be evaluated. Furthermore, this study did not assess the diagnostic accuracy (sensitivity, specificity, likelihood ratio and receiver operating characteristics) of these cut-off values. Hence, how accurate they are in diagnosing iron deficiency in young children is not known.

*Optimizing early child development for young children with non-anemic iron deficiency in the primary care practice setting (OptEC): study protocol for a randomized controlled trial.*

The protocol for the OptEC trial was developed following the 2013 SPIRIT Statement checklist (204). This checklist provides recommendations for a minimum set of scientific, ethical and administrative elements that should be addressed in a clinical trial protocol. SPIRIT intends to facilitate the consistency and rigor of trial conduct and full appraisal of the conduct and results after trial completion (204). Thus by following this checklist in the development of this protocol, the intention was to conduct an unbiased trial and improve the validity of the future findings from the trial.
In addition to providing the guidance to perform a methodologically rigorous and unbiased clinical trial, this protocol has also reported on the methods used to select the parameters needed to calculate the sample size of this trial. Evidence has shown that the sample size of a clinical trial plays a vital role in evaluating the efficacy of the intervention being investigated (244, 247). Thus, according to modern clinical research guidelines, determining the appropriate sample size of a trial is a crucial step in the protocol development stage (204). In order to identify appropriate parameters to calculate the sample size for this trial a review was undertaken to identify clinically meaningful differences (effect estimates) for the primary outcome; and an internal pilot study was performed to obtain a reliable estimate of the standard deviation of the primary outcome which was then used to recalculate the sample size. Thus, instead of a fixed sample size, a range of samples were calculated, so that the trial would have enough power to detect an array of effect estimates.

This protocol was approved by the Research Ethics Board (REB) of the Hospital for Sick Children, Toronto and St. Michael’s Hospital, Toronto (REB file number: 1000027782). It was developed based on a study proposal that was funded by an operating grant of the Canadian Institute of Health Research (FRN 115059).

This protocol describes the design of a randomized trial aimed at evaluating the effectiveness of iron interventions in children with NAID. The current study assesses the concurrent effect of iron deficiency on children’s neurodevelopment; it does intend to assess the long-term effect of iron deficiency on children’s neurodevelopment. However, evidence has shown through longitudinal studies, significant long-term neurodevelopmental effect of IDA in children identified and treated with iron interventions during infancy (134). Hence, in order to have a complete understanding of the impact of iron deficiency on children’s developmental trajectory, longitudinal assessment is very important. Thus, the absence of longitudinal assessment in the design of the OptEC trial is a limitation of the OptEC trial.

Plans for communicating important protocol modifications, itself is an item in the SPIRIT checklist (204). The OptEC trial has a 4 year recruitment period. Within this time, concepts related to the disorder (such as the diagnostic criteria, methods of assessment), treatment, outcome measures,
recruitment strategy or study management process may change or need to be changed. In the presence of a REB approved protocol, the implementation and communication of amendments are time consuming, potentially costly and sometimes burdensome.

An internal pilot study for a randomized trial aimed at evaluating the effectiveness of iron interventions in children with non-anemic iron deficiency: the OptEC trial.

Pilot studies play an important role in health research, in providing information for the planning and justification of randomized controlled trials. Pilot studies are important to ensure that large randomized trials are rigorous, feasible, and economically justifiable (238, 248).

Use of inaccurate estimates of parameters (such as the standard deviation of the primary outcome) to calculate sample sizes of randomized trials may lead to underestimation of the necessary sample size, inadequate statistical power and consequently an unanswered study question (244). An internal pilot study is a unique design innovation that allows more reliable and accurate determination of the sample size of randomized clinical trials (229, 238). It allows a data-driven recalculation of the sample size of clinical trials by using estimates of parameters generated from the internal pilot study (230). Since internal pilot studies are an integral part of the main trial (the early phase), the new estimates of the parameters actually represent the population of the clinical trial. Hence, if knowledge about the parameters used to calculate sample size are weak, performance of an internal pilot is superior to an ordinary fixed sample design, because the initial sample size can be appropriately adjusted.

Information generated from our internal pilot study has been used to recalculate the sample size for the currently ongoing OptEC trial (187). The recalculated sample ($N_r = 32-56$) was 70% smaller than the initially projected one ($N_a = 112-198$). Despite being considerably smaller, the recalculated sample of the OptEC trial had the same power as the initial sample size, by taking into account the correlation between the baseline and follow-up assessment of the primary outcome. This method of sample size calculation is referred to as the ANCOVA (analysis of covariance) method (224). Use of the ANCOVA method keeps the statistical power the same while reducing the number of subjects required for a trial. In situations where patient recruitment is a challenge, application of the ANCOVA method
is a powerful and valid approach to minimize the sample size in clinical trials without affecting the power of the trial.

Another valid use of internal pilot data is to determine the adherence of the participants to the study intervention (229). Patient non-adherence with assigned treatment presents a considerable challenge in the conduct and analysis of clinical trials. Non-adherence may decrease the probability of detecting treatment differences and affect the interpretation of observed differences (234). Through the assessment of adherence of the participants in our internal pilot study, we were able to anticipate the adherence level of participants in the OptEC trial and identify the main reasons for non-adherence. The results from our internal pilot study found that there was sufficient adherence to evaluate the effectiveness of the study intervention. We implemented strategies to enhance the adherence in the trial by targeting the reasons of non-adherence. These strategies will strengthen the rigor of our clinical trial and enhance the validity of the results at the end of the trial.

At the end of the trial participants in the internal pilot study are included in the final analysis. However, because there has been an interim look at the data, there is a risk of inflating the trial’s type I error (229, 244). Our internal pilot was only used to estimate a parameter (SD of the primary outcome) and significance testing of the treatment effect was not performed; in this case the inflation will be negligible (229, 244). The adherence rate reported from our study may be an overestimate of the actual adherence, due to the fact that it was based on parent reported measures. Considering the setting we used for our trial (offices of primary care practices participating in the TARGet Kids! practice-based research network), we had few other possibilities than to rely on the parent’s statement for the clinical assessment of adherence.
9.3 Implication for clinicians and policy makers

*Risk factors, practice variation and hematological outcomes of children identified with non-anemic iron deficiency, following screening in primary care setting.*

Findings from our study were found to be generalizable to populations with similar demographic and socio-economic characteristics. In the absence of more nationally representative data for NIAD in young Canadian children, findings from our study can be used to guide decisions in clinical practice or in public health (246). We have identified risk factors significantly associated with NAID (younger age, higher zBMI, longer duration of breastfeeding and greater intake of cow’s milk per day). These modifiable risk factors being associated with the early stage of iron deficiency can be considered by primary care physicians in preventing the progression of NAID to IDA (severe iron deficiency) in young children.

Previous Canadian recommendations have declared iron deficiency to be an inadequately addressed and significant public health problem among Canadian infants and children that has short and long term health consequences (71). Due to outdated guidelines for screening of iron deficiency, a gap in evidence existed in regards to the current prevalence, risk factors, clinical impact and management of iron deficiency in young Canadian children. Hence, an invitation was made by a prominent researcher (Hartfield) in the field to develop a Canadian National strategy to address infant and childhood nutrition and iron deficiency (71). Our study addresses most of these gaps in evidence and has identified high prevalence, presence of modifiable risk factors, significant physician practice variation and poor resolution of hematological outcomes over time in children with NAID. In order to develop an optimal strategy to control NAID in the Canadian pediatric population its epidemiology and determinants need to be known.

Current US recommendations (American Academy of Pediatrics) suggest screening for iron deficiency using hemoglobin as the indicator of choice. Hemoglobin may not be an appropriate test for screening as it is a late indicator of iron deficiency (40). Screening for anemia with hemoglobin determination neither identifies children with NAID nor specifically identifies those with IDA (40). Our study has
used iron specific indicators (SF with CRP) to screen for NAID in our population. From our study it appears that it is feasible to use SF to screen for NAID in young children in the primary health care setting.

NAID is the early latent stage of iron deficiency which provides an opportunity for early detection. Furthermore, in the absence of clinical guidelines poor resolution of NAID in children was also identified where it was shown children with NAID may progress to develop IDA (see figure 11). Evidence has shown that NAID may cause developmental delay and IDA to cause irreversible impairment in children’s development. Therefore, the NAID stage fulfills several of the principles for effective screening programs presented by WHO (84). Stakeholders of pediatric health in Canada may consider the findings from our study useful in the development of an optimal strategy to prevent, treat and follow-up iron deficiency, specifically whether young Canadian children should be screened for NAID. Such statistics should be useful to health agencies charged with planning for the provision of primary health care services, maintenance of surveillance programs and policy development for preventive health care.

Re-evaluation of serum ferritin cut-off values for the diagnosis of iron deficiency in infants aged 12 to 36 months.

SF cut-offs (17.9 µg/L and 4.6 µg/L) determined from this study should be taken as preliminary findings, because their direct clinical effect and their diagnostic accuracy in diagnosing iron deficiency have not been established. Thus, at this stage, these values cannot be recommended to clinicians and policy-makers in their practice or during prevention strategy development decisions. However, the aim of this study was not to call for change in current policy or practice, rather the aim of this study was two-fold. First, to bring into focus the evidence associated with SF cut-offs currently used to diagnose iron deficiency and their lack of clinical relevance. Second, to identify clinically important SF cut-offs, through its association with clinically important cut-offs of Hb. Thus, the suggested SF cut-offs may carry great meaning for researchers working in this field. However, the direct clinical impact of these cut-offs need to be established. Appropriate SF cut-offs will greatly influence the prevalence and prevention strategy for iron deficiency in young children. This study may be considered the initial step
towards finding cut-off values of SF that show clinical relevance rather than being merely distribution-based.

*Optimizing early child development for young children with non-anemic iron deficiency in the primary care practice setting (OptEC): study protocol for a randomized controlled trial.*

The OptEC trial is a randomized controlled clinical trial that aims to evaluate the effectiveness of iron interventions in treating the developmental effect of NAID in young children. Results from this trial will provide high quality evidence to justify the development of clinical guidelines for this condition (187). The OptEC trial protocol ensures that the trial is conducted in a transparent and unbiased manner. The validity of the findings from the OptEC trial will greatly depend on how well the protocol is followed. Protocol adherence will ensure the authenticity of the results when the trial is complete. Hence, the importance of the protocol lies in its implementation on the conduct of this randomized trial and this will generate high quality evidence that may influence the decision of clinicians and policy makers involved in the treatment and prevention of non-anemic iron deficiency in young children.

*An internal pilot study for a randomized trial aimed at evaluating the effectiveness of iron interventions in children with non-anemic iron deficiency: the OptEC trial.*

Our internal pilot study has great implications for stakeholders involved in the development and promotion of modern research standards for the design, conduct and reporting of clinical trials. This is especially important for the pediatric population where the scarcity and well documented shortcomings of clinical trials has been reported (249, 250). It is now recognized that the quantity, quality and relevance of data involving children are substantially lower than for adults, despite data demonstrating that inadequate testing of interventions in children can result in ineffective or harmful treatments being offered or beneficial treatments being withheld (251-254).

To address the paucity and shortcomings of pediatric clinical trials a global initiative (StaR Child Health) has been undertaken by international expects who are dedicated to developing practical, evidence-based standards to enhance the reliability and relevance of pediatric clinical research (255).
Leading child health methodologists and regulators have identified determination of adequate sample sizes as one of the priority issues that can greatly enhance the design and conduct of pediatric clinical trials. Guidance on this priority issue has encouraged the use of internal pilot studies to overcome challenges faced when attempting to derive sample size estimates in pediatric research. The design and objectives of the internal pilot study we conducted meet the recommendations made by the StaR standard development group on sample size calculation. Therefore, our research provides validation of the StaR guidelines for pediatric clinical trials. Furthermore, the data generated from this internal pilot can inform future trials to be performed in similar population, particularly those in developed country settings.

This internal pilot study is a testament to how methods-based research can be performed using a primary health care platform (TARGet Kids!). Using the same data-source to perform clinical and methods based research will greatly reduce the cost associated with pediatric research. This information would be very useful to child health researchers and methodologists intending to perform clinical and/or methods based research. It alsohighlights the fact that being pragmatic and cost-effective in the conduct of clinical trials is attainable.
9.4 Unanswered questions and future directions

*Risk factors, practice variation and hematological outcomes of children identified with non-anemic iron deficiency, following screening in primary care setting.*

Despite the similarity in the epidemiological findings of the TARGt Kids! data and those of other developed countries, there has not yet been a direct assessment of the generalizability of the data collected through the TARGt Kids! research initiative. Thus, future research includes comparing TARGt Kids! data with those of regional and national databases with the aim to understanding the generalizability of the TARGt Kids! data. This comparison will include an epidemiological assessment of children’s iron status, prevalence rates and risk factors for NAID and IDA. A systematic approach will be undertaken to assess and compare the generalizability of the populations using seven key determinants: (a) population definition, (b) definition of outcome, (c) recruitment of subjects, (d) inclusion and exclusion criteria, (e) data collection, (f) subject retention and (g) length of follow-up (256). To strengthen the evidence identified from our study on possible risk factors associated with NAID, these associations need to be investigated in special populations where NAID/iron deficiency has been found to have greater frequency (e. g. aboriginal population, low SES population). Potential causes of disease may be even more evident in such populations (257).

*Re-evaluation of serum ferritin cut-off values for the diagnosis of iron deficiency in infants aged 12 to 36 months.*

The assessment of clinically important cut-offs for SF in young children requires the determination of their direct clinical effect on important clinical outcomes (such as children’s neurodevelopment). Through assessment of the effect of SF on the neurodevelopment of young children, optimal SF levels below which child development is affected / impaired can be identified. It has been shown that the most efficient indicators to assess the iron status of populations are those that can detect change in important clinical outcomes in response to iron interventions (57, 80). Hence experimental study designs where the association between specific SF cut-off values and neuro-development are assessed,
can allow us to identify their true clinical impact. The current study has identified SF cut-offs (17.9 µg/L and 4.6 µg/L) that may have clinical relevance through their association with physiologically and clinically important Hb cut-offs. Thus a future research aim is to establish the direct clinical importance of these SF cut-off values using the OptEC trial platform (187).

Furthermore, the diagnostic accuracy of the SF cut-offs identified from the current study needs to be determined. The AAP in addition to recommending SF (with CRP), also recommends using CHr (reticulocyte hemoglobin) for diagnosing iron deficiency in young children (40). The TARGed Kids! research program intends to include CHr as an iron status indicator. Hence, future research will include the assessment of diagnostic accuracy of SF cut-offs using CHr as the gold standard and determination of likelihood ratios and performing Receiver Operating Characteristics (ROC) analysis (77, 78).

*Optimizing early child development for young children with non-anemic iron deficiency in the primary care practice setting (OptEC): study protocol for a randomized controlled trial.*

The current OptEC trial protocol describes the design and conduct of a randomized trial aimed at assessing the concurrent effect of NAID on children’s developmental outcomes. It was designed to detect meaningful changes in tests of children’s cognition and motor development over a 4 month period (127). However, evidence from longitudinal studies performed by Lozoff and colleagues has shown the importance of assessing developmental trajectories in children (134, 137, 139, 140). Leveraging the infrastructure and funding from the OptEC trial, a long term assessment of children’s development (at 5 years of age) has been added to the trial design. The OptEC trial protocol will be amended to include this additional assessment. Longitudinal assessment of children’s development will be a unique contribution to understanding the influence of iron status on the developmental trajectory of young Canadian children.

*An internal pilot study for a randomized trial aimed at evaluating the effectiveness of iron interventions in children with non-anemic iron deficiency: the OptEC trial.*
TARGet Kids! is a primary care practice-based research network (PBRN) created to advance the scientific basis for chronic disease prevention and develop innovative interventions for primary healthcare providers to overcome common health and nutritional problems that limit children’s potential (163). One of the research priorities of this initiative is to implement pragmatic primary health care based randomized trials of interventions for the treatment and prevention of common nutritional disorders affecting the Canadian pediatric population (163). The OptEC trial and by extension the internal pilot study uses the TARGet Kids! research platform to investigate the effectiveness of iron interventions in treating children identified with NAID. Internal pilot studies are an innovative method in the design of clinical trials that allow the recalculation of sample size and refine the conduct of clinical trials. Performance of an internal pilot study within the TARGet Kids! research platform emphasizes the feasibility of using primary health care set-up to perform methods based research. I believe a variety of methodological research can be performed using this research platform. I am greatly enthusiastic on the creation of a methods research group within the TARGet Kids! research initiative. The methodological research conducted through this group will be tailored to meet the challenges and overcome the shortcomings of the clinical research performed through this research initiative.
Reference:


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Appendices
Appendix 1: List of excluded studies

<table>
<thead>
<tr>
<th>N</th>
<th>Author, date, country</th>
<th>Reason for exclusion</th>
<th>Citations</th>
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<tbody>
<tr>
<td>1</td>
<td>Aukett MA et al, Britain</td>
<td>Did not meet haemoglobin inclusion criteria</td>
<td>Arch Dis Child. 1986; 61(9):849-57</td>
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<td>2</td>
<td>Ayala R et al, Mexico</td>
<td>Did not meet haemoglobin inclusion criteria; Did not meet age inclusion criteria</td>
<td>Nutr Neurosci. 2008;11(2):61-8.</td>
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<td>6</td>
<td>Engle PL et al, Guatemala</td>
<td>Did not meet intervention inclusion criteria</td>
<td>Early Hum Dev. 1999;53(3):251-69</td>
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<td>7</td>
<td>Friel JK et al, Canada</td>
<td>Did not meet NAID definition</td>
<td>J Pediatr. 2003;143(5):582-6</td>
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<tr>
<td>8</td>
<td>Gonzalez HF et al, Argentina</td>
<td>Did not meet intervention inclusion criteria</td>
<td>Biological Trace Element Research. 2007;120(1-3):92-101</td>
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<tr>
<td>11</td>
<td>Hokama T et al, Japan</td>
<td>Did not meet intervention inclusion criteria; Did not meet NAID definition</td>
<td>Asia Pac J Public Health 2005;17(1):19-21</td>
</tr>
<tr>
<td>13</td>
<td>Lozoff B et al, Guatemala</td>
<td>Did not meet NAID definition</td>
<td>J Pediatr. 1982;100(3):351-7</td>
</tr>
<tr>
<td>15</td>
<td>Lozoff B et al, Costa Rica</td>
<td>Did not include a control group of children with NAID</td>
<td>Pediatrics 1987;79(6):981-95</td>
</tr>
<tr>
<td>18</td>
<td>Lozoff B et al, Costa Rica</td>
<td>Did not include a control group of children with NAID</td>
<td>J Pediatr. 1996;129(3):382-9</td>
</tr>
<tr>
<td>No.</td>
<td>Authors</td>
<td>Country</td>
<td>Study Details</td>
</tr>
<tr>
<td>------</td>
<td>---------------------------</td>
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<td>-------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>21</td>
<td>Lozoff B et al,</td>
<td>Chile</td>
<td>Did not meet intervention inclusion criteria</td>
</tr>
<tr>
<td>23</td>
<td>Lozoff B et al,</td>
<td>India</td>
<td>Did not include NAID group</td>
</tr>
<tr>
<td>25</td>
<td>Olney DK et al,</td>
<td></td>
<td>Did not meet serum ferritin inclusion criteria; Did not meet study design criteria (a cross sectional analysis)</td>
</tr>
<tr>
<td>26</td>
<td>Otero GA et al,</td>
<td>Mexico</td>
<td>Did not meet age inclusion criteria</td>
</tr>
<tr>
<td>30</td>
<td>Pollitt E et al,</td>
<td>Indonesia</td>
<td>Did not meet healthy child inclusion criteria; Did not meet intervention inclusion criteria</td>
</tr>
<tr>
<td>33</td>
<td>Soemantri AG et al,</td>
<td>Indonesia</td>
<td>Did not meet age inclusion criteria; Did not include NAID group</td>
</tr>
<tr>
<td>35</td>
<td>Steinmacher J et al,</td>
<td>Germany</td>
<td>Did not meet healthy child inclusion criteria; Did not meet age inclusion criteria</td>
</tr>
<tr>
<td>36</td>
<td>Stoltzfus RJ et al,</td>
<td>Zanzibar</td>
<td>Did not meet intervention inclusion criteria; Did not include NAID group</td>
</tr>
<tr>
<td>37</td>
<td>Sungthong R et al,</td>
<td>Thailand</td>
<td>Did not meet intervention inclusion criteria; Did not include NAID group</td>
</tr>
<tr>
<td>No.</td>
<td>Authors, Location</td>
<td>Inclusion Criteria Issue</td>
<td>Journal/Reference</td>
</tr>
<tr>
<td>-----</td>
<td>-------------------</td>
<td>--------------------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>39.</td>
<td>Walter T et al, Chile</td>
<td>Did not meet duration and dose of intervention inclusion criteria</td>
<td>Pediatrics 1989;84(1):7-17</td>
</tr>
<tr>
<td>40</td>
<td>Walter T, Chile</td>
<td>Did not meet duration and dose of intervention inclusion criteria</td>
<td>Am J Clin Nutr. 1989;50(3 Suppl):655-61</td>
</tr>
<tr>
<td>41</td>
<td>Yalcin SS et al, Turkey</td>
<td>Did not meet age inclusion criteria</td>
<td>Pediatr Int. 2000;42(6):625-30</td>
</tr>
<tr>
<td>43</td>
<td>Gupta SK et al, India</td>
<td>Did not include NAID group</td>
<td>Indian J Pediatr. 2010;77(4):375-9</td>
</tr>
<tr>
<td>44</td>
<td>Lozoff B et al, Chile</td>
<td>Did not meet age inclusion criteria; Did not meet intervention criteria</td>
<td>Pediatr 2010;126(4):e884-94</td>
</tr>
</tbody>
</table>
Appendix 2: Responses to peer review (Risk factors, practice variation and hematological outcomes of children identified with non-anemic iron deficiency, following screening in primary care setting.)

Our response to specific comments:

Reviewer: 1

1) Is it possible that there could be some confounding if physicians chose not to order follow-up lab tests because they knew a patient was enrolled in TARGet Kids! and would be offered a repeat blood test? Do you think this was likely to have a large effect or not? Are you able to estimate how large (or small) this effect may be?

_We are not able to estimate whether participating in TARGet Kids! influenced physicians test-ordering practice behavior. We have added this to our limitations section (page 10): “It is possible that physicians laboratory test-ordering behavior was influenced by participating in TARGet Kids!, however we are unable to confirm this.”_

2) Methods – Second paragraph –just a little more information on ‘TARGet Kids!’ would be helpful. Also is the data given in this paragraph for all TARGet Kids!? When did enrolment begin? – I’m just trying to figure out why the 5062 listed here differs from the 3814 reported in this study. Maybe it might be best to leave out these ‘TARGet Kids!’ numbers as it relates to the whole enrolment as it is potentially distracting/confusing – or at least reword to improve clarity.

_We have made some revisions for clarity. We have added the link to TARGet Kids! website in this section and also added the website as a reference (# 22). Furthermore the details of the TARGet Kids! cohort has been published as a manuscript which has been cited in reference #23. The data given in the stated paragraph (methods- second paragraph), are for all children enrolled in TARGet Kids! between the period, June, 2008 to September 2013. We have also added the age range of children included in this cohort (0-72 months) (page 4, methods, 2nd paragraph). The sample for our study was collected between June, 2008 to June, 2012 and included children 12-60 months of age._

3) Methods - Need just a little more discussion as to why ferritin >12ug/L was chosen as a cut off for iron sufficiency, though this is a fairly accepted value? Also some short discussion on why it was chosen not to use other tests including the reticulocyte Hb concentration (CHr) content, total iron-binding capacity, transferrin saturation, or serum transferrin receptor 1 (TfR1) concentration; though I
recognize use of Hgb along with serum ferritin in children with normal CRP is fairly well accepted to measure iron status, there is still some debate around this. Just a couple of lines might help position this better for the reader.

We have added the following section on page 6 –

“Iron status was measured using indicators suggested by the American Academy of Pediatrics (AAP) in their guideline for assessment of iron deficiency in young children (hemoglobin and serum ferritin with CRP (2). Values commonly considered low for serum ferritin are 10-12 µg/L (9, 11, 18). In our study NAID was defined as a serum ferritin level of ≤12 µg/L with CRP <10 mg/L and a normal hemoglobin level (≥110 g/L). IDA was defined as a serum ferritin level of ≤12 µg/L with CRP <10 mg/L and a low hemoglobin level (<110 g/L). Iron sufficiency (IS) was defined as having both a normal serum ferritin and hemoglobin level (serum ferritin >12 µg/L with CRP <10 mg/L and hemoglobin ≥110 g/L) (1, 2, 9, 27). Serum ferritin was measured using a Roche Modular platform, Roche Diagnostics and hemoglobin was measured using Sysmex platform, Sysmex Diagnostics.”

4) Most important, while I assumed from the exclusion criterion that a CRP was measured on all the studied patients, I just wanted to confirm that it was?
Yes, CRP was measured in all participants. Please see response #3.

5) Methods – Was MCV assessed? Why not?
We do assess MCV. However, this indicator was not used for the current study for reasons described in response #3.

6) Methods – why not assess nutrition intake including iron containing foods – was this not captured in the assessments?
Our current study was not designed to assess intake of iron containing food. We did however collect data on several feeding practices that have previously been found to be associated with iron deficiency, specifically duration of breastfeeding, volume of cow’s milk consumption, bottle use.

7) Statistical analysis: Approach seems fair but interesting that the variables found on univariate analysis did not seem to influence variables that were chosen to use in multivariate analysis. Other
than age of child the other variables found significant differed between the univariate and multivariate analysis. I think this deserves some discussion as to why this may have been. Potentially one explanation for this may be that some of the variables found significant in the univariate analysis were highly correlated with some other ones and dropped out. However, if so then this should be stated for the reader to understand what happened. One should also define what is meant by not ‘highly correlated’.

We tested whether the variables were correlated with each other by estimating their variance inflation factor (VIF) (SAS procedure) and found that the variables were not highly correlated with each other based on their individual VIF values. In Methods–Statistical Analysis (page 7) we have added the definition of “not highly correlated” variables by stating “variance inflation factor (VIF) for all variables was < 2.5”. A variable having a VIF value >2.5 indicates that it is highly correlated with another variable in the model, hence cannot be used in the same model. None of the variables included in our model for multivariate analysis had VIF > 2.5.

In our manuscript we have specifically stated (statistical analysis, page 7) “The association between NAID (reference group) and all clinically important variables (based on previous evidence) were examined using a multivariate logistic regression analysis where clinically important and not highly correlated variables (variance inflation factor for all variables were < 2.5) were tested simultaneously.” Thus we selected variables for inclusion in the multivariate analysis based on their clinical importance, not their significance level in the univariate analysis. This approach has been recommended for multivariate regression modeling in Harrell’s book on Regression Modeling Strategies (chapter 4). We have added this as reference (#30) after the above statement (statistical analysis, page 7).

Variables found to be not significant in the univariate analysis (zBMI, duration of breast feeding and volume of cow's milk) may be significant in the multivariate analysis, due to confounding of unknown nature.
8) Statistical analysis: Also, why did the multivariate analysis not use only those variables found significant in the univariate analysis (or if they did it was not stated)? Not that the approach used is incorrect, but some clarification would be useful for the reader.

_In choosing our co-variates for the multivariate analysis, all variables in the univariate analysis was included regardless of their p value, because evidence has shown all of these variables to have clinical importance (please see methods section, page 6 and reference #2,20,21). Thus our selection of co-variates was based on evidence of clinical importance not their significance level in the univariate analysis. We have clarified in the statistical analysis section (page 7): “The association between NAID (reference group) and all clinically important variables were examined using a multivariate logistic regression analysis...”_

Reviewer: 2

1) Page 3 line 40 “recommend screening “NOT “recommends”

_Corrected._

2) Page 9 line 26 “our study findings indicate that serum ferritin as an iron specific indicator may be used to screen for NAID in pre-school children” – the purpose/design of this study was not to answer this question. You have no other diagnostic criteria (MCV, RDW, iron indices) to compare with, and ferritin may be elevated following illness – your rate of NAID may be underestimated! I would suggest not commenting on this in this work, and leave this to the follow-up research you describe in your conclusion examining the best screening tests for NAID and IDA.

_We have revised this section on page 9 as follows -_

_“Our study findings indicate that it is feasible to use serum ferritin to screen for NAID in pre-school children in community practice setting. In developed countries where the prevalence of IDA is low, sole use of hemoglobin is inadequate to identify iron deficiency in children. However, further research is necessary to evaluate and compare the diagnostic properties of serum ferritin with other indicators (such as transferrin receptor and reticulocyte hemoglobin) used to screen NAID in young children (2, 33)”_
3) Page 10 Conclusion – you weaken your conclusion and the importance of the work when you state that “stronger evidence is needed” about causality before you can create a guideline. NAID is the precursor to IDA. There is certainly some evidence that NAID is bad for children, and it is clear that IDA is. If IDA can be prevented by treating NAID appropriately, why shouldn’t a guideline be provided for physicians to improve care? Particularly as this seems to be a “grey area” for many, and the interventions are very simple.

*We appreciate the reviewer’s point of view. However, we feel that guidelines need to be based on evidence and according to current methodological standards high quality evidence is needed for guideline and policy development. Hence we have revised this section on page 11 as follows –

“We assessed more than 2,000 young children (1-5 years), following laboratory screening for NAID and identified high prevalence, presence of modifiable risk factors, significant physician practice variation and poor resolution of hematological outcome in children with NAID. To strengthen the findings from our current study, a randomized controlled trial is currently being conducted through our research group to further evidence related to screening of NAID using iron specific indicators and the effect of NAID on the neurodevelopment of young children (34)”*

Reviewer 3

1) Page 8, lines 6-15: For continuous variables, specify what the odds ratio represents (increase in odds for each one-month increase in duration of breastfeeding, for example).

*We have revised this section on page 8 as follows -

“In the multivariate analysis, factors found to be significantly associated with NAID: age (with each month decrease in age, children had 1.08 times greater odds of NAID); zBMI (with each unit increase in zBMI, children had 1.22 times greater odds of NAID); duration of breastfeeding (with each month increase in breastfeeding duration children had 1.05 times greater odds of NAID); and volume of cow’s milk intake (with each cup/day increase in cow’s milk intake, children had 1.13 greater odds of NAID) (see table 2 for 95% odds ratio and p-value)”.

2) Table 2, column 3: The authors have not specified what type of estimate they are alluding to in this column. I imagine it has to do with the Beta coefficient. I am not sure that including this column
is necessary in the table; the readership will most likely not use this information. This being said, if the authors wish to leave it in, I would specify what they mean by estimate.

*Table 2 has been changed accordingly. We have retained the column and provided a heading: \( \beta \) coefficient*

3) **Table 3:** The presentation of percentages using a different denominator in this Table is confusing. I would remove the first line (Proportion of children with NAID for whom an intervention was recommended - N= 57), as this information is already given in the text and as it uses a denominator that is different from all the other proportions given in the table. The title of the table could be changed to something along the lines of 'Interventions recommended by physicians to treat NAID (N=57)', as this is the denominator used in the rest of the Table.

*Table 3 has been changed accordingly.*

Editors' comments:

1) All editorial changes have been accepted.

2) **Comment on page 8:**

The percentage has been corrected as follows -

“This was 23% of all those who were recommended a treatment (n = 57”).

3) **Comments on page 9 (discussion, 2nd paragraph):**

*There are many indicators for iron status assessment and among them serum ferritin is one. We have chosen serum ferritin because this indicator has been recommended by the American Academy of Pediatrics in their guideline for management of iron deficiency in young children. See page 6 in our manuscript where we justify our selection.*

*We have also revised the 2nd paragraph in the discussion section on page 9 as follows -*
“Our study findings indicate that it is feasible to use serum ferritin to screen for NAID in pre-school children in community practice setting. Specially, in developed countries where the prevalence of IDA is low and sole use of hemoglobin becomes less effective to identify iron deficiency in children. However, further research is necessary to evaluate and compare the diagnostic properties of serum ferritin with other indicators (such as transferrin receptor and reticulocyte hemoglobin) used to screen NAID in young children (2, 33).”

4) Comments on page 9 (discussion, 3rd paragraph):

Due to the design we used in our study we were only able to identify documented hematological outcome, we are not able to comment on the compliance of the children to the recommended intervention. We state this as one of our limitation on page 10 (discussion, last paragraph).
Appendix 3: Responses to peer review (Optimizing Early Child development for young children with non-anemic iron deficiency in the primary care practice setting (OptEC): study protocol for a randomized controlled trial.)

Our response has been included after each reviewer comment.
Response to reviewer:

1. Perhaps the authors could include the SPIRIT checklist as an appendix
   
   *We have included the SPIRIT checklist as an appendix*

2. The background section is possibly rather too long and includes an overview of IDA which appears to be less relevant than the detail about NAID – the background could more effectively focus on the research context around childhood NAID and move more quickly to the rationale for this treatment trial.
   
   *The evidence related to IDA and child development forms the foundation for building evidence related to NAID and child development. Hence we feel it is necessary to include this information in the background. However, in the current draft we have condensed the information in this section (page 6).*

3. One query I had about the rationale and flow of background literature was whether in fact the links between NAID and developmental problems have been definitively established or whether the data from this treatment trial will also contribute data on whether there is a connection between NAID and poorer outcomes, thus building a case for screening.
   
   *Our trial will contribute to understand whether there is an association between NAID and poorer developmental outcomes in young children and the effectiveness of iron interventions to improve the development of children with NAID. We have established in the background that there is significant lack of evidence in relation to the effect of NAID on children’s development. Performance of high quality, adequately powered clinical trials in developed country setting will begin to establish an evidence base for screening for iron deficiency with an aim to improve developmental outcomes. (See page 7, line 207-211)*

4. The first sentence of the trial objectives and hypotheses section could be more clearly written so it is clear that the trial is a comparison of oral iron plus dietary advice versus placebo plus dietary advice. At the moment it says the trial will assess oral iron plus advice with placebo and advice which is confusing.
   
   *We have changed the trial objectives and hypotheses as follows -*
The primary objective of this trial is to assess the effectiveness of 4 months of oral iron plus dietary advice versus placebo plus dietary advice, in children with NAID aged 12-40 months to improve their developmental outcomes. We hypothesize that children receiving 4 months of oral iron plus dietary advice will have better developmental outcomes than those who receive placebo plus dietary advice (page 9).

Response to editorial request:

1. Please ensure the title conforms to journal style for study protocol articles. The title should follow the format "__________: study protocol for a randomized controlled trial."

The title has been changed according to the journal style as below-

“Optimizing early child development for young children with non-anemic iron deficiency in the primary care practice setting (OptEC): study protocol for a randomized controlled trial.”

2. Please move your statements of consent and ethical approval to the Methods section.

Consent and ethical approval section has been moved to the Methods section (page 22).

3. Please mention each author individually in your Authors? Contributions section. We suggest the following kind of format (please use initials to refer to each author's contribution): AB carried out the molecular genetic studies, participated in the sequence alignment and drafted the manuscript. JY carried out the immunoassays. MT participated in the sequence alignment. ES participated in the design of the study and performed the statistical analysis. FG conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript?

Author's contribution has been changed as instructed -

KA and PP was responsible for the design and conception of the study, development of study protocol, nutritional guidance and data collection instruments, drafting and reviewing the manuscript for important intellectual content, and approved the final manuscript. KT ensured the accuracy of the statistical analysis and sample size calculation and approved the final manuscript. JM, CB, AH, DF, EM, CM, SZ was responsible for critical review of the manuscript and approved the final draft.

4. Please move the additional file section below the reference list.

The additional file section has been moved below the reference list (page 34) and another file has been included as appendix 3, the SPIRIT checklist.

5. Please include a figure title and legend section after the reference list.
A figure title and legend section has been added after the reference list on page 34.
Appendix 4: Iron intake guideline

<table>
<thead>
<tr>
<th>Types of food rich in iron (According to food types containing high to low level of iron)</th>
<th>Food guide for Pre-school Children to Improve Iron In-take</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meat and eggs (highest source of iron)</td>
<td>1 serving = 1/2 cup cooked fish/poultry/lean meat or 2 eggs</td>
<td>Beef (burger, beef liver, corned beef, steak)</td>
</tr>
<tr>
<td>Meat alternatives (medium source of iron)</td>
<td>1 serving = 3/4 cup cooked beans or tofu</td>
<td>Chicken and turkey (breast, thigh, wings, liver)</td>
</tr>
<tr>
<td>Grain Products (lower source of iron)</td>
<td>3 servings = 3/4 cup of cereal/oatmeal / cream of wheat or 1/2 cup cooked pasta/rice</td>
<td>Fish (haddock, halibut, salmon, tuna) fresh or canned in water</td>
</tr>
<tr>
<td>Vegetables and Fruit (lower source of iron)</td>
<td>4 servings = 1/2 cup of fresh, frozen or canned vegetable/fruit/fruit juice or 1 cup of leafy raw vegetable</td>
<td>Lamb and pork</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Eggs (especially egg yolks)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Beans (chick peas, lima beans, navy beans, kidney beans, lentils); Baked beans (canned)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tofu (firm)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cream of wheat; Oatmeal; Iron-enriched breakfast cereals (cheerios, corn flakes)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Enriched pasta</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Enriched Rice</td>
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<tr>
<td></td>
<td></td>
<td>Broccoli</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Spinach</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Baked potato with skin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fresh or dried fruits (apricots, figs, raisins)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Prune juice</td>
</tr>
<tr>
<td>Foods that increase iron absorption</td>
<td>Along with foods rich in iron 4 servings/day of vegetable and fruits that contain vitamin C is essential</td>
<td>Vitamin C containing foods:</td>
</tr>
<tr>
<td>-----------------------------------</td>
<td>-----------------------------------------------------------------------------------------------</td>
<td>-------------------------------------------------</td>
</tr>
<tr>
<td>1 serving =</td>
<td></td>
<td>• Citrus fruits (orange, grapefruit, tomatoes) and juice</td>
</tr>
<tr>
<td>• 1/2 cup of fresh, frozen</td>
<td>• • Centaloupe</td>
<td>• Centaloupe</td>
</tr>
<tr>
<td>or canned vegetable/fruit/fruit</td>
<td>• Kiwifruit</td>
<td>• Kiwifruit</td>
</tr>
<tr>
<td>juice</td>
<td>• Leafy greens (spinach, cabbage), cauliflower</td>
<td>• Leafy greens (spinach, cabbage), cauliflower</td>
</tr>
<tr>
<td>OR</td>
<td>• Brocoli, Brussels sprouts</td>
<td>• Brocoli, Brussels sprouts</td>
</tr>
<tr>
<td>• 1 cup of leafy raw vegetable</td>
<td></td>
<td>• Green and red peppers</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dietary habits that prevent</td>
<td></td>
<td>• Limit cow's milk to 2 cups (16 ounces, or 450 mL) per day</td>
</tr>
<tr>
<td>development of iron deficiency</td>
<td></td>
<td>• Limit juice to 1/2 to 1 cup (4 to 8 ounces, or 115 to 225 mL) per day</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Remove all baby bottles and offer milk, juice and water from a cup</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Do not give any tea. Commercial black tea contains substances that bind to iron so it cannot be used by the body.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tips to increase iron intake</td>
<td></td>
<td>• Adding beef to tomato or pasta sauce</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Adding chunks of ham to macaroni and cheese</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Serving baked beans with pork and tomato sauce</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Sprinkling dried fruit (dates, raisins, prunes, apricots) on cereal</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Add dried peas or beans to soups and casseroles</td>
</tr>
<tr>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

References:
- Health information for families from The Hospital for Sick Children. URL: [www.aboutkidshealth.ca](http://www.aboutkidshealth.ca) (accessed on September 26, 2011).
Appendix 5: 4 month follow-up data collection form

Follow-up Data Collection Form – Data linking sheet
(to be stored separately from study data)

ID String # ________________  Date _____________________
Age _____ (months)  Child’s Sex:  Male [ ]  Female [ ]
Home Telephone #_________________  Work/cell Tel # _______________________
Name of caregiver interviewed ____________________________________________________
Relationship to child ______________________________________________________________
OptEC Trial: Optimizing early child development in the primary care practice setting: Pragmatic randomized trial of iron treatment for young children with non-anemic iron deficiency (NAID)

Follow-up Data Collection Form

1. How many days last week did your child receive the provided study drug (circle one)?
   0  1  2  3  4  5  6  7

2. Since starting this study, has your child taken any iron supplements regularly other than the ones provided for this study (fill in all that apply)?
   - No
   - Iron: Ferinsol ________ ml per ____________ (day, week, month, year)
   - Iron: Other ________ ml per ____________ (day, week, month, year)
   - Multivitamin with iron ________ ml per ____________ (day, week, month, year)

3. Since starting this study, has your child taken any other vitamins or supplements regularly (fill in all that apply)?
   - No
   - Vitamin D: Drops ________ ml per ____________ (day, week, month, year)
   - Vitamin D: Liquid ________ ml per ____________ (day, week, month, year)
   - Multivitamin (without iron) ________ ml per ____________ (day, week, month, year)
   - Other—Please explain___________________________________________________

4. How hard or easy has it been to give the provided study drug?
   Easy
   0  1  2  3  4  5  6  7

5. Did your child like taking the study drug (circle one)?
   - Yes
   - No
   - Indifferent

6. In a typical week, how many days did your child receive the study drug (please circle)?
   0  1  2  3  4  5  6  7

7. If your child received the study drug 6 days/week or less, please state the reason (circle all that apply)?
   - Takes too long  Yes  No
   - Too messy    Yes  No
8. Did your child experience any of the following while administering the study drug (circle all that apply)?

- Coughing
- Spitting up
- Choking, gagging
- Unhappy with the taste

9. Did your child experience any of the following during the past 4 months (circle all that apply)?

- Staining of the teeth
- Constipation
- Loose stool
- Passage of black stool

10. Is your child currently breastfeeding (please circle)?

- Yes
- No — at what age did you stop breastfeeding? ________________ months
- Not applicable, did not breastfeed

11. Please specify your child's diet for the past 3 days. Please check all that apply.

- Breast milk
- Infant formula
- Red meat (beef, veal, pork, lamb, etc.)
- Poultry (chicken, turkey, duck, etc.)
- Fish (salmon, halibut, haddock, cod, tuna, etc.)
- Shellfish (lobster, crab, shrimp, etc.)
- Eggs
- Milk [ ] Skim [ ] 1% [ ] 2% [ ] Homo
- Fruits
- Vegetables
- Cheese
- Yogurt
- Margarine
- Honey
- Whole grain products (bread, bagel, bun, cereal, pasta, rice, roti, tortillas, etc.)
- Fast Food
- Infant cereal
- Vegetarian: does not eat red meat, poultry, fish or shellfish
- Vegan: does not eat red meat, poultry, fish, shellfish, eggs, dairy or honey
12. Circle how many cups of each drink your child has currently in a typical day. (1 cup = 8 ounces = 250 ml)

<table>
<thead>
<tr>
<th>Drink</th>
<th>0</th>
<th>½</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cow’s milk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infant formula</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infant cereal</td>
<td></td>
<td>¼</td>
<td>½</td>
<td>¾</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Soy milk</td>
<td></td>
<td>½</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5+</td>
</tr>
<tr>
<td>Other milk (rice, goat etc)</td>
<td></td>
<td>½</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5+</td>
</tr>
<tr>
<td>100% Juice (apple, orange etc)</td>
<td></td>
<td>½</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5+</td>
</tr>
<tr>
<td>Sweetened drinks (Kool aid, Sunny D, etc.)</td>
<td></td>
<td>½</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5+</td>
</tr>
<tr>
<td>Tea</td>
<td></td>
<td>½</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5+</td>
</tr>
<tr>
<td>Soda or Pop</td>
<td></td>
<td>½</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5+</td>
</tr>
</tbody>
</table>

13. Did your child’s diet include the following foods during the last 4 months? Please check all that apply?

- Whole grain products (example – iron enriched breakfast cereals, enriched pasta and rice, beans such as chick peas, kidney beans, lentils and canned baked beans)
  
  _______ times per ____________ (day, week, month)

- Tofu
  
  _______ times per ____________ (day, week, month)

- Citrus fruits (example - oranges, grapefruit, lemon juice, tomatoes, cantaloupe, kiwi fruit)
  
  _______ times per ____________ (day, week, month)

- Citrus vegetables (example – spinach, cabbage, broccoli, Brussels sprouts, bell pepper, cauliflower)
  
  _______ times per ____________ (day, week, month)

14. Has your child been ill within the past 4 months?

- No

- Yes, (complete all that apply below)
  
  • Colds or flus, how many times? _____
  • Asthma attack, how many times? _____
  • Pneumonia, how many times? _____
  • Ear infection, how many times? _____

For office use only

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Height</td>
<td>________ cm</td>
</tr>
<tr>
<td>Weight</td>
<td>________ kg</td>
</tr>
<tr>
<td>BMI</td>
<td>________ kg/m²</td>
</tr>
<tr>
<td>Waist circumference</td>
<td>________ cm</td>
</tr>
</tbody>
</table>
Appendix 6: Response to review (An internal pilot study for a randomized trial aimed at evaluating the effectiveness of iron interventions in children with non-anemic iron deficiency: the OptEC trial.)

Our response has been included after each reviewer/editorial comment.

Response to reviewer:

1. It is worth clarifying on page 6 that the overall aim of the internal pilot is to inform the design of the full and definitive evaluation and to make clear whether or not the full trial follows on seamlessly from the internal pilot.

   We have addressed this comment on page 6, by adding the following sentences -

   *The overall aim of the internal pilot was to inform the design of the full and definitive trial.*

   *At the end of the pilot study, if necessary the sample size will be recalculated and compliance measures may need to be enhanced. Otherwise, the OptEC trial will continue following the protocol as previously reported (1). During the collection and analysis of data for the internal pilot study the recruitment of the trial continued.*

2. The primary outcome of the OptEC trial is the ELC - page 7 - but this is for the full trial and not the internal pilot. So perhaps just say this clearly here.

   We have followed the checklist of items to include when reporting a pilot study as per Thabane et al. This includes reporting the primary outcome measures for the main study. We have addressed this matter issue on page 6, by adding the following sentences –

   *The internal pilot study is an integral part of the OptEC trial, which consisted of the first few participants enrolled in the trial. Hence, it follows the same design and conduct of the main trial. In the following sections we describe methods that are particularly relevant to the internal pilot study as check list recommended by Thabane et al. for reporting of pilot study results (14).*

3. Did the internal pilot in effect have stopping rules? That is if the criteria for success were not met would the full trial been halted?

   This comment has been addressed on page 8 by adding the following -
There was no stopping rule for the internal pilot study that would halt the OptEC trial.

4. Did the authors treat the adherence threshold of 90% among 40% participants as a pre-specified success criteria in the same way as recruiting the relevant number of participants? I note that this was not achieved but the authors say that the rate of 86% among 44% is adequate - was this a decision reached by the study team alone or by an independent trial steering committee too? We have addressed this comment in the Discussion, page 10, by revising the sentence as follows: Since our adherence assessment was based on the number of days per week the child received the study intervention, our study team modified this criteria for success to ≥86% adherence, as this corresponds ≥6 days in a week.

5. Is data from the internal pilot going to contribute to the overall sample and outcomes analysis of the full trial? If not is this an external pilot? We have added the following on page 10 - At the end of the trial, participants in the internal pilot study will contribute to the overall sample and outcome analysis of the full trial.

Response to editorial request:

1. Please restructure the Abstract. This should be composed of the following sub-sections: Background, Methods, Discussion and Trial Registration. The abstract has been changed according to the journal style (see page 4).

2. Please provide a heading of 'Update' for the main body of text after the Abstract. Heading ‘Update’ has been included on page 5.

3. Please ensure and state that all authors meet point 1 of the ICMJE criteria for authorship: "To qualify as an author one should 1) have either made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data".
Author’s contribution has been written as follows on page 12 –
“KA was responsible for the conception, design and analysis of the data for the internal pilot study. KA and PP were responsible for interpretation of the data, drafting and reviewing the manuscript for important intellectual content, and approved the final manuscript. KT ensured the accuracy of the statistical analysis and sample size calculation and approved the final manuscript. JM, CB, AH, DF, EM, CM, SZ were responsible for interpretation of data, important intellectual content, critical review of the manuscript and approved the final draft”.

4. Please include a figure title and legend section after the reference list.

A “Figure title and legend” section has been added on page 15.

5. For additional files, please ensure that you list the following information after your reference section in your manuscript:

- Additional files: File name (e.g. Additional file 1)
- File format including the correct file extension for example .pdf, .xls, .txt, .pptx (including name and a URL of an appropriate viewer if format is unusual)
- Title of data
- Description of data

An “Additional files” section has been added on page 15.
Appendix 7: Formula to calculate pooled standard deviation

\[(S_2)^2 = \left[ (n_1 - 1) S_A^2 + (n_2 - 1) S_B^2 \right] / (n_1 + n_2 - 2)\]

$S_2 =$ pooled SD

$S_A =$ observed SD in group A

$S_B =$ observed SD in group B

$n_1 =$ sample size for group A

$n_2 =$ sample size for group B