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Development and Determinants of Iron Status and Anemia, and Relationship to Inflammation, in a Cohort of Bolivian Infants

By

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Doctor of Philosophy

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An abstract of
A dissertation submitted to the Faculty of the
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Abstract

Development and Determinants of Iron Status and Anemia, and Relationship to Inflammation, in a Cohort of Bolivian Infants
By Rachel M. Burke

Iron deficiency is the most common micronutrient deficiency worldwide, and has been associated with preterm birth and cognitive delays in children. However, measurement of iron status can be complicated: biomarkers must be measured in blood, and the effect of inflammation must be accounted for. These considerations underscore the importance of understanding status during early infancy, while adjusting for inflammation.

The Nutrición, Inmunología, y Diarrea Infantil (NIDI) study followed 456 healthy mother-infant pairs over the first year of life, collecting several blood samples as well as clinical and sociodemographic data. The study took place in El Alto, Bolivia, where the population is primarily indigenous with low socioeconomic status.

In the first aim of this dissertation, we estimated the effects of acute and chronic morbidities and pathogen exposure (represented by water, sanitation, and hygiene [WASH] resources) on the inflammatory biomarkers CRP and AGP over these infants' first year of life. Inflammation was more prevalent in older infants and significantly associated with recent illness.

In the second aim, we calculated the prevalence of iron deficiency (ID), anemia, and iron deficiency anemia (IDA) at 3 time points, correcting for the effect of inflammation. Though infants were born with normal iron stores, ID was nearly universal in the cohort by 12 – 15 months. Inflammation correction affected prevalence estimates.

In a third aim, we used multiple imputation to account for the uncertainty introduced into models by linear regression correction of ferritin. We found a minimal effect of random error on effect estimates.

In the fourth aim, we assessed the effect of infant feeding practices on iron status at 6 – 8 months of age. Although the length of exclusive breastfeeding was significantly negatively associated with iron status, the evidence did not support changes to currently recommended practices of 6 months of exclusive breastfeeding.

Taken together, these studies suggest that even healthy, breastfed infants in developing countries are highly vulnerable to early declines in iron status, implying a need for early interventions to improve maternal and infant iron status (e.g., delayed cord clamping, improved maternal nutrition). Further, the importance of measuring and accounting for inflammation is underscored.

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I must also thank the NIDI study participants and their families, as well as our very dedicated Bolivian and Emory study teams.

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Introduction

Background and Motivation

Iron is a micronutrient critical to various human functions, with an especially important role as a component of hemoglobin, the protein responsible for transporting oxygen within red blood cells. When iron usage outpaces iron stores and intake, depletion and eventually deficiency can result. If uncorrected, iron deficiency can lead to anemia, characterized by a decrease in the blood's ability to carry oxygen (caused by low hemoglobin in the case of iron deficiency). Iron deficiency can also have numerous other deleterious effects, however, even in the absence of anemia, and has been associated with labor complications, preterm birth, and impairments in infant cognitive development.

Iron deficiency is the most common micronutrient deficiency worldwide. Yet, iron status can be complex to measure (requiring a bone marrow aspiration for the gold standard measurement, and a serum sample for commonly used surrogates). Further, serum measurements of iron markers are substantially affected by even sub-clinical inflammation. Anemia (which can be assessed by finger stick) is often used as a surrogate for iron status, but this is neither a sensitive nor a specific indicator.

Given that infants are born with high iron stores, few studies have longitudinally assessed iron status in healthy infants followed from below six months of age, and fewer have done so while employing sophisticated adjustments for the effects of inflammation on iron biomarkers. Data is particularly scarce in developing countries, though these populations are the most vulnerable to iron deficiency and the most often affected by sub-clinical inflammation. Infant iron status has multiple determinants, but few have been

specifically investigated, especially in an Andean, high-altitude population, where competing causes of anemia may be different from low-altitude settings.

Study Setting

The *Nutrición, Inmunología, y Diarrea Infantil* (NIDI) study followed 456 healthy mother-infant pairs over the first year of life and collected several blood samples as well as numerous clinical and sociodemographic data. The study took place in El Alto, Bolivia (altitude ~13,500 ft.), where the population is primarily indigenous, with low socioeconomic status and a high prevalence of malnutrition.

Goal and Specific Aims

The overall goal of this work is to examine the development of iron deficiency (ID), anemia, and iron deficiency anemia (IDA) in a cohort of Bolivian infants followed over the first year of life, while accounting for the effect of inflammation. Specifically,

1. Estimate the prevalence of inflammation and the effect of recent illness, adiposity, and pathogen exposure on inflammation among Bolivian infants over the first year of life, and assess whether these effects change over time.
2. Estimate the prevalence of iron deficiency (ID), anemia, and iron deficiency anemia (IDA) in Bolivian infants at 2, 6 – 8, and 12 – 18 months of age, while correcting for inflammation.

3. Estimate the effect of postpartum maternal iron status on infant iron status at 6 months, as a test case to explore the impact of uncertainty introduced by linear regression-based inflammation-correction methods.
4. Estimate the effect of feeding practices on iron deficiency (ID), anemia, and iron deficiency anemia (IDA) in Bolivian infants at 6 – 8 months of age, while correcting for the effect of inflammation.

Chapter 1: Literature Review

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Review

Identification, Prevention and Treatment of Iron Deficiency during the First 1000 Days

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Abstract: Iron deficiency is a global problem across the life course, but infants and their mothers are especially vulnerable to both the development and the consequences of iron deficiency. Maternal iron deficiency during pregnancy can predispose offspring to the development of iron deficiency during infancy, with potentially lifelong sequelae. This review explores iron status throughout these "first 1000 days" from pregnancy through two years of age, covering the role of iron and the epidemiology of iron deficiency, as well as its consequences, identification, interventions and remaining research gaps.

Keywords: iron deficiency; iron supplementation; pediatrics; infants; first 1000 days

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1. Introduction

The importance of early life experiences to subsequent health outcomes is increasingly being recognized and studied, with special attention often paid to nutrition and growth. Early nutritional insults can lead to irreversible linear growth restriction (stunting), particularly in the first two years of life [1]. Together with pregnancy, this critical period of "1000 days" is associated with adverse effects much later in the life course, such as increased risk of non-communicable disease, as well as reduced cognitive capacity and economic productivity [2,3]. Given that iron deficiency in the first two years may also cause irreversible deficits in cognitive development, among other potential adverse effects [4,5], we propose that the "first 1000 days" framework can also be useful for the discussion of the identification, prevention and treatment of iron deficiency. Similar to the observation that maternal stunting may lead to intrauterine

growth restriction of the fetus, progressing to a stunted infant [6], maternal iron deficiencies may also lead to low iron status for newborns, progressing to iron deficiency in infants [5,7]. This intergenerational cycle provides several potential points for intervention for at-risk mothers and infants. The present review will thus focus on iron deficiency in pregnant, lactating and infant populations (though the first two years of age), describing the role of iron, the epidemiology of iron deficiency, its consequences, identification, interventions and remaining research gaps.

2. Iron Metabolism and Requirements

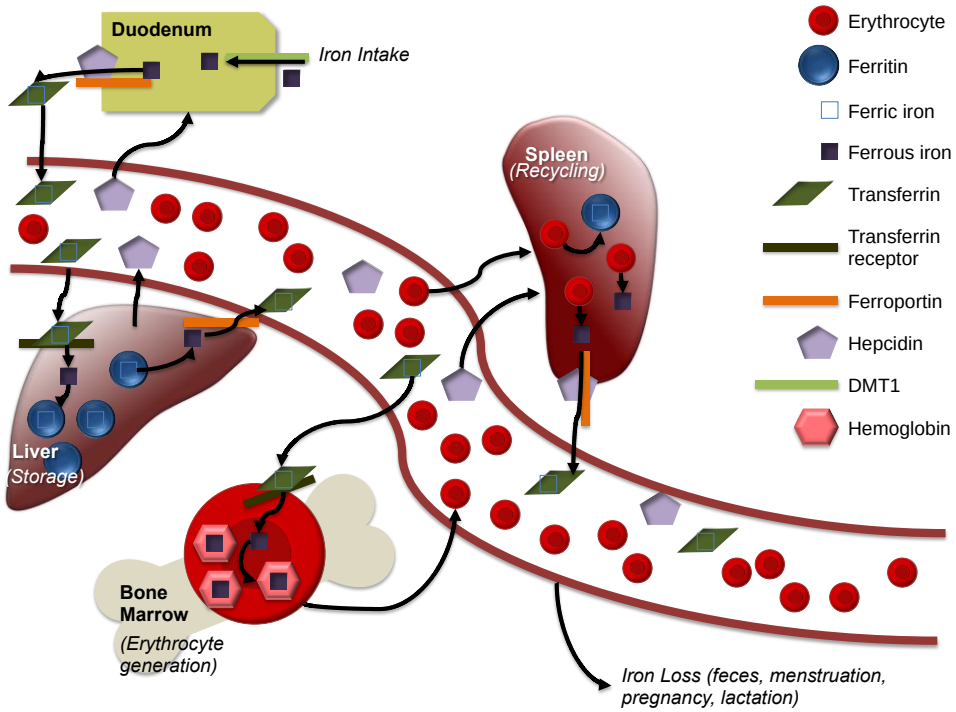
2.1. Iron Metabolism

Iron is one of the most important micronutrients for human populations, given its central role in key biological processes. One key process is that of tissue oxygenation, which is accomplished by red blood cells (RBCs); generation of RBCs requires hemoglobin, of which iron is a key component [8]. New RBCs are also created to replace RBCs that are lost from normal turnover, shedding (of skin cells or from the intestinal lining) or via hemorrhage [8]. Situations that require an increase in RBCs (such as the increased tissue mass of a growing fetus or infant) will consequently increase iron requirements. Absorption of iron is primarily regulated within the intestine, and once iron has been absorbed, there is no mechanism of excretion from the kidneys or liver [9]. For this reason, iron homeostasis is tightly regulated. After absorption, iron is either stored within ferritin, which keeps the iron in a nonreactive state within cells, or within transferrin, which also keeps the iron in a nonreactive state, but maintains it in aqueous circulation, so that it can be delivered to cells [9]. Within cells, stored iron (as ferritin) can be located in the cytoplasm, nucleus or mitochondria

[10]. Iron is transported across cell membranes with the assistance of divalent metal transporter 1 (DMT1; importing function) and ferroportin (exporting function) [9]. Ferroportin can be bound by the protein hepcidin, preventing ferroportin's export function and, thus, decreasing levels of serum iron [9]. (Figure 1) Whether in serum or within cells, most stored iron is present as ferritin [11].

Figure 1.1. Simplified representation of iron metabolism.

Adapted from [12]. Iron is absorbed in the intestine, with non-heme iron being imported by divalent metal transporter 1 (DMT1). Ferrous iron is reduced to ferric iron and then exported by ferroportin. Within an aqueous solution, iron is stored within transferrin. Transferrin-bound iron is imported with the help of the transferrin receptor into the liver, heart and other storage areas, where it is stored within ferritin. Hepcidin, produced by the liver, helps to regulate iron metabolism by binding to ferroportin and, thus, inhibiting iron export. Within the bone marrow, iron is incorporated into hemoglobin for incorporation into erythrocytes. Macrophages recycle iron from erythrocytes, largely in the spleen. There is no mechanism for iron excretion by the kidneys or liver, though small amounts are lost via feces. Menstruation, pregnancy and lactation result in iron loss in women.



2.2. Requirements in Pregnant and Lactating Women

Iron is especially critical during pregnancy given the rapid cell and tissue development involved in fetal growth. Pregnancy has a net iron cost in the range of 600–800 mg [13,14]. Nearly 300 mg of iron are needed just for the fetus, at least 25 mg for the placenta and nearly 500 mg for the increased volume of red blood cells [15,16]. These ~800 mg of pregnancy-associated iron are a requirement over and above the 230 mg of iron that the woman would have required even if she had not been pregnant, and the 150 mg that she may lose through blood loss at delivery [15,16]. Though this iron expenditure is offset by the lack of menstrual blood loss during this time, the net cost is still high enough that iron recommendations during pregnancy (27 mg per day) far exceed those for non-pregnant, non-lactating women (18 mg per day) [13]. Further, because fetal iron requirements take precedence over maternal needs and storage [14], adequate iron intake is important to both mother and fetus.

While iron recommendations for lactating women are much lower than those for non-pregnant,

non-lactating women (9 mg *vs.* 18 mg), this number is based on the assumption of lactation-induced amenorrhea and does not take into account that many women enter or conclude pregnancy with iron insufficiency or deficiency [13].

2.3. Requirements in Infants of Zero to 24 Months of Age

Iron requirements for infants under the age of six months are generally not well defined, because needs are difficult to estimate in the context of exclusive breastfeeding [17]. Furthermore, during the first four to six months of age, most infants benefit from iron stores present at birth, most of which are accumulated during the last 10 weeks of gestation [5,18]. Though breastfed infants do not receive large quantities of iron through breast milk (iron concentration < 1 mg/L [19–22], equivalent to < 0.5 mg per day for a typical infant [23], they are able to absorb a large proportion of this iron [24,25]. For this reason, iron supplementation is typically not recommended for breastfed term infants below the age of six months [23]. However, because preterm infants and low birth weight infants are born with lower birth iron stores, supplementation in the range of 2–4 mg/day is advised in order to avoid development of iron deficiency [4,23]. Supplementation may also be advised for infants that have lower birth iron stores of other etiology. Infant formula typically contains 10–12 mg/L of iron, though this iron is much less bioavailable [26], and evidence to support the benefits of this level of iron intake is inconclusive [23].

For older infants (over six months of age), different methods lead to different estimates of daily requirements, with a commonly used factorial approach generating a recommendation of 11 mg/day for 7–12-month-olds and 7 mg/day for 1–3-year-olds [23]. It is worth noting that requirements likely do not suddenly increase from ~2 mg/day (recommendations for infants <6 months) to ~11 mg/day (recommendations

infants 7–12 months), exactly at six months, in a stepwise fashion. This "jump" is rather an artifact generated by the use of the factorial method, which calculates recommendations based upon assumptions about the amount of iron required to accommodate growth (e.g., increased blood volume and tissue mass) and the amount of iron lost (e.g., from skin cells, from intestinal and urinary tracts). However, these assumptions are based on what is known about adult iron metabolism, as no data specific to infant populations is available [17]. Nonetheless, iron requirements certainly do increase from birth through childhood, in order to keep pace with rapid growth during this time, and other methods of calculation (e.g., linear interpolation from breast milk content to adult requirements, extrapolation based on energy expenditure) tend to generate similar results for iron requirements of older infants and toddlers [17].

3. Epidemiology of Iron Deficiency

3.1. Pregnant and Lactating Women

Iron deficiency (ID) is the most common micronutrient deficiency among pregnant women, leading to iron deficiency anemia (IDA) if uncorrected. However, ID can be difficult to measure in populations due to the lack of availability of field-friendly iron biomarkers (Table 1). In contrast, anemia is less time- and resource-intensive to assess, and thus, anemia is often used as an ersatz surrogate for iron status. However, anemia is neither a sensitive nor a specific indicator of iron status: a loss of up to 20%–30% of body iron would be necessary for some individuals to exhibit anemia based on hemoglobin cut-offs [27], and only up to half of anemia can be attributed to iron deficiency in most settings [28]. However, the prevalence of iron deficiency can be estimated at 2.5-times the prevalence of iron deficiency anemia in many settings [29].

Anemia may affect up to 56% of pregnant women in developing countries, implying a relatively high prevalence of IDA (up to ~25%) and, thus, an even higher prevalence of ID [30]. In settings with endemic malaria, such as many countries in Sub-Saharan Africa, the prevalence of anemia can be much higher—up to 65% in pregnant women and 75% in non-pregnant women of reproductive age [31]. In India, another area of high malaria endemicity, anemia may affect >75% of pregnant women [32], with a prevalence >50%, even in areas with iron supplementation programs [33]. Pregnant women in developed countries are also subject to ID and IDA, with approximately 16% suffering from anemia and more likely suffering from some degree of iron deficiency [29,30]. This is equivalent to approximately 56 million women worldwide [31]. To prevent iron deficiency, it is generally recommended that women enter pregnancy with iron stores of at least 500 mg [5]; however, over 20% of women in developed countries enter pregnancy with extremely low iron stores, with figures in developing countries likely even higher [15].

Although lactating women are not typically considered separately from non-pregnant and non-lactating women in iron deficiency and anemia calculations, the prevalence of anemia among women of reproductive age may provide a useful proxy. Anemia affects 46% of this population in developing countries and 14% in developed countries, indicative of a high prevalence of iron deficiency [30]. Anemia in recently postpartum women may reach 15%–50% in developed country settings and 50%–80% in developing country settings [34].

3.2. Infants of Zero to 24 Months of Age

Given that a majority of young term infants are largely protected from iron deficiency by their birth iron stores, iron deficiency is not often considered in infants

less than six months of age. However, even in the context of the exclusively breastfed, term infant, iron deficiency and iron deficiency anemia may be observed, with population estimates in the range of 0%–15% (ID) and 0%–4% (IDA) of six-month-old infants in different settings worldwide [35].

Older infants (6–12 and 13–24 months of age) are typically not considered as separate populations in global estimates of ID and IDA. Nonetheless, research indicates that these groups are at even higher risk of ID and IDA than younger infants. In a study of Icelandic infants, Thorisdottir *et al.* found 9% of infants to be iron deficient at 12 months; ID was highest (21%) among infants fed a diet of primarily cow milk, and much lower among infants fed primarily breast milk (2.6%) or formula (1.4%) [36]. In a German study comparing the iron status of infants that had been primarily breastfed during their early months to infants that had been primarily formula fed, 19% of the breastfed infants were iron deficient at seven months, with 4% diagnosed with iron deficiency anemia. At 10 months, 21% suffered ID and 2% suffered IDA [37]. None of the infants who had been formula-fed developed iron deficiencies. The 2006 National Health and Nutrition Survey in Mexico found low iron stores in 32% of children 12–24 months of age and tissue iron deficiency in 19% of the same group [38]. Meanwhile, a population-based study of Indonesian children found that 54% of urban children 6–24 months and 57% of their rural counterparts suffered from anemia, implying a high prevalence of iron deficiency [39]. Data from India indicates that 70%–90% of children 6–59 months of age may be anemic, with prevalence even higher in those under two years of age [40]. Recent demographic and health survey (DHS) data in other developing countries indicate even higher prevalence of anemia in these age groups, with especially high prevalence in West Africa, an area of high malaria endemicity: in Côte d'Ivoire, anemia was found in 90% of 6–9-month-olds, 94% of 9–10-month-olds

and 84% of 11–23-month-olds; in Guinea, the prevalence of anemia was about 85% in each of these groups. Similar results were seen in other areas of Sub-Saharan Africa, as well [41].

4. Risk Factors for Iron Deficiency

4.1. Pregnant and Lactating Women

Pregnant women are especially vulnerable to iron deficiency, not only because of the large quantities of iron required for fetal and placental growth (825 mg for fetus, placenta and increased blood volume [15,16]), but also due to the fact that so many enter pregnancy without adequate iron stores, especially in developing countries [42]. In some of these countries, e.g., India, vegetarianism may be more common due to religious beliefs; these women are also more vulnerable to the development of iron deficiency, because iron is much more efficiently absorbed in heme form (found in animal products). Iron status can also be affected by the intake of other nutrients that may inhibit (e.g., calcium, phytates) or promote (e.g., vitamin C) iron absorption [43]. Other proximal risk factors for low iron status during pregnancy include low intake of bioavailable iron, infections (e.g., intestinal helminthic infections, malaria), multiple pregnancies and adolescent pregnancy, while intermediary factors include low socioeconomic status and membership in certain ethnic groups, depending on the country of residence [5,15,42]. Social and psychological factors may also affect development of iron deficiency anemia, via reducing iron supplementation adherence and compliance [44]. Distal factors, such as food security and access to healthcare, play important roles, as well; "underlying determinants," such as the existence of anemia control programs (e.g., universal supplementation), fortification policies and the economic situation and agricultural productivity of the locale should also be considered [45]. Iron deficiency factors for lactating women include all of the risk factors identified for pregnant women, in addition to low iron status prior to and during pregnancy, as well as delivery-induced bleeding [34]. Obesity has also been associated with iron

deficiency in adult populations, with dietary deficiency, elevated blood volume and subclinical inflammation as the suggested mechanisms [46].

4.2. Infants of Zero to 24 Months of Age

Given the potentially severe and irreversible consequences of severe iron deficiency and iron deficiency anemia in infants, many studies have sought to assess potential risk factors in addition to treatment and prevention strategies. Infant iron status may be affected by factors arising either prior to the time of birth or following birth, as described below.

4.2.1. Pre- and Peri-Natal Risk Factors

In the absence of supplementation and complementary feeding, young infants are dependent upon iron from only two sources: their birth stores and breast milk or formula. Although breast milk has highly bioavailable iron [24,47], its iron content is not high [19–22], making birth iron stores critical in the prevention of early iron deficiency [35]. While the importance of birth iron stores is well recognized, determinants of infant birth iron stores remain incompletely understood [48], and the regulation of iron transport from mother to fetus is complex [5,14].

Nonetheless, given the fact that maternal iron status is a known intervention target, multiple studies have assessed the effect of maternal iron status on infant levels, and numerous studies have assessed other outcomes (e.g., low birth weight). There is some suggestion that maternal iron status may affect birth stores in the infant, and infants born to anemic mothers have been shown to be more vulnerable to anemia during their first year of age [49]; correlations between maternal and infant measures of iron status near the time of birth have also been demonstrated [50]. Further, a study of iron status in U.S. infants from birth through 12 months demonstrated that infants maintained their

iron ranking over time, again supporting the hypothesis that iron status is influenced by factors that act *in utero* [48]. This is in contrast to some earlier literature, such as a 1961 study by Lanzkowsky *et al.*, which did not demonstrate an effect of maternal anemia on infant anemia [51].

Congruent with the importance of the birth iron stores and the fact that these are generated primarily during the last 10 weeks of gestation, preterm birth and gestational age have consistently been implicated as risk factors for impaired iron status in young infants [52–55]. Similarly, low birth weight has also been associated with increased risk of iron deficiency in young infants [36,56–58].

4.2.2. Postnatal Risk Factors

While pre- and peri-natal risk factors (e.g., low birth weight) can have large effects on infant susceptibility to iron deficiency, infant experiences after birth can also play a large role.

Although iron deficiency is observed in normal-weight, term, exclusively breastfed infants, it is much less common in that population than among preterm or low-birth-weight infants. Furthermore, though breast milk is known to vary widely in exact nutritional content [59], it has generally been assumed that, except in cases of severe maternal nutritional deficiency, breast milk is adequate to meet infant micronutrient requirements, including iron, provided that infants are born with sufficient birth iron stores. Therefore, few studies have studied the impact of postpartum maternal nutritional status on infant nutritional status during the first six months of life, especially in normal birth weight, term infants. Findings of the several studies that have been done appear to be mixed. In a longitudinal study of Peruvian mother-infant pairs, Finkelstein *et al.* found that maternal postpartum hemoglobin and iron status were significantly

associated with infant hemoglobin and iron status at two and five months of age, regardless of maternal prenatal supplementation [25]. In contrast, a recent study by Ziegler *et al.* in U.S. infants found no association between maternal and infant plasma ferritin levels at one month postpartum [48], and a randomized clinical trial (RCT) in Turkish mother-infant pairs also found no significant effect of postpartum maternal supplementation on infant parameters of iron status at four months, with the exception of serum iron binding capacity [60].

Once infants reach the age of 4–6 months, iron needs start to outpace iron intake, and stores begin to be exhausted, making infants much more vulnerable to iron deficiency [48]. Infants that grow rapidly, as in the case of low birth weight infants that experience catch-up growth, are also at elevated risk of iron deficiency given their enhanced needs [61]. Further, common complementary foods, especially in developing countries, may be low in iron [35]. Additional factors associated with lower iron intake or absorption (and thus increased risk of iron deficiency) include lower socioeconomic status, cow milk intake and exclusive breastfeeding without additional iron supplementation [36,37,54,58,62–66]. Frequent enteric infections (bacterial, viral or parasitic) are also associated with the development of environmental enteropathy, an intestinal pathology that can negatively affect the absorption of nutrients, such as iron, and cause subsequent deficiencies [67]. Social factors that have been associated with anemia prevalence in young children include rural location [41,68], family structure [69] and sanitation (also related to enteric infections) [45], among others.

5. Consequences of Iron Deficiency

5.1. Pregnant and Lactating Women

Iron deficiency during pregnancy can have severe consequences, not only for the mother, but also for her infant. Low iron stores and low intake during pregnancy not only cause anemia, associated with weakness, fatigue, reduced cognitive performance and diminished immune response, but may also increase the risk of delivery complications and perinatal maternal mortality [42]. Maternal iron deficiency has also been implicated as a risk factor for preterm delivery, small-for-gestational-age and neonatal mortality [42]. Underscoring the importance of iron to fetal brain development, maternal iron deficiency has also been associated with cognitive and behavioral deficits [42,70,71], likely mediated through reduced birth iron stores and subsequent iron deficiency in the infant [5]. Iron deficiency during pregnancy also increases the risk of iron deficiency anemia during lactation [13]. Among lactating women, iron deficiency has the same effects as on non-pregnant, non-lactating women of reproductive age: increased risk of iron deficiency anemia, reduced work and mental capacity, increased risk of postpartum depression and other emotional disorders, as well as reduced quality of mother-child interactions [42].

5.2. Infants Zero to 24 Months

Infants are vulnerable to the effects of low iron status, even before the first moments of birth: low birth iron stores have been associated with both iron deficiency and with increased risk of cognitive and psychomotor developmental deficits later in infancy [35]. Iron deficiency that develops later in infancy and leads to iron deficiency anemia has similarly been associated with impaired cognitive, behavioral and motor development; the effect of iron deficiency without iron deficiency anemia is less clear [61,66]. Iron deficiency in young children has also been associated with elevated blood lead levels, with some evidence of a causative relationship, wherein iron deficiency

makes children more vulnerable to higher blood lead levels; elevated blood lead levels, like iron deficiency, can also cause cognitive impairment [72]. The cognitive deficits of iron deficiency may be irreversible, even if iron supplementation is begun within the critical period of zero to 24 months [4,7].

6. Screening and Measurement of Iron Deficiency

6.1. Commonly Used Indicators

Several biomarkers are used to assess iron deficiency, and each has its own advantages and disadvantages (Table 1). While hemoglobin is commonly used to assess anemia, it is neither a sensitive nor a specific indicator of iron status. For instance, hemoglobin (Hb) levels may not fall below anemia cut-offs until iron stores fall by up to one-third, and anemia can also be caused by several other micronutrient deficiencies (e.g., B12, folate) or conditions [11,27,73]. Serum ferritin (SF), a marker of iron storage, has the advantage of being a sensitive indicator of iron deficiency, but because it is increased in the presence of inflammation, ferritin is not a specific indicator of iron deficiency [11,27,73]. Transferrin saturation (Tfs), a marker of circulating iron, has also been widely used, but levels are depressed in the presence of inflammation, decreasing its specificity [74,75]. Though less affected by inflammation than SF or Tfs, soluble transferrin receptor (sTfR) levels begin to change relatively late in iron deficiency; further, levels can also be affected by other causes of altered rates of red blood cell generation [11,27,73]. Nonetheless, this marker may be preferred for infant populations with high levels of background infection, as it has been shown to have good accuracy in this setting [76]. Zinc protoporphyrin (ZPP) is another indicator and can reflect a shortage of iron in the last step prior to hemoglobin formation; however, it is not a

specific indicator of iron status [11,27,73]. The ratio of TfR to SF can be a useful marker of iron status, but is again limited by SF's response to inflammation and has also not been fully validated in children or infants [27]. Hepcidin is a liver-produced hormone that is active in iron homeostasis and, thus, has potential as a biomarker of iron status and function [5]. Levels of hepcidin are decreased in conditions leading to or resulting from iron deficiency (e.g., erythropoiesis), and increased in conditions of iron sufficiency (high iron stores) or inflammation [5,75]. However, the relationship of hepcidin to inflammation, as well as the fact that normative levels are still ill-defined, limits the utility of hepcidin alone in the definition of iron status [5]. Reticulocyte hemoglobin (CHr; mean cellular hemoglobin content of reticulocytes) has also recently been recommended for the diagnosis of iron deficiency in infant populations [23]. CHr is a measure of the iron incorporated into the hemoglobin of red blood cells and, thus, provides a fairly direct measure of iron availability to cells; it has also been shown to be highly accurate when compared with other biomarkers [77]. While this marker also has the advantage of not being affected by inflammation, the required assay is not yet widely available [23]. Thus, it is most useful to assess iron status with several markers, as opposed to just one. For instance, in CDC analysis of the National Health and Nutrition Examination Survey (NHANES), iron deficiency is usually defined as abnormal values on at least two of the following three indicators: ZPP, SF, sTfR [78].

Table 1.1. Summary of iron indicators.

Iron indicators are ranked from easiest and most economical to measure, to most expensive and most invasive.

Biomarker	Advantages	Limitations	Normal Range/Cut-offs
Hemoglobin (Hb)	• Easy, economical to measure (can be assessed with handheld device) • Good screening tool for severe iron deficiency	• Neither sensitive nor specific for iron status • Better measure of function rather than status	• Pregnant women: anemia <11.0 g/dL (1T, 3T) or <10.5 g/dL (2T)* • Newborns: anemia <13.0 g/dL (venous), <14.5 g/dL (capillary) • Infants 6–24 months: anemia <11.0 g/dL
Hematocrit (Hct)	• Relatively easy to measure	• Provides no additional information above Hb	• Pregnant women: anemia <33% • Infants 6–24 months: anemia <32%

Red blood cell indices (mean cell volume (MCV), red cell distribution width (RDW))	• Low MCV and increased RDW characteristic of iron deficient erythropoiesis
	• Useful clinically
		• Late finding, not representative of iron status	• MCV
			  ○ Pregnant and lactating women: <82 fl
			  ○ Infant reference ranges (age-dependent):
			  ▪ Neonates: 100–112 fl
			  ▪ <2 months: 85–98 fl
			  ▪ 2–12 months: 73–84 fl
			  ▪ 12–24 months: 72–85 fl
			• RDW
			  ○ Abnormal: <11.5%, >14.5%

Serum or plasma iron	• Measure of circulating iron	• Easily contaminated by iron from other sources • Variation by time of day, post-prandial state • Does not detect iron in Hb	• Adults: <40–50 µg/dL • Infants <24 months: <50–60 µg/dL

Table 1.1 *Cont.*

Serum ferritin (SF)	• Sensitive indicator of iron deficiency ○ Proportional to liver stores of iron • Responds well to iron interventions	• Increases with the acute phase response (not specific in the presence of inflammation) •	• Pregnant women: <12.0 µg/L (1T) • Reference range (women): 0–230 µg/L (trimester-dependent) • Newborns: <34.0 µg/L (cord blood) • Infants 6–24 months: <12.0 µg/L
Transferrin saturation (Tfs)	• Marker of circulating iron	• Levels are depressed by inflammation	• Pregnant women: <16% • Infants <24 months: <10%
Transferrin receptor (TfR)	• Less sensitive to inflammation than SF ○ Useful in populations with high levels of background infection	• Not very sensitive; levels change only late in ID • Not as specific as other measures; other conditions may cause restriction of iron to RBCs	• Pregnant women: >8.5 mg/L or >4.4 mg/L • Infants <24 months: >20 mg/L
TfR:SF ratio	• Proportional to stored iron or iron deficit • Sensitive indicator of response to iron supplementation	• Vulnerable to effects of inflammation on SF • Not validated in children or infants • Assay dependent (based on Ramco assay for TfR)	• Pregnant women: >500 consistent with iron deficiency or depleted iron stores • Can be used to calculate body iron stores: -[log (TfR/ferritin ratio) -2.8229]/0.1207 ○ Negative values defined as tissue iron deficit
Total iron binding capacity (TIBC)	• More stable than other measures • Measures iron-binding sites on transferrin	• Changes only with depletion of iron stores • Not typically used in newborns	• Adults: >400 µmg/dL
Zinc protoporphyrin (ZPP)	• Sensitive indicator of severe iron deficiency, but not of moderate iron deficiency • Can be measured with very little blood volume	• Not specific as levels can be increased due to lead poisoning, inflammation, and other situations • Cut-off levels not well established for infant populations	• Pregnant women: >70 µg/dL RBCs (1T) • Infants <24 months: >70–80 µg/dL RBCs

Table 1.1 *Cont.*

Hepcidin (Hep)	• Reflects iron homeostasis • May be measured in blood or in urine	• Also increases in conditions of inflammation • Normative levels not well defined	• Pregnant and lactating women: Mean levels immediately prior and following delivery have ranged 2.5–17.5 µg/dL • Newborns: Mean levels in cord blood have ranged 48.5–69.3 µg/dL
Reticulocyte hemoglobin (CHr)	• Measure of iron availability to cells • Not affected by inflammation	• Assay not yet widely available	• Adults: reference range 28–35 pg/L • Infants <24 months: reference range 23–35 pg/L
Stainable bone marrow	• Gold standard for diagnosis of iron deficiency	• Invasive • Subject to observer error	• Units: Observer assesses stained iron content according to a semi-quantitative scale

* 1T: first trimester; 2T: second trimester; 3T: third trimester. References: [5,11,23,27,28,79–88].

6.2. Impact of Inflammation on Indicators of Iron Status

Measurement of iron status is further complicated by the fact that infection and its resulting inflammation, even at a sub-clinical level, can affect serum levels of markers commonly used to assess iron deficiency [75]. Though this effect plays a role in the assessment of most micronutrients, it is particularly important for the measurement of iron status. While serum iron and transferrin (which binds iron and becomes low in the case of iron deficiency) are depressed in the presence of inflammation, ferritin (which decreases with iron deficiency) rises in the presence of inflammation [75]. These changes are thought to reflect an evolutionary effort to sequester iron away from pathogen use during infection (for instance, by bacterial or parasitic agents) [89]. Other measures of iron status are also affected by infection and inflammation.

The body's initial inflammatory reaction (the acute phase response; APR) activates a number of proteins (acute phase proteins; APP) and other mediators that help the body to recover from the instigating trauma [75]. This response typically lasts nine to 10 days, with the levels of different APP varying over this time. C-reactive protein (CRP) is one of the first to peak, reaching its maximum within one to two days after the initial insult, and with a half-life of two days. Alpha-1-glycoprotein (AGP) is also commonly measured, but is slower to peak than CRP (maximum reached at four to five days) and has a longer half-life, as well (5.2 days). While ferritin is itself considered an APP, the exact biological mechanisms of the APR-driven changes in other iron biomarkers are not as well understood. However, given that these effects have been widely observed, it is critical to adjust for inflammation status when assessing prevalence of iron deficiency in a population [90].

While many nutritional studies commonly use C-reactive protein (CRP) to adjust for inflammation (e.g., by calculating correction factors based on nutrient levels in inflamed *vs*. non-inflamed groups), this may not be sufficient to fully correct for the effect of inflammation on iron status, given the short half-life of CRP and the timing of its peak relative to inflammation-induced changes in iron measures. Transferrin and serum iron both fall relatively rapidly in the presence of inflammation, while ferritin rises quickly, much like CRP. However, other measures of iron status (and anemia status), such as hematocrit, hemoglobin and zinc protoporphyrin (ZPP), change much more slowly, and even ferritin takes longer to return to normal levels than CRP does [75]. Thus, adjustment for inflammation based solely on CRP levels may not fully account for the changes in micronutrient levels that occur as a result of the APR. More recently, investigators have measured both CRP and AGP in order to categorize the stage of inflammation with more precision, thus adjusting for four different inflammation groups instead of a simple dichotomy [75,91]. While other proteins, such as alpha-1-antichymotrypsin (ACT) and ceruloplasmin, have also been measured and used in some studies to adjust micronutrient status for inflammation [92,93], they are not as commonly used as either AGP or CRP.

7. Interventions

7.1. Pregnant and Lactating Women

The most common and effective intervention to combat iron deficiency in pregnant women is supplementation with iron, often combined with folic acid (given to prevent neural tube defects). As described in previous sections, given the number of women that enter pregnancy with insufficient iron stores, iron supplementation may need to begin prior

to conception to ensure prevention of maternal iron deficiency. Indeed, the World Health Organization (WHO) recommends weekly iron-folic acid supplementation programs in areas with a high prevalence of anemia in women of reproductive age (WRA) [94]. Daily iron supplementation is generally recommended for pregnant women worldwide, though in some developed country settings, universal iron supplementation is not recommended due to uncertain benefit and adverse effects (e.g., GI upset) in iron-replete women [5,95]. The success of iron supplementation programs is also often limited by poor adherence (as low as 50% in some settings [96]), which can be associated with gastrointestinal side effects, as well as lack of reliable access to supplements [97,98]. Further, these supplements often incorporate iron of lower bioavailability [98]. While fortification of foods would also help reduce iron deficiency among pregnant women, programs are sparsely implemented in many countries and may be insufficient to meet iron needs without additional supplementation [99]. Multiple micronutrient powders containing iron may also have good potential to prevent iron deficiencies, but more research is needed in order to determine their level of benefit [100,101]. The risk of postpartum iron deficiency can also be reduced by antenatal iron supplementation, as well as by prevention of perinatal hemorrhage [102].

7.2. Infants Zero–24 Months of Age

Infants, particularly preterm or low-birth-weight infants, will also benefit from iron supplementation, regularly recommended for these high-risk populations [4]; infant iron supplementation is often provided in liquid form and has been shown to be effective in reducing anemia [45], as well as iron deficiency [4,54]. However, these drops have several disadvantages: they frequently cause gastrointestinal side effects and also create the

potential for overdose [103]. Multiple micronutrient powders (MNPs) containing iron for home fortification of complementary food have also been shown to be highly effective in reducing anemia, as well as iron deficiency in infant populations 6–24 months of age [103]. These have the advantage of providing not only iron, but other critical micronutrients, as well. Further, MNPs are easy for mothers to use and incorporate into complementary feeding and carry a lower risk of overdose, as they are packaged in single-dose packets [104].

Nonetheless, iron supplementation (in various forms) has been associated with certain risks, particularly in iron-replete populations. Initial controversy was sparked in 2006, when an RCT of iron supplementation of preschoolers in malaria-endemic Zanzibar was published showing an increased risk of severe adverse events and death in the supplementation arm [105]. Though a Cochrane review has since concluded that there is no excess risk in settings with regular malaria surveillance and control [106], the development of an appropriate universal policy remains a topic of discussion [107]. Iron supplementation of iron-replete infants has also been associated with reduced linear growth and increased risk of other infections in some populations [61,103,108].

Delayed cord clamping has also been shown to have a protective effect against iron deficiency in infants: a recent Cochrane review found that infants whose cord clamping was not delayed were over twice as likely to later be found iron deficient as compared to infants whose cord clamping was delayed [109]; this is likely related to the amount of blood transferred to the infant during this time, with the delayed cord clamping allowing approximately 30% more blood to be transferred to the infant [5].

8. Research Needs

Although our understanding of iron function and metabolism and ability to assess and improve iron status in diverse populations has vastly improved in the last decades, much progress remains to be made. In particular, several gaps remain in the assessment of iron status, especially in resource-limited settings, as well as in the safe and cost-effective prevention and treatment of iron deficiency.

While it is now widely recognized that inflammation affects commonly used markers of iron status, there is still a lack of consensus on how to best adjust for these effects. There are numerous markers of inflammation that could be measured, each with different behavior (signaling a different role or stage in the inflammatory process), and each requiring a different assay, with some assays being more expensive or less widely available than others. Appropriate cut-offs for each inflammatory biomarker are also not well defined. Furthermore, the exact approach to adjust for inflammation using these markers is also still under consideration: while correction factors are most commonly used, other mechanisms of adjustment, such as linear regression, may also be useful [90].

Furthermore, currently available iron biomarkers have numerous other limitations apart from sensitivity to inflammation: they are not field-friendly, must be used in concert (*i.e.*, the use of a single biomarker to assess iron status is not recommended) and do not correlate well to iron function or iron exposure. Accurate measures of iron function are critical in order to be able to better understand the impact of supplementation and the effects of deficiency. For instance, brain imaging has been explored as a potential way to elucidate the mechanisms behind iron's role in early cognitive development [110]; this and other functional biomarkers should be further researched. Additionally, biomarkers of iron

exposure would be useful during pregnancy, since pregnancy often depletes maternal iron stores in the last trimester, when the fetus is building its birth iron store [74]. Lastly, measures of iron status also need to be better defined and validated for specific populations, such as young infants.

In terms of prevention and treatment of iron deficiency, additional research should assess the risk-benefit of iron supplementation and clearly communicate this to policy makers. This is particularly important in the supplementation of young infants, given the mixed evidence as to the potential adverse effects of supplementation on growth and vulnerability to infection. The risk-benefit calculation for supplementation of pregnant and lactating women is less ambiguous, though more work needs to be done on ways to improve adherence and bioavailability of supplements. Further, the potential advantages of moving away from a supplementation-focused approach to a food-based approach (e.g., staple food fortification, promotion of dietary diversity) should be explored. Nutrition-sensitive interventions, e.g., home gardens, biofortification or conditional cash transfers, could also support a whole-foods-based approach, while simultaneously addressing social and economic determinants of iron status discussed above; however, additional research is needed to determine the effectiveness and feasibility of such programs for improvement of iron status [111].

9. Conclusions and Recommendations

Iron deficiency is undeniably a critical public health issue given its high prevalence and potentially life-altering consequences. Infants are particularly vulnerable to iron deficiency

due to their rapid growth, and consequences of iron deficiency in this population can be wide-ranging and long lasting. However, because of the links between maternal iron status and neonatal iron status, interventions on infants alone will be insufficient to reduce levels of infant iron deficiency; the improvement of maternal iron status (before, during and after pregnancy) is also critical. While many advances have been made, knowledge gaps still remain, not only in the best approach to intervention, but also in the most correct approach to screening and diagnosis of iron deficiency itself.

This review summarizes the importance, development and diagnosis and treatment of iron deficiency in maternal and infant populations. We have placed special emphasis on the influence of early and fetal life experiences on iron deficiency and its consequences, as well as the interplay between mother and infant before and after birth. With our treatment of iron deficiency from fetal life through the first two years of age, we hope that we have demonstrated the "first 1000 days" framework to be highly useful in considering iron status and planning interventions.

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Author Contributions

Rachel M. Burke drafted the manuscript. Parminder S. Suchdev and Juan S. Leon helped to review the manuscript. All authors read and approved the final version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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Chapter 2: Prevalence and Predictors of Inflammation

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Predictors of Pediatric Inflammation in a Cohort of Bolivian Infants, Using CRP and AGP

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Predictors of Inflammation in Bolivian Infants

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ABSTRACT

Inflammation has been associated with cardiovascular disease and other health outcomes in children and adults, yet few longitudinal data are available on the prevalence and predictors of inflammation in infants. We aimed to identify the prevalence of inflammation in a cohort of Bolivian infants and estimate its association with acute (recent illnesses) and chronic (overweight, stunting) morbidities and pathogen exposure (represented by water, sanitation, and hygiene [WASH] resources). We measured the plasma concentrations of two acute phase proteins [C-Reactive Protein (CRP), marking acute inflammation, and alpha(1)-acid-glycoprotein (AGP), marking chronic inflammation] at three time points (target 2, 6 – 8, and 12 – 18 months). Of 451 singleton infants enrolled in the parent study, 272 had the first blood draw and complete data. Anthropometric measurements and sociodemographic and recent illness data (2-week recall of cough, diarrhea, and fever) were collected at each visit. Inflammation was defined as CRP > 5 mg/L or AGP > 1 g/L. The prevalence of inflammation increased from early infancy (3% at 1st blood draw) to later infancy (15 – 22% at later blood draws). Recent cough, recent fever, and age in months were significantly associated with relative increases in CRP (7 – 44%) and AGP (5 – 23%), while recent diarrhea was only significantly associated with a relative increase in CRP (48%). Neither anthropometry nor WASH were significantly associated with inflammation. Results confirm the role of recent acute illness in inflammation in infants, and indicate that adiposity and WASH are not as important to inflammation in this age category.

TEXT

Introduction

Inflammation has been associated with numerous adverse health outcomes ranging from cardiovascular disease[1-3] to psychiatric and mood disorders,[4, 5] and even potentially some cancers.[6, 7] Inflammation is often described by the acute phase response (APR), a process activated by the body in response to stress, trauma, or infection. The APR is triggered by cytokines such as interleukin-1 (IL-1) and interleukin-6 (IL-6), which in turn induce the liver to produce C-reactive protein (CRP), alpha(1)-acid glycoprotein (AGP), and other proteins that can be measured in serum. CRP rises rapidly (within 1 – 2 days) and remains elevated for about 1 week after symptom resolution, while AGP rises more slowly (after 4 – 5 days) and remains elevated for several weeks.[8] Although this response resolves within 1 – 2 weeks in a healthy system, it can remain activated in situations of chronic stress or repeated immunological insult.[9, 10] Further, in minor illnesses or in cases where the body's immune response is particularly robust, this inflammatory process may be subclinical and not manifest overt symptoms of illness such as fever; however, it can still affect nutritional biomarkers and other health outcomes.[11] For example, inflammation can contribute to invalid nutrition measurements given that multiple biomarkers of micronutrient status (e.g., ferritin, retinol) are affected by inflammation.[8, 11] Since various stimuli may cause clinical or subclinical inflammation, inflammatory status cannot be predicted simply by the presence or absence of recent infection or trauma.

Various studies have sought to assess different correlates of inflammation, in order to better understand the role of acute as well as chronic exposures in this process,

and identify points of intervention so as to prevent harmful consequences of inflammation. Adiposity is one known correlate of inflammation, and it is thought that adipose tissue is in fact pro-inflammatory; this association has been demonstrated in both children and adults.[12-14] Socioeconomic status (SES) has also been frequently studied as a potential correlate of inflammation, with measures of increasing household wealth typically associated with reduced likelihood of elevated CRP.[15-18] In developing-country settings, models of inflammation also often incorporate potential sources of pathogen exposure (and thus, infection), often represented by access to water, sanitation, and hygiene (WASH) resources such as where the family obtains water (and whether it is treated), the type of sanitation facilities, and the general cleanliness level of the house and surrounding area, with mixed results.[15, 17-19] Indoor and outdoor pollutants have also been associated with inflammation in children and adults.[19-21]

Much research on correlates of inflammation has taken place in developed-country settings, which may have not only a different distribution of inflammation and risk factors, but also potentially a different relationship of risk factors to inflammation.[18] Further, some studies have suggested that prevalence and correlates of inflammation may differ by sex as well as by age.[15, 17, 18, 22, 23] Although a number of studies have examined inflammation in schoolchildren, and others have described extreme states such as sepsis in neonates, very few studies have been published on prevalence or correlates of inflammation in healthy infants in community settings, particularly in developing countries where inflammation may be prevalent.[15, 24] Nonetheless, this is an important population to study given the potential adverse consequences of inflammation and immune dysregulation even in infants.[25, 26]

The present study leverages data from a longitudinal birth cohort of Bolivian infants to identify the prevalence and correlates of inflammation in young infants and examine how these may change with increasing infant age. Bolivia, as a lower-middle-income country in the midst of the epidemiologic transition and suffering the "double burden" of malnutrition and overweight, is a location well suited to studying varied contributors to inflammation.[27] This work will focus on the role of recent illness, anthropometry, and pathogenic exposures (represented by access to WASH resources); these factors have been shown to be correlated with inflammation in adults and schoolchildren, but there are very few data in infants. The present work will help to elucidate different inflammatory factors in a community population of healthy infants in a developing-country urban setting. Further, it will add to knowledge regarding correlates of inflammation in infants, whose innate immune function differs from that of adults.[28] Also, nearly all studies of correlates of inflammation have only taken into account CRP, a marker of the earlier stages of inflammation. In the present study, we also add AGP, a marker of later stages of inflammation, as a way to better understand how correlates may differ by acute versus chronic inflammatory processes.

Methods

Study population and design

Data for the present study were drawn from the *Nutrición, Inmunología, y Diarrea Infantil* (NIDI) study, the primary aim of which was to assess the effect of nutritional status on response to the rotavirus vaccine. For this reason, all enrolled infants

were required to receive both doses of the rotavirus vaccine (scheduled at 2 and 4 months of age). In brief, 461 healthy infants (2 – 4 weeks of age) and their mothers were recruited from 2 hospitals in El Alto, Bolivia (altitude 4000m), during well-child or vaccination visits. This is a primarily urban and largely indigenous population; while most have access to sanitation and improved water, socioeconomic resources are typically low. Exclusion criteria included infant illness at recruitment, infants suspected to have immunodeficiency (e.g., HIV), congenital malformations, and maternal inability to speak and understand Spanish or Aymara. Recruitment took place from May 2013 through March 2014, and infant-mother dyads were followed for 12 – 18 months, with final data collected in March of 2015. The study comprised 7 hospital visits and 2 in-home visits, with blood drawn from mothers at a target schedule of 1 and 6 – 8 months postpartum, and from infants at a target schedule of 2 months, 6 – 8 months, and (optionally) 12 – 18 months of age. However, despite interviewer efforts, some blood draws fell outside the target ranges due to participant family travel or faulty contact information. The third blood draw, added to support a newly funded secondary aim, was approved only after 50% of infants had already completed the study; these mothers were contacted to participate optionally in an extra visit and blood draw. Although infants were required to be healthy at the first visit (0 – 2 months of age), no infants were excluded from blood draws based on concurrent or recent illness. For the present study, only singleton infants who had completed at least the first blood draw (N = 365) were eligible, and of these 272 had non-missing data for exposures and outcomes.

Ethical approval

The protocol and instruments for this study were approved by the Emory University IRB (IRB00056127) and the Bolivian "Comité de Etica de la Investigación" (Research Ethics Committee). Mothers provided written informed consent in Spanish or Aymara.

Laboratory analysis and definitions of inflammation

Venous blood was collected by trained phlebotomists using sterile, disposable equipment. Blood was drawn into zinc-free microtainers using butterfly needles, and stored at 2 – 8 C before transport later that day to our partner lab in La Paz. Samples were then frozen before shipping to Emory, where they were aliquoted and shipped for further analyses. Plasma was analyzed by sandwich ELISA for C-Reactive Protein (CRP; a marker of inflammation; limit of detection [LOD]: 0.5 mg/L) and alpha(1)-acid-glycoprotein (AGP; a marker of inflammation; LOD: 0.1 g/L) [29].

Data collection

Sociodemographic data were collected by trained Bolivian interviewers at the first study visit via questionnaire. At each visit, interviewers collected data on maternal report of infant morbidities and feeding practices over the two weeks prior to the interview. Anthropometry was conducted by a two-person team of trained interviewers. Infants were weighed nude or with light clothing (no diaper) on a Seca scale (weight measured to the nearest 0.1kg), and measured on a ShorrBoard® (length measured to the nearest 0.1cm). Weight-for-length and length-for-age Z scores were calculated based on WHO references and using the WHO SAS macro.[30] In the field, stunted (Length-for-age Z [LAZ] < -2)

and wasted (Weight-for-length Z score [WLZ] < -2) infants were identified by study staff according to WHO growth charts and were referred as appropriate.

Variable definitions for outcomes, exposures, and covariates

Inflammation was defined as CRP > 5 mg/L or AGP > 1 g/L.[8, 31] As described above, acute illness, anthropometry, and WASH resources were considered as primary exposures of interest. Acute illnesses were defined as positive two-week maternal recall of infant symptoms; diarrhea, fever, and respiratory symptoms were included in each model as indicator variables. Anthropometric variables included overweight or obesity (Weight-for-length Z score [WLZ] > 1) and stunting (Length-for-age Z [LAZ] < -2).[32] Several measures of WASH resources (a proxy of pathogenic exposure) were considered: sewer type (piped vs. other, e.g., pit latrine), private toilet (vs. shared or no access), water source (piped indoors vs. other, e.g., piped outdoors), water treatment (any vs. none), trash disposal (picked up vs. other, e.g., thrown onto patio), crowding (>2 people sleeping per bedroom). Potential covariates included preterm birth (< 37 completed gestational weeks), Caesarian section (vs. vaginal) birth, months of exclusive breastfeeding completed at the time of visit (defined as months during which infant had received only breast milk, with no other liquids or solids), maternal employment, maternal education (categorized as primary or less, at least some secondary, or at least some superior [reference]), and sample-specific wealth index (created via principal components analysis of assets and house materials, divided into quintiles with the highest quintile as reference[33]). Cow's milk intake was considered for the models but could not be included due to low prevalence (0% at 2 months, though 14 – 30% at later visits).

Concurrent or recent vaccination was not considered as a confounder given its potential role as an intermediate between age and inflammation.

Statistical methods

Given that the same infants were followed over time and contributed multiple measurements, methods appropriate for clustered data were applied. AGP and CRP were considered first separately and as continuous variables, and then as categorical variables (elevated / non-elevated), and finally together (dichotomized inflammation defined as either elevated AGP or CRP). Potential confounders of the relationships of acute illnesses, sanitation, and obesity, to AGP and CRP were selected a priori based on literature review and Directed Acyclic Graph (DAG) analysis, and were then included in the initial models based on significant bivariate association with the exposure or the outcome.

Continuous AGP and CRP were log-transformed (base 10) to meet normality assumptions, and linear models were used to test relationships of predictors to the outcome; percent relative changes in the outcome are reported for each predictor. Percent relative change was calculated as $100*(10^{\beta} - 1)$, where β is the coefficient associated with predictor X and thus 10^{β} is equal to the ratio of $E(Y \mid X = 1)$ to $E(Y \mid X = 0)$. For categorized AGP and CRP, logistic regression was used. In each case, mixed models were first applied, and a random intercept was included if significant (Approximate Likelihood Ratio Test). If non-significant, Generalized Estimating Equations (GEE) were fit using an appropriate distribution (binomial for dichotomized outcomes) and an exchangeable correlation structure. Interactions of acute illnesses with time were tested

using Likelihood Ratio Tests (with Maximum Likelihood Estimates [MLE]) for mixed models and Wald tests for GEE models, and $p < 0.05$ was considered significant. Collinearity was assessed for each model using Condition Indices and Variance Decomposition Proportions, and models reduced as necessary until collinearity was no longer present.[34] Final linear mixed models used Restricted Maximum Likelihood Estimation (REML) and were fit using the lme4 package in the R Environment for Statistical Computing.[35, 36] Marginal R^2 (reflecting the proportion of variance explained by fixed factors) and conditional R^2 (reflecting the proportion of variance explained by fixed and random factors) were calculated for linear mixed models using the piecewise SEM R package.[37, 38] Fixed effects were tested using F tests with the Satterthwaite approximation for denominator degrees of freedom. GEE models were fit using the R gee package[39], and Wald tests based on robust variances are presented for fixed effects. Data were cleaned and analyzed using SAS v9.4 (Cary, NC) and the R Environment for Statistical Computing.[35]

Results

Characteristics of the study sample

Out of 451 enrolled singletons, 365 infants (80.9%) had at least the first blood draw (target schedule 2 months), and 272 of these (74.5%) had non-missing data for exposures and outcomes. The most common missing data were for wealth index (16% missing) and type of sewer (8% missing). Of these 272, 239 (87.8%) also completed a second blood draw (at a target schedule of 6 – 8 months), while 126 infants (46.3%)

completed a blood draw at the final study visit (target schedule of 12 – 18 months), and 121 had all three blood draws. Although some blood draws took place outside the target age range, the majority were within the target ranges (Figure 2).

Infants were fairly evenly distributed in terms of gender, nearly one third were born via Caesarian section, and one fifth were born preterm (Table 1). At least 60% of the sample had access to improved water, sanitation, and hygiene (WASH) for at least one measure, though only 40% had water that was piped inside their home (the majority of the rest had access to water that was piped to a point outside their home but close by, often in the same lot). Mothers had a mean age of 26 years, one quarter were employed, and most had at least some secondary education. About one quarter of households owned a refrigerator, and one-third had high-quality flooring material. The prevalence of exclusive breastfeeding was 60% at 1-5 months and declined with age (Table 2). Stunting varied between 14% and 18%, while overweight varied from 32% at the first blood draw to 19% at the third blood draw.

Presence of Inflammation and Recent Illness

Both CRP and AGP were right-skewed at all time points, with both also right-shifted at the second and third blood draws (at approximately 6 – 10 and 10 – 18 months of age) as compared to the first blood draw (at approximately 2 months of age; Figure 2). Almost no inflammation (elevated CRP or AGP) was detected at the first blood draw when infants were 1-5 months old (3%), but the prevalence of inflammation rose to 22% among infants assessed at 6-10 months old and 15% among the infants assessed at 10-18 months. The prevalence of recent illness among infants varied by age group, with

younger infants tending to have lower prevalence of recent illness (Table 2). Specifically, the prevalence of diarrhea increased with increasing age from 14% among infants 1-5 months to 24% among infants 10-18 months old. Cough or respiratory illness was highly prevalent, with 40% of the youngest infants affected and around 50% of the older age groups affected. The prevalence of fever was also high, with 21% among the youngest infants and around 40% for older infants and toddlers.

Results of Regression Models

AGP. Continuous (log-transformed) infant AGP was significantly associated with recent cough (relative increase of 18%, $p = 0.0001$), recent fever (relative increase of 23%, $p < 0.0001$), and age in months (relative increase of 5% per month, $p < 0.0001$; Table 3). Marginal and conditional R^2, representing the proportion of variance explained by fixed and fixed and random effects, respectively, were 27% (marginal) and 35% (conditional). Elevated AGP (> 1 g/L) was also significantly associated with recent cough (OR 3.9, $p = 0.001$), recent fever (OR 2.8, $p = 0.013$), and age in months (OR 1.1, $p < 0.0001$, for a one-month increase; Table 4). Interactions between acute illnesses and age were tested for each model and found to be non-significant (data not shown).

CRP. Continuous (log-transformed) infant CRP was significantly associated with recent diarrhea (relative increase of 48%, $p = 0.01$), recent cough (relative increase of 44%, $p = 0.003$), recent fever (relative increase of 35%, $p = 0.021$), and age in months (relative increase of 7% per month, $p < 0.0001$). Marginal and conditional R^2 were 13% and 29%, respectively. Elevated CRP (> 5 mg/L) was significantly associated with recent diarrhea (OR 2.2, $p = 0.015$) and age in months (OR 1.1, $p = 0.001$, for a one-month increase), but

not with other recent illnesses (Table 4). Interactions between acute illnesses and age were tested for both models and found to be non-significant (data not shown).

Inflammation. Dichotomized infant inflammation (elevated AGP or CRP) was significantly associated with recent fever (OR 2.1, $p = 0.019$) and age in months (OR 1.1, $p < 0.0001$, for a one-month increase; Table 4.) Interactions between acute illnesses and age were tested and found to be non-significant (data not shown).

Discussion

In this prospective cohort study of Bolivian infants, the prevalence of any inflammation increased from 3% at the first assessment (approximately 2 months of age) to 15 – 22% at later time points (approximately 6 – 8 and 12 – 18 months of age for most infants). While this is much lower than the prevalence of elevated CRP (> 5 mg/L) in a cohort of Tanzanian infants 6 – 35 months of age,[15] the overall geometric mean CRP in our older infants (0.80 mg/L at the third blood draw, ~12 – 18 months old) is comparable to, though somewhat higher than, that in a cohort of European and South Asian British infants at 12 months of age (0.69 mg/L for European origin infants, 0.51 mg/L for South Asian origin infants).[24] These comparisons potentially reflect population differences in socioeconomic status, rurality, age, and other correlates or potential modifiers of inflammation. Our study also measured AGP and found it to be elevated in only 2% of infants at the first blood draw, but > 10% of infants at later blood draws. This is a new finding, as previous studies of inflammation in healthy infants have not measured AGP.

In our study of Bolivian infants, inflammation was significantly associated with maternal recall of recent illnesses and increased age in months regardless of biomarker. The association of recent illness with inflammation and the larger magnitude of the effect in linear CRP models as opposed to linear AGP models are consistent with the biology of the APR. Specifically, CRP experiences a much more dramatic relative increase as compared to AGP during the APR [8]. We did not see significant interactions of acute illness and age, which may suggest that the infant inflammatory reaction to infectious illness does not change significantly between 2 and 18 months of age. While two other identified studies demonstrated a significant effect of age in the opposite (negative) direction, these studies included older children: one compared children 6 – 35 months to those 36 – 59 months [15], while the other included only children 2 – 15 years [17]. We hypothesize that inflammation in developing-country settings may peak at 12 – 24 months of age, and then decline as children's immune systems are trained. Another study in British infants more similar in age to our study population did not show any significant effect of age, but took place in a low-inflammation, high-resource, developed-country setting [24]. We hypothesize that our finding of increasing inflammation with age may be related to the fact that older infants and toddlers will have much more opportunity for inflammatory exposures (e.g., pathogens) given their increased mobility as well as the incorporation of new foods and potentially unsafe liquids during weaning. Although our models did not demonstrate a significant effect of cumulative months of exclusive breastfeeding, a sensitivity analysis assessing current breastfeeding practices within each age group did show a trend towards a protective effect of exclusive breastfeeding among

younger infants (data not shown). A study in Tanzanian children also suggested a "protective" effect of breastfeeding [15].

Neither marker of anthropometry (stunting nor overweight, both at low-to-moderate levels in the population) was significantly associated with inflammation in this cohort. While an association of adiposity and inflammation has been well established in adults [18, 19, 23, 40], and other authors have found similar associations in children [16, 19, 41-51], we did not see a significant association of WLZ with inflammation in our cohort. Alternative definitions of adiposity (using BMI-for-Age Z score as well as cut-offs for obesity [WLZ > 2] vs. normal – overweight) also failed to yield significant results in our models, suggesting that differences in adiposity definition were not the explanation for our results. Our results may be due to a lack of power or to the lower prevalence of overweight in our cohort as compared to developed-country children. Alternatively, it may be that our infants were too young for the inflammatory effect of adiposity to have presented, or that WLZ is not a good measure of obesity or adiposity in children this young; the studies showing significant associations were all in children of at least preschool age. Although a study of Ecuadorian children found a negative association between CRP and attained growth in children 2 – 7 years old [52], two other studies of inflammation that included infants (one in British infants 3 – 24 months of age, one in Tanzanian children 6 – 59 months of age) did not find significant associations between body size measures, including length, and CRP [15, 24].

Markers of WASH resources were also not significantly associated with individual markers of inflammation in bivariate or multivariable analyses. Though this was contrary to expectations, as we hypothesized that lack of access to WASH resources

would reflect increased pathogen exposure, it is consistent with results from Hadley et al. and Thompson et al. [15, 19], who also failed to see significant associations of WASH resources (e.g., private toilet, water quality) in multivariable models of child inflammation. It may be that the available measures of WASH were not granular or specific enough to capture true pathogen exposure, or that young infants are more protected from these effects given their lower mobility, restricted eating, and close maternal supervision. It may also be that WASH measures are more important to a longer-term process of immune development as opposed to short-term markers of inflammation in young infants, whose immune systems may still be undeveloped.

Calculated R^2 for linear models indicated that fixed effects explained a larger proportion of AGP variance as compared to CRP variance, but that no more than a third of variance was explained by the parameters in either model. This suggests that factors outside of our measured morbidities, anthropometry, and WASH resources may be important to the development of inflammation in infants. The similar R^2 (marginal and conditional) for the model of AGP implies that within-subject correlations are less important (supported by the small magnitude of the variance of the random intercept term), and that other environmental factors or behavioral factors may be important. Conversely, the large jump from the marginal to the conditional R^2 for the model of CRP suggests that within-subject factors are important for CRP levels, potentially implying constitutional differences or other factors present from birth. To our knowledge this is the first that this type of analysis has been applied to the study of inflammation in healthy infants.

Although the inflammation cut-off of 5 mg/L for CRP is widely used [11], it has been suggested that this cut-off is inappropriate for healthy or pediatric populations [11, 46, 53]. Therefore, we reran the model of elevated CRP with a cut-off of > 3 mg/L, which has previously been suggested and used as a "high-risk" cut-off [11, 54, 55]. This analysis demonstrated similar results in terms of significance to the model with the original cut-off (5 mg/L), although the effect of recent diarrhea became non-significant, while recent cough became significant (OR 2.2 [1.2 – 4.0], p = 0.012). We also tested a cut-off of > 1.1 mg/L as suggested by Wander et al.'s analysis of Tanzanian children.[53] This model also showed similar results to the models with cut-offs of 3 and 5 mg/L. However, recent fever (OR 1.8 [1.2 – 2.7], p = 0.005) and trash picked up (OR 0.6 [0.3 – 1.0], p = 0.043) became significant (recent diarrhea was again non-significant). We could not perform an analysis using a cut-off of CRP > 10 mg/L (often suggested as a marker of acute inflammation or infection [56]), because not enough infants at all time points exhibited CRP values this high.

This study has several strengths. First, we were able to follow infants over the course of their first year of life, gathering data on inflammation and its potential correlates at multiple time points. This enabled us to test whether the effects of acute illness varied over time as infants' immune systems matured. We also had access to a rich variety of data, including not only recent illness recall, but also anthropometry, birth characteristics, WASH resources, and other sociodemographics. Further, we were able to test two separate markers of inflammation—CRP and AGP—that reflect different time points in the APR. However, our study is limited by our decreased sample size at the third visit, which negatively impacts our power to test associations with many predictors

at once. Comparison of the full analytical sample with the subset with data at all three visits revealed similar prevalence of elevated inflammatory biomarkers (though cough and diarrhea were slightly less prevalent among younger infants) and few differences in characteristics—the prevalence of preterm infants was slightly lower in the subset (13% vs. 18%), while the prevalence of overweight among 2-month-olds was slightly lower (24% vs. 32%) and mothers were slightly more educated (32% with superior education vs. 26%). Linear and logistic models for both AGP and CRP gave similar conclusions with the subset as compared to the analytic dataset, though the significance of some results varied (the effects of flushing toilet and preterm birth became significant, but with a wide CI, in several models, while the effects of wealth index and water treatment became significant in the logistic model of CRP, and the effect of fever became non-significant in two models; data not shown). Although this may point to a mild selection bias resulting from drop-out at the third visit, it is reassuring that our reported results are in the same direction and towards the null as compared to those in the subset. Another limitation is that we did not have information on other potential markers of pathogen exposure, such as the presence of feces around the home.

Conclusions

In this study, we found that inflammation increased from early infancy to later infancy and early toddlerhood, potentially reflecting increased exposure to pathogens and other inflammatory agents in this cohort of Bolivian infants. We also found that recent illnesses were significantly associated with CRP and AGP, but did not fully explain differences in either APP. This cohort of infants did not demonstrate significant

associations between inflammation and WASH or anthropometry, suggesting that these exposures may be more relevant to older children. Overall, results underscore the importance of biochemical measures of subclinical inflammation in infants, given that inflammation cannot be identified by sociodemographic or morbidity information alone.

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Conflicts of Interest

The authors declare they have no conflicts of interest.

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TABLES

Table 2.1: Characteristics of the Study Sample (N = 272)

	Frequency* or Mean (±SD)	Percent
Infant Characteristics		
Age (days) at enrollment	34 ± 8	–
Male	144	52.9
C-section	81	29.8
Preterm (< 37 weeks gestational age)	48	17.6
Water and Sanitation		
Water piped indoors	108	39.7
Treats water before drinking	176	64.7
Private toilet (vs. shared or none)	170	62.5
Piped sewer (vs. pit latrine)	226	83.1
Trash picked up (vs. thrown onto patio, other)	226	83.1
Crowding (> 2 people per bedroom)	200	73.5
Other Sociodemographics		
Maternal age (years)	25.5 ± 6.3	–
Maternal employment	70	25.8
Household owns refrigerator	77	28.3
Higher quality floor material (hardwood, carpet, or tile vs. cement)	98	36.0
Maternal Education		
Primary or less	35	12.9
At least some secondary	167	61.4
At least some superior	70	25.7

*****Of singleton infants with plasma available for at least the 2-month visit and no missing data for exposures or outcomes.

Table 2.2: Sample Characteristics by Age

	1st assessment (range 1 – 5 mo.)		2nd assessment (range 6 – 10 mo.)**		3rd assessment (range 10 – 19 mo.) †	
	Frequency or Mean (±SD)	Percent	Frequency or Mean (±SD)	Percent	Frequency or Mean (±SD)	Percent
N	272	–	243	–	127	
Age and Nutrition						
Age (months)	2.1 ± 0.3	–	6.7 ± 0.9	–	14.1 ± 2.3	–
Exclusively breastfed	162	59.8	21	8.6	0	0.0
Months of exclusive breastfeeding	1.9 ± 1.3	–	3.1 ± 2.3	–	3.0 ± 2.4	–
Any breastfeeding	267	98.2	235	96.7	107	84.3
Stunted (LAZ < -2)	44	16.2	36	14.2	22	17.5
Overweight (WFLZ > 2)	86	31.6	70	29.3	24	19.0
Inflammation and Morbidities						
Elevated AGP (> 1 g/L)	4	1.5	30	12.3	14	11.0
Elevated CRP (> 5mg/L)	7	2.6	40	16.5	16	12.6
Any Inflammation*	7	2.6	54	22.2	19	15.0
Recent (2-week) diarrhea	38	14.0	38	15.6	30	23.6
Recent (2-week) cough	110	40.4	136	56.0	60	47.2
Recent (2-week) fever	56	20.6	107	44.0	49	38.6

*Inflammation: CRP > 5 mg/L or AGP > 1 g/L. **Table includes all infants in analysis (all infants with at least first blood draw and no missing data on key predictors or outcomes).

Table 2.3: Mixed Linear Regression Models of Associations of Predictors and (log-transformed) AGP and CRP

	AGP* (N = 626 observations of 272 infants)			CRP** (N = 626 observations of 272 infants)		
	Percent Change †	95% CI	P-Value‡	Percent Change †	95% CI	P-Value‡
Recent Illness (2-week recall) and Age						
Diarrhea	7.6	(-2.9, 19.1)	0.16	47.7	(10.0, 98.2)	0.01
Cough or respiratory illness	17.7	(8.5, 27.8)	0.0001	44.2	(13.7, 82.7)	0.003
Fever	23.3	(13.0, 34.6)	< 0.0001	34.6	(4.7, 73.1)	0.021
Age (one-month increase)	4.9	(4.0, 5.7)	< 0.0001	7.3	(4.8, 9.8)	< 0.0001
Anthropometry						
Stunted (LAZ < -2)	-2.0	(-12.0, 9.0)	0.70	6.7	(-21.9, 45.8)	0.68
Overweight (WFLZ > 1)	1.3	(-6.9, 10.2)	0.77	-11.5	(-30.9, 13.2)	0.33
Water and Sanitation						
Water piped indoors (vs. other source)	7.1	(-2.2, 17.3)	0.14	8.5	(-17.8, 43.2)	0.56
Treats water	5.5	(-3.5, 15.3)	0.24	10.3	(-15.8, 44.4)	0.48
Private toilet (vs. shared with other households or none)	6.1	(-3.2, 16.3)	0.21	20.0	(-9.2, 58.6)	0.20
Flush toilet (vs. other)	7.5	(-4.5, 20.9)	0.23	28.1	(-10.4, 83.3)	0.18
Trash picked up (vs. burned or otherwise disposed of)	-4.6	(-14.9, 6.9)	0.42	-14.4	(-39.4, 20.8)	0.38
Crowding (> 2 people per bedroom)	-2.5	(-12.3, 8.5)	0.65	3.8	(-24.9, 43.5)	0.82
Other Sociodemographics						
Preterm birth (< 37 weeks gestational age)	7.8	(-3.6, 20.4)	0.19	28.9	(-7.9, 80.4)	0.14
Months of exclusive breastfeeding (one-month increase)£	-0.6	(-2.6, 1.4)	0.55	-1.3	(-7.1, 4.8)	0.67
Maternal Education						

Primary or less	0.7	(-12.5, 16.0)	0.95	23.1	(-19.8, 88.9)	0.28
At least some secondary	1.5	(-7.9, 11.9)	–	27.4	(-5.4, 71.4)	–
At least some superior (reference)	0.0	–	–	0.0	–	–
Wealth Index[+]						
First (lowest) quintile	9.8	(-6.2, 28.5)	0.71	31.7	(-18.4, 112.6)	0.20
Second quintile	6.3	(-8.4, 23.4)	–	25.0	(-20.6, 96.6)	–
Third quintile	10.0	(-3.9, 26.0)	–	64.8	(9.0, 149.1)	–
Fourth quintile	4.5	(-8.1, 19.0)	–	30.4	(-12.0, 93.2)	–
Fifth (highest) quintile (reference)	0.0	–	–	0.0	–	–

*All available observations with non-missing data included for all infants with at least first blood draw. Random intercept with variance 0.004 was significant with P = 0.013 (approximate likelihood ratio test). The marginal R^2, reflecting variance explained by fixed effects, was 0.27, and the conditional R^2, reflecting variance explained by fixed and random effects, was 0.35. ** All available observations with non-missing data included for all infants with at least first blood draw. Random intercept with variance 0.065 was significant with P = 0.0004 (approximate likelihood ratio test). The marginal R^2, reflecting variance explained by fixed factors, was 0.13, and the conditional R^2, reflecting variance explained by fixed and random factors, was 0.29. [†]Percent relative change calculated as $100*(10^{\beta} - 1)$, where β is the coefficient associated with predictor X and thus 10^{β} is equal to the ratio of E(Y | X = 1) to E(Y | X = 0). [‡]F tests with Satterthwaite approximation for df. [£] Months of exclusive breastfeeding defined as total months, at time of assessment, that infant was fed only with breast milk, with no other liquids or food. One month of formula was allowed as long as breastfeeding was concurrent. [+]Constructed using principal components analysis of household assets and construction materials.

Table 2.4: GEE Models of Associations of Predictors and Elevated AGP and CRP

	Elevated AGP* (N = 626 observations of 272 infants)			Elevated CRP* (N = 626 observations of 272 infants)			Elevated AGP or CRP* (N = 626 observations of 272 infants)		
	OR	95% CI	P-Value**	OR	95% CI	P-Value**	OR	95% CI	P-Value**
Recent Illness (2-week recall) and Age									
Diarrhea	1.24	(0.56, 2.75)	0.59	2.18	(1.16, 4.10)	0.015	1.78	(0.99, 3.20)	0.053
Cough or respiratory illness	3.86	(1.71, 8.72)	0.001	1.58	(0.80, 3.14)	0.19	1.78	(0.97, 3.25)	0.062
Fever	2.78	(1.24, 6.23)	0.013	1.67	(0.86, 3.24)	0.13	2.05	(1.13, 3.73)	0.019
Age (one-month increase)	1.15	(1.08, 1.22)	< 0.0001	1.09	(1.03, 1.15)	0.001	1.10	(1.05, 1.15)	< 0.0001
Anthropometry									
Stunted (LAZ < -2)	1.79	(0.75, 4.28)	0.19	0.79	(0.36, 1.75)	0.56	1.28	(0.66, 2.50)	0.47
Overweight (WFLZ > 1)	1.17	(0.55, 2.48)	0.68	0.89	(0.46, 1.69)	0.71	1.12	(0.64, 1.95)	0.70
Water and Sanitation									
Water piped indoors (vs. other source)	0.57	(0.27, 1.19)	0.14	1.00	(0.52, 1.93)	1.00	0.80	(0.45, 1.43)	0.45
Treats water	0.88	(0.41, 1.90)	0.75	1.45	(0.80, 2.62)	0.22	1.06	(0.62, 1.80)	0.83
Private toilet (vs. shared with other households or none)	0.85	(0.39, 1.82)	0.67	1.21	(0.64, 2.28)	0.55	1.02	(0.58, 1.82)	0.93
Flush toilet (vs. other)	1.30	(0.46, 3.72)	0.62	2.12	(0.79, 5.66)	0.13	1.56	(0.73, 3.35)	0.25
Trash picked up (vs. burned or otherwise disposed of)	0.84	(0.34, 2.06)	0.71	1.10	(0.46, 2.63)	0.83	0.92	(0.46, 1.85)	0.81
Crowding (> 2 people per bedroom)	0.75	(0.33, 1.70)	0.49	0.85	(0.42, 1.73)	0.65	0.72	(0.39, 1.35)	0.31
Other Sociodemographics									
Preterm birth (< 37 weeks gestational age)	1.05	(0.46, 2.43)	0.90	1.76	(0.92, 3.35)	0.088	1.44	(0.79, 2.62)	0.23
Months of exclusive	0.92	(0.79, 1.07)	0.27	1.03	(0.90, 1.19)	0.65	1.03	(0.91, 1.16)	0.67

breastfeeding (one-month increase)†									
Maternal Education									
Primary or less	0.78	(0.36, 1.67)	0.68	2.13	(0.88, 5.16)	0.12	1.28	(0.59, 2.74)	0.57
At least some secondary	0.64	(0.22, 1.83)	–	2.16	(1.02, 4.55)	–	1.37	(0.76, 2.47)	–
At least some superior (reference)	1.00	–	–	1.00	–	–	1.00	–	–
Wealth Index‡									
First (lowest) quintile	0.36	(0.09, 1.44)	0.56	1.50	(0.48, 4.67)	0.11	0.82	(0.30, 2.24)	0.14
Second quintile	0.66	(0.23, 1.93)	–	1.95	(0.70, 5.44)	–	1.26	(0.54, 2.95)	–
Third quintile	1.04	(0.35, 3.06)	–	3.23	(1.24, 8.42)	–	2.17	(0.98, 4.82)	–
Fourth quintile	1.03	(0.41, 2.60)	–	2.43	(1.05, 5.66)	–	1.66	(0.81, 3.42)	–
Fifth quintile (reference)	1.00	–	–	1.00	–	–	1.00	–	–

* All available observations included for all infants with at least first blood draw. Elevated AGP defined as AGP > 1 g/L. Elevated CRP defined as CRP > 5 mg/L. GEE models used, with binomial link function and exchangeable correlation structure. **Wald tests used. †Exclusive breastfeeding defined as not having received any other liquid besides breast milk or any food at the time of the assessment. Partly breastfed defined as receiving complementary foods as well as breast milk (within last 24hr.) at time of assessment. Infants not breastfed were receiving complementary foods at the time of the assessment but had not breastfed within the last 24hr. ‡Constructed using principal components analysis of household assets and construction materials.

FIGURES AND FIGURE LEGENDS

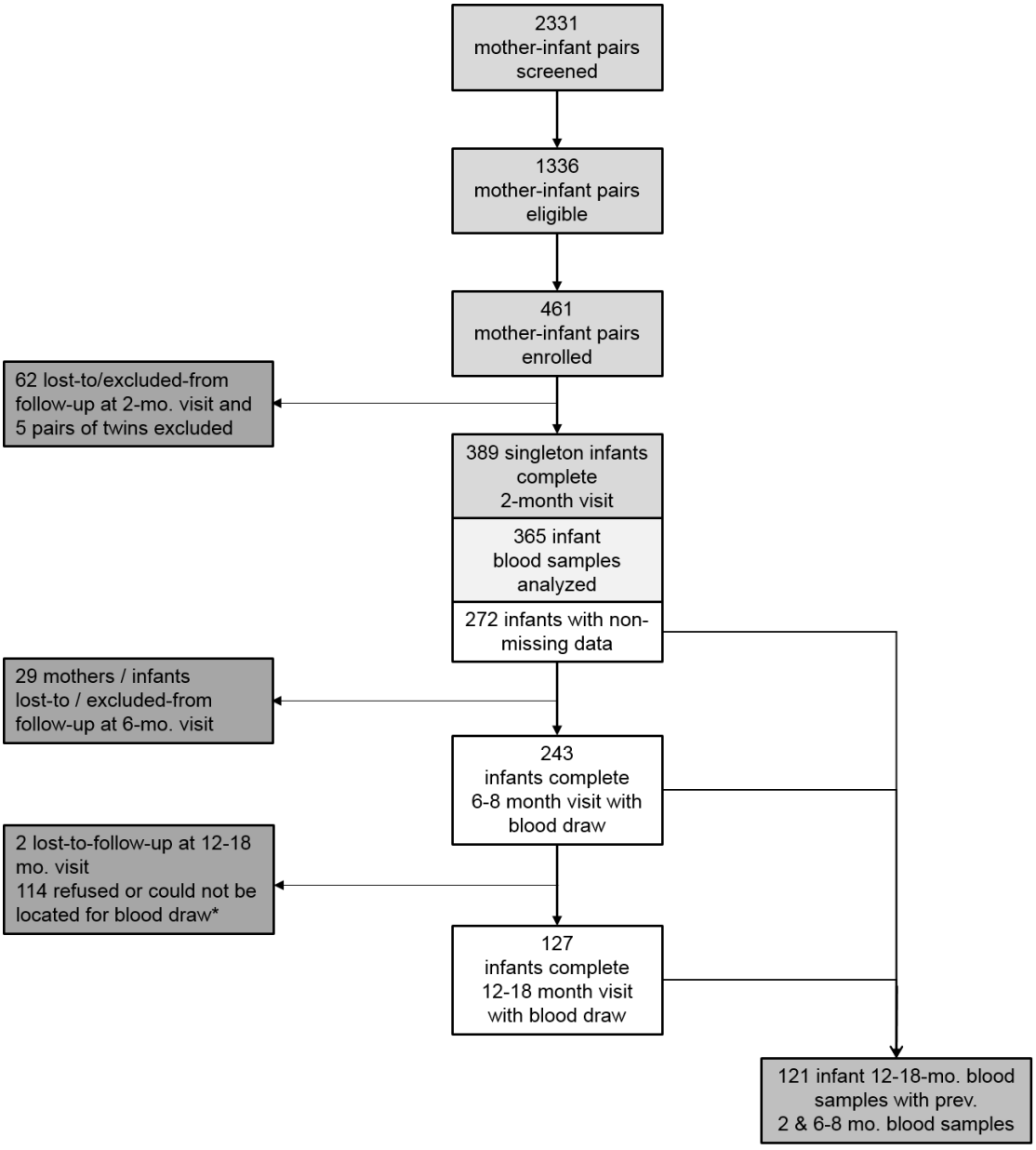

Figure 2.1: Participant Flow.

Of 2331 screened mother-infant pairs, 1336 were eligible and 461 enrolled. A total of 365 singleton infants provided samples at 2 months, but only 272 were included in the present analysis due to missing data. Of these, 243 had blood drawn at 6 – 8 months, and 127 at 12 – 18 months of age. There were 121 infants with samples at all three time points. *Third blood draw was added after a secondary aim was funded, see Methods.

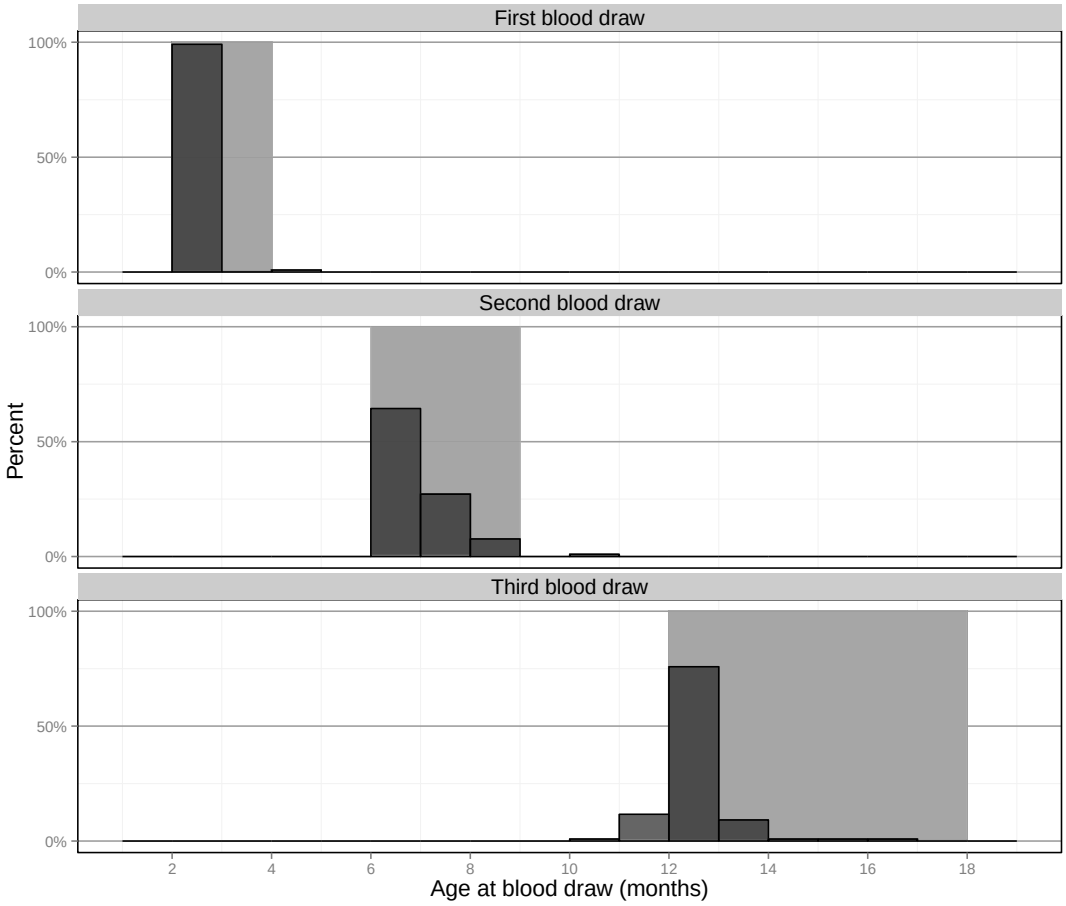

Figure 2.2: Infant age distribution.

Most of the blood draws (dark gray bars) fell within the target age ranges (light gray bands), with the highest success in the first blood draw.

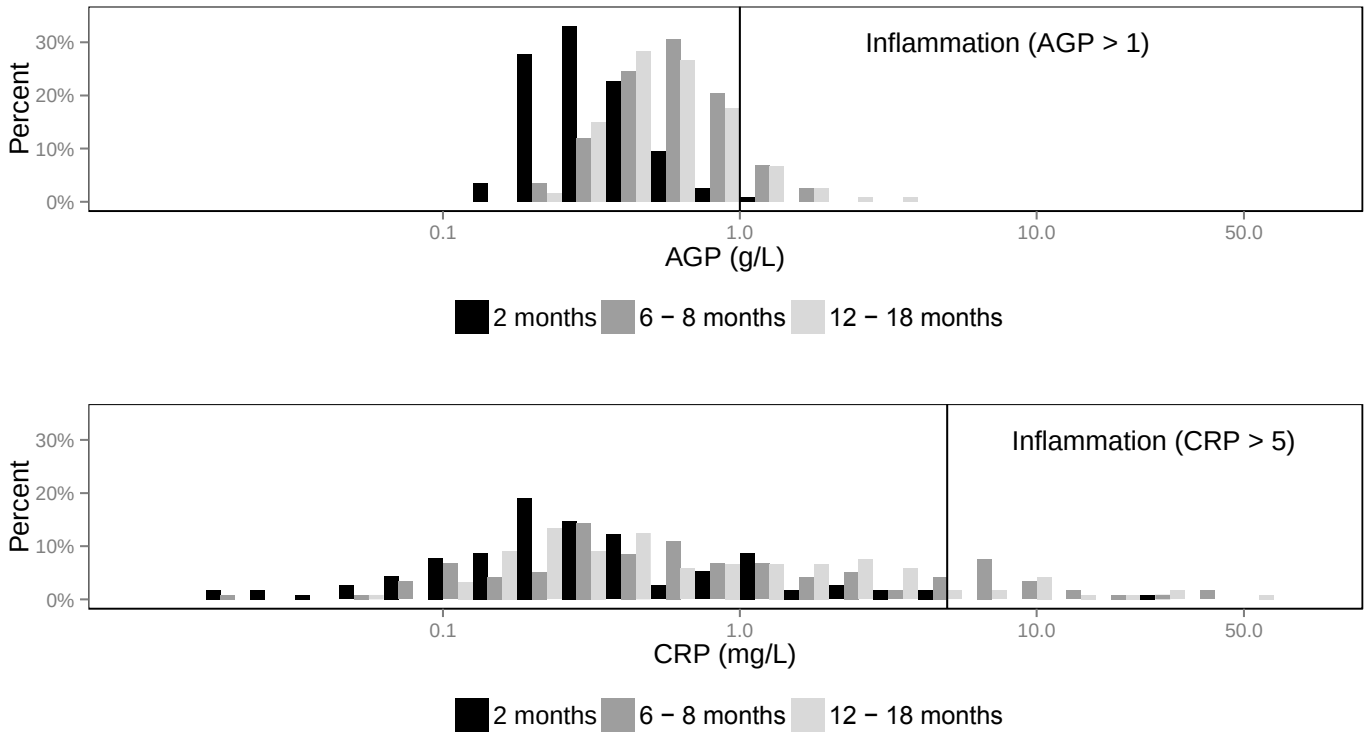

Figure 2.3: Distribution of inflammatory biomarkers.

The distribution of infant AGP and CRP was right-shifted for ages older than 2 months. Both biomarkers of inflammation were right skewed. The X axes are shown on the log scale. The Y axes indicate the percent of observations within each visit at each level of AGP and CRP. Abbreviations include AGP (alpha(1)-acid glycoprotein) and CRP (C-reactive protein).

Chapter 3: Prevalence of Iron Deficiency, Iron Deficiency Anemia, and Anemia

In preparation.

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The findings and conclusions in this article are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

Abstract

Background. Iron deficiency (ID) is a major contributor to infant and maternal morbidity worldwide, with potentially severe consequences if uncorrected. There is limited research on the trajectory of iron status in young infants, as well as the necessity of adjusting iron biomarkers for inflammation in diverse populations, important for guiding iron interventions.

Objective. We aimed to quantify the prevalence of inflammation-adjusted ID, anemia, and iron deficiency anemia (IDA) over the first year of life in a cohort of primarily breastfed Bolivian infants and their mothers.

Methods. This prospective cohort study recruited healthy mother-infant dyads from two peri-urban hospitals. Three blood draws were taken (1 – 2, 6 – 8, and 12 – 18 months). Blood was analyzed for hemoglobin (Hb), ferritin (Fer), soluble transferrin receptor (sTFR), C-reactive protein (CRP), and alpha(1)-acid glycoprotein (AGP). Linear regression was used to adjust for the effect of inflammation (measured via CRP and AGP). Standard cut-offs were used (with altitude adjustment of hemoglobin).

Results. The prevalence of inflammation (elevated CRP or AGP) among infants and toddlers was low (15% in 12 – 18-month-olds). The prevalence of ID was <1% (inflammation-adjusted Fer) in 2-month-olds, but significantly increased to 56% in 6 – 8-month-olds and 78% in 12 – 18-month-olds. ID estimates using sTFR or Body Iron (BI) were lower but displayed similar patterns. Anemia and IDA were highly prevalent in infants, increasing over time from 72% and <1% in 2-month-olds to 82% and 68% in 12 – 18-month olds. Maternal ID declined from 41% at one month postpartum to 28% at 6 –

8 months postpartum. Inflammation-adjusted ID prevalence was different from unadjusted estimates by up to 15 percentage points.

Conclusions. ID, anemia, and IDA were common in this cohort of healthy Bolivian infants beginning at 6 – 8 months and increased by one year of age, suggesting that early interventions may be necessary in populations where mothers have low iron stores. Ignoring inflammation tended to substantially underestimate prevalence of ID even in this low-inflammation population, underscoring the importance of adjusting iron biomarkers for this effect.

Key words: Iron deficiency, anemia, iron deficiency anemia, infant nutrition, micronutrient deficiencies, global micronutrient malnutrition

Introduction

Iron deficiency (ID), which can progress into iron deficiency anemia (IDA) if uncorrected, is one of the most common micronutrient deficiencies globally: an estimated 40% of children under 5 and 38% of pregnant women worldwide suffer from ID [1]. ID and IDA can have especially severe consequences in young children as well as pregnant women, and have been associated with reduced cognitive development in infants, preterm delivery, and maternal death [2, 3].

Infants are typically born with high stores of iron, which are accumulated during the third trimester of gestation and are thought to last for at least 4 – 6 months [4]. While multiple studies have gathered data on iron status on infants at different ages [5-12], few have provided data for healthy infants followed from breastfeeding age through weaning and up to one year of life [13-15]. These published studies suggest that iron status declines significantly over the first year of life, potentially leading to ID in vulnerable populations. Longitudinal studies, especially in developing-country settings where ID is prevalent, can be particularly helpful in understanding iron status over the course of early life [5, 16]. Further, while ID cut-points have been suggested for young infants (< 6 mo.), none have been universally agreed upon [5, 16]. It may therefore be useful to assess cut-points in young, breastfeeding infants to predict later ID at weaning and afterwards.

Similarly, mothers also undergo changes in iron metabolism during and following pregnancy [17]. During pregnancy, blood volume increases to support fetal life, also

leading to changes in hemoglobin concentration and thus appropriate cut-offs for ID and anemia. Delivery itself can cause large blood losses, which will also affect iron and hemoglobin concentration. Following pregnancy, mothers begin a process of returning to normal blood volume and composition; as part of this process, hemoglobin iron is recycled to restore body iron stores. These processes are generally thought to normalize by 6 – 8 weeks postpartum [17]. However, while results of some studies have been consistent with this hypothesis [18], others have demonstrated that some changes may last past this time [19-22], suggesting that the duration of the "postpartum period" may be longer, and that cut-points for ID in non-pregnant women may not be the best predictors of ID risk for very recently postpartum women. New cut-points indicating at-risk mothers may be useful. Further, additional data regarding iron status biomarkers in postpartum women, and their evolution over time, will inform policies regarding the timing and target population for postpartum iron supplementation.

Iron status and response to iron interventions can be assessed through a variety of biomarkers, which are also used to diagnose ID and IDA [16]. Ferritin (Fer), a sensitive measure of iron stores, is highly affected by inflammation (typically defined by elevated levels of the acute phase reactants C-reactive protein [CRP] and alpha(1)-acid glycoprotein [AGP]). Ferritin values may double during the inflammatory response, even when the inflammation is subclinical [23-25]. Though soluble transferrin receptor (sTFR) is less affected by inflammation, it may also increase [23-25]. BI is another measure commonly used to describe iron status, and as a function of Fer and sTFR, is also affected by inflammation [26]. Therefore, it is critical, particularly in areas with high background

levels of inflammation, to adjust iron biomarkers for the effect of inflammation [24]. Several different methods are typically considered for the adjustment of Fer (and thus, estimates of ID) in the presence of inflammation, each with advantages and disadvantages; there are no international guidelines on how to best adjust iron biomarkers for the effects on inflammation [23, 24]. Adjusting for inflammation in high-inflammation areas, such as those with high malarial and HIV burden, may change estimates of ID by up to 30 percentage points, depending on the adjustment method used [27, 28]. Yet there is limited data on how different inflammation-adjustment methods perform across populations with different ages, sociodemographics, background inflammation, and from different geographies. In particular, more data in different settings will add to understanding of the more-recently developed linear regression method, which will be used in the present analysis [27, 29]. Further, it is necessary to better understand the impact of adjusting for inflammation even in areas with lower inflammation, given that studies to-date focus on high-inflammation settings.

To address these needs, the present study seeks to quantify the prevalence of ID, anemia, and IDA at several time points during the first year of life, while adjusting for inflammation, in a cohort of mother-infant dyads in El Alto, Bolivia. A secondary objective will be to investigate the predictive value of early iron status on later iron status among mothers and their infants. This high-altitude population, while under-resourced, does not suffer a high prevalence of malaria or HIV, and will thus be an important comparator to past research, which was mainly in African settings where these diseases are endemic. Further, this is a generally healthy population that benefits from a national

supplementation program that provides multiple micronutrient powder (MNP) sachets at regular intervals to children 6 – 59 months [30]. Understanding the trajectory of iron status among young infants from breastfeeding through weaning age will inform supplementation policies for infants and mothers. Results comparing inflammation-adjusted to unadjusted values will also inform preferred methods of adjustment particularly in non-malarial settings, enabling more-accurate assessment of the iron status of populations.

Methods

Study population and design

Data for the present study were drawn from the *Nutrición, Inmunología, y Diarrea Infantil* (NIDI) study, the primary aim of which was to assess the effect of nutritional status on response to the rotavirus vaccine. A total of 461 infants were enrolled to reach a sample of 422, calculated to enable detection of an 18% difference in seroconversion between malnourished and adequately nourished infants, with 80% power. In brief, healthy infants (2 – 4 weeks of age) and their mothers were recruited from 2 hospitals in El Alto, Bolivia (altitude 4000m), during well-child or vaccination visits. This is a peri-urban and largely indigenous population; while most have access to sanitation and improved water, socioeconomic resources are typically low. Exclusion criteria included infant acute illness, suspected immunodeficiency (e.g., HIV), congenital malformations, and maternal inability to speak and understand Spanish or Aymara. Recruitment took place from May 2013 through March 2014, and infant-mother dyads were followed for

12 – 18 months, with final data collected in March of 2015. The study comprised 7 hospital visits and 2 in-home visits, with blood drawn from mothers at a target schedule of 1 and 6 – 8 months postpartum, and from infants at a target schedule of 2 months, 6 – 8 months, and (optionally) 12 – 18 months of age. Mothers were also offered anemia testing for themselves via finger stick at 12 – 18 months postpartum. This third blood draw, added to support a newly funded secondary aim, was approved only after 50% of infants had already completed the study; mothers were contacted to participate optionally in an extra visit and blood draw.

Ethical approval

The protocol and instruments for this study were approved by the Emory University IRB (IRB00056127) and the Bolivian Comité de Etica de la Investigación (Research Ethics Committee). Mothers provided written informed consent in Spanish or Aymara.

Laboratory analysis and definitions of anemia, iron deficiency, and inflammation

Venous blood was collected (1mL) from mothers and infants using zinc-free syringes and tubes. Hemoglobin (Hb) was measured at point-of-care using a HemoCue® Hb system. Plasma was analyzed by sandwich ELISA for C-Reactive Protein (CRP; a marker of inflammation; limit of detection [LOD]: 0.5 mg/L), alpha(1)-acid-glycoprotein (AGP; a marker of inflammation; LOD: 0.1 g/L), ferritin (Fer; LOD: 2 µg/L), and soluble transferrin receptors (sTFR; LOD 0.5 mg/L; (VitMin Lab, Germany) [31]. BI was calculated from Fer and sTFR utilizing Cook's formula [26]:

$$Body\ Iron = -\frac{1}{0.1207} * \left(\left(\log_{10}\frac{sTFR}{Fer}\right) - 2.8229\right)$$

Hb was adjusted for the high altitude (3500 – 4000m) of El Alto and the surrounding area of La Paz [32]. Anemia was defined as corrected Hb < 11 g/dl for infants and pregnant mothers (< 10 mothers were pregnant by the last visit), and as corrected Hb < 12 g/dl for non-pregnant mothers, based on WHO guidelines [33]. Low iron stores were defined as Fer < 12 µg/L for infants and Fer < 15 µg/L for mothers [16]. Values of sTFR > 8.3 mg/L were considered indicative of tissue ID for both mothers and infants. BI was also used to assess ID, with a cut-off of BI < 0 mg/kg for both mothers and infants. Inflammation was defined as CRP > 5 mg/L or AGP > 1 g/L [23, 34].

Adjustment of iron marker values

Given the well-known effect of inflammation on Fer, it was deemed important to adjust all iron marker values for CRP and AGP [23]. As in the Biomarkers Reflecting Inflammation and Nutrition Determinants of Anemia (BRINDA) project [29], we explored various methods for accounting for inflammation: exclusion of inflamed individuals, calculation and use of correction factors [23], and linear regression [27]. For the present paper, we present the crude (unadjusted) results as well as those adjusted with a linear regression method. Briefly, the marker of iron status (log-transformed to meet normality assumptions) was modeled as a function of continuous CRP and AGP (also log-transformed to improve model fit):

$$\ln(Ferritin) = \beta_0 + \beta_1 \ln(CRP) + \beta_2 \ln(AGP).$$

Estimated coefficients of AGP and CRP were then used in the adjustment equation:

$$\ln(Fer_{corr}) = Fer_{obs} - \hat{\beta}_1\left(\ln(CRP_{obs}) - \ln\left(CRP_{ref}\right)\right) - \hat{\beta}_2(\ln(AGP_{obs}) - \ln(AGP_{ref})).$$

For observations where neither CRP nor AGP exceeded the referents, ferritin was not corrected. For observations where only CRP was above the referent value, only CRP was used in the equation. For observations where only AGP was above the referent value, only AGP was used in the equation. Although it would be possible to correct values with APP below the referent values, this could result in APP below biologically plausible levels, and so it was determined to adjust only those participants with APP above referents. Results for adjustment of Fer for all APP provide extremely similar results and are included in the Appendix (Figure A3). As there are no established reference values for normal CRP and AGP in the literature, the first deciles of CRP and AGP in the non-inflamed population were used as the referent values for the reported results. Adjusted BI was calculated by applying Cook's formula to the adjusted Fer and sTFR values. Alternative reference values were also tested and results are reported in the Appendix (Figure A4).

Statistical methods

To assess differences between continuous variables (e.g., Fer) at different time points or comparing adjusted to unadjusted values, the Wilcoxon signed rank test was used given non-normal distributions and paired data. To assess differences between categorical variables (e.g., ID) at different time points, the McNemar test for paired data was used. Fisher's exact chi square test was used to test unpaired categorical data. Alpha was set to 0.001 to correct for multiple comparisons. Cut points were examined and ROC curves were plotted using the ROCR package in the R statistical computing environment [35]. Reported cut-points were calculated to maximize accuracy while maintaining sensitivity

(Se) of at least 60% and specificity (Sp) of at least 35%. Data were cleaned and analyzed using SAS v9.4 (Cary, NC) and the R Environment for Statistical Computing [36].

Results

Study population

The study population included 374 infants (81.1% of 461 enrolled and 93.7% of 399 completing requirements for the second visit) with at least one successful blood draw (from the first blood draw, at approximately 2 months) available for micronutrient analysis (Figure 1). Of these 374, 315 (84.2%) had the first two blood draws, and 162 (43.3%) had all three blood draws (last blood draw was optional; Figure 1). Infants were recruited at an average age of one month, and were relatively evenly distributed in terms of gender (Table 1). Nearly one-third were born via Caesarian section, one-fifth preterm, and less than one-tenth low birth weight; fewer than one fifth were born with a birth interval < 36 months. Virtually all infants had been breastfed at some point in their life, and most received some form of micronutrient supplementation by the age of one year. Approximately half of the infants were first-born children; the average age of mothers was 25 years, the average maternal BMI was 26, and most mothers were married or cohabiting with the infant's father. Based on maternal education and household characteristics, the study sample was of generally low socioeconomic status (Table 1).

Feeding practices and global nutrition

Nearly all infants were partly breastfed for at least 6 months, with 82% still continuing to breastfeed after one year of age (Table 2). Exclusive breastfeeding was lower, with only 58% of infants exclusively breastfed at the first assessment of iron status (around 2 months of age). Stunting was present in 21% of infants at the first blood draw, 14% at the second blood draw, and 21% at the last blood draw. Overweight varied across time points from 8% to 4%. Inflammation reached a maximum of 21% at 6 – 8 months of age. Maternal inflammation was highest (35%) in the first month after delivery. Anemia was present in most infants, with 82% classified as anemic by one year of age; however, the prevalence of anemia was not statistically significantly different by time point. Maternal anemia was highest one month after pregnancy (32%), and then decreased over time (with a significant reduction from 1 to 6 – 8 months postpartum).

Infant iron status

At the first blood draw (around 2 months), most infants had age-appropriate stores of iron (as indicated by Fer and BI above ID thresholds), as well as adequate tissue iron (measured by sTFR $\leq$ 8.3 mg/L). These stores declined significantly ($p < 0.0001$) by the second blood draw (around 6 – 8 months), with median adjusted Fer falling from 156 μg/L to 10 μg/L, and then declined further to 6 μg/L by the third blood draw (around 12 – 18 months of age; Table 3). Iron status also worsened over time as measured by sTFR and BI. Since differences between adjusted and unadjusted values were meaningful, results will primarily report adjusted values.

These temporal patterns of declining iron status were also reflected in prevalence estimates of ID (Table 4). By the second blood draw (around 6 – 8 months), 56% of infants had low iron stores, defined as inflammation-adjusted Fer < 12 μg/L (unadjusted ID was 41%). By the third blood draw (12 – 18 months), 78% (65% for unadjusted Fer) of infants had low iron stores. Low tissue iron, defined as inflammation-adjusted sTFR > 8.3 mg/L, also steadily rose in prevalence (27% at the third blood draw), as did ID as defined by inflammation-adjusted BI < 0 (67% at the third blood draw). All differences between time periods were statistically significant ($p \leq 0.0001$), with the exception of unadjusted tissue ID from the first to second blood draws.

Neither preterm birth nor low birth weight were significantly associated with infant ID as measured by Fer or BI at the second or third blood draws (data not shown). For infants at the second blood draw (6 – 8 months), low birth weight (OR 6.3 [1.8 – 20.3], p = 0.002), but not preterm birth, was significantly associated with low tissue ID (sTFR > 8.3 mg/L). For infants at the third blood draw (12 – 18 months), neither low birth weight nor preterm birth was significantly associated with tissue ID (sTFR > 8.3 mg/L). Among infants at the second blood draw (6 – 8 months old), exclusive breastfeeding was significantly associated with tissue ID (OR 4.5 [1.3 – 13.6], p = 0.008), but not with low iron stores as defined by BI < 0 (OR 2.3 [0.9 – 5.7], p = 0.06) or Fer <12 μg/L (OR 2.3 [0.9 – 6.6], p = 0.10). The effect of exclusive breastfeeding could not be assessed at other time points due to low prevalence of ID at the first blood draw and no exclusive breastfeeding at the third blood draw.

Given that anemia was present in nearly all infants, the prevalence of IDA closely tracked the prevalence of ID (Table 4). Differences between time periods were again statistically significant.

Maternal iron status

Mothers' iron status improved from the first blood draw (one month postpartum) to the second blood draw (6 – 8 months postpartum); all differences were statistically significant ($p < 0.0001$) with the exception of unadjusted Fer. At the first visit, median adjusted Fer was 18.4 µg/L, while at the second visit, median adjusted Fer was 24.7 µg/L (Table 3). Similarly, iron status as measured by sTFR and BI also significantly improved.

Maternal ID decreased in the second blood draw as compared to the first blood draw. At the first blood draw (one month postpartum), 40% of mothers had low iron stores as defined by adjusted Fer < 15 µg/L (Table 4). At the second blood draw (6 – 8 months postpartum), this prevalence dropped to 28%. Patterns for sTFR and BI were similar (Table 4). Differences in ID between the first and second blood draws were statistically significant ($p < 0.0001$) for all adjusted prevalence estimates.

As in infants, mothers' IDA also tracked their ID prevalence closely (Table 4). Adjusted estimates of IDA were significantly different ($p < 0.0001$) by time period for all three markers (Fer, sTFR, BI).

Exploration of cut-points

In order to examine the utility of different potential Fer cut-offs in identifying infants and mothers at high risk of developing ID, we constructed ROC curves to assess Fer as a predictor of later ID. Because not all iron biomarkers may always be accompanied by inflammatory biomarkers used for adjustment, we examined both adjusted and unadjusted biomarkers. Unadjusted values are presented for ease of comparison to other studies. Unadjusted infant Fer at the first blood draw (approximately 2 months of age) was a fair predictor [37] of infant ID at the second blood draw (approximately 6 – 8 months), with an AUC of 77%. A threshold of 167 µg/L predicted later ID with 81% sensitivity and 65% specificity (Figure 2). Infant Fer at the first blood draw was not very predictive of ID at the third blood draw (12 - 18 months; AUC of 57%). However, Fer at the second blood draw was a moderately good predictor of ID at the third blood draw (AUC of 72%), with a threshold of 28 µg/L providing 76% sensitivity and 63% specificity. Maternal Fer was not a good predictor of infant ID at any time point (data not shown). Maternal Fer at the first blood draw was predictive of maternal Fer at the second blood draw (AUC of 74%; threshold of 17 µg/L provided 60% sensitivity and 76% specificity; Figure 3). Results were very similar for curves using inflammation-adjusted Fer for infants and mothers (Figures 2 – 3).

Discussion

Infant iron status

In this cohort of Bolivian infants, ID was extremely common early in life; by the age of 6 – 8 months, approximately half of infants had low iron stores, and by the age of 12 – 18

months, approximately three quarters of infants had low iron stores. While tissue ID was less common, it too increased from infancy into young toddlerhood. Anemia was also very common (>70%), and nearly all iron-deficient infants were also anemic, leading to a high prevalence of IDA (46% at 6 – 8 months and 67% by 12 – 18 months). At the first blood draw, < 1% of anemia was associated with ID (data not shown). However, by the second blood draw, 62% of anemia was associated with ID, and at the third blood draw, 81% of anemia was associated with ID. This suggests that ID is a primary cause of anemia in this population.

The general pattern of decreasing infant iron stores and increasing prevalence of ID is consistent with iron biology and other research. Even in well-resourced populations (e.g., USA, Denmark, Norway), Fer has been shown to decrease from birth through at least 6 months of age, as birth iron stores run out faster than they are replenished through diet [8, 13, 15, 38]. The prevalence of ID in our cohort was comparable to that in a cohort of Nigerian infants (59% at 6 months [9]) and that in a cohort of Korean infants (70% of 6 – 24-month-olds)[39], though higher than cohorts of Peruvian (32% at 5 months [6]) and Brazilian infants (26% at 6 months [7]). Both ID and IDA in our cohort were much higher than the prevalence reported in a recent review of studies of European infants and preschoolers (6 – 36 mo.; ID generally ≤ 25% and IDA generally < 10%)[40]. Prevalence of IDA was comparable to that in a cohort of Saudi Arabian infants (49% at 6 – 24 months of age)[41]. Among Latin American countries in a recent review, Bolivia has the highest national prevalence of anemia among preschoolers (6 – 59-month-olds; 61%) [42], and so it is not surprising that our study also showed an extremely high prevalence

of anemia in infants. The high prevalence of anemia in our cohort is also consistent with (though somewhat higher than) studies of anemia in low-resource populations of Chinese infants (54 – 60% of 6 – 11-month-olds [43, 44], 51% of 12 – 17-month-olds [44]). Overall, these comparisons suggest that our population showed a high prevalence of ID, IDA, and anemia even among other low-resource populations.

The high prevalence of ID in our cohort is concerning, especially in the context of a national supplementation program that regularly provides iron-containing multiple micronutrient powder (MNP) sachets ("Chispitas") to all infants and children 6 – 59 months of age (60 sachets, to be taken 1 per day, provided once per year) [30]. Given that > 90% of our sample reported receiving Chispitas by the age of 12 months, and over 90% of these reported some use of Chispitas, it is possible that the dose (12.5 mg per sachet as ferrous fumarate [30]) was not sufficient, or the iron was not adequately absorbed, to prevent ID in most infants. While preterm birth is a known risk factor for ID [4], we did not observe this difference in our cohort (perhaps because most infants were of normal birth weight and > 34 weeks gestational age). However, only 25% of these infants received iron drops according to recommendations (data not shown). Consistent with other reports [7, 45, 46], we observed increased odds of ID for exclusively breastfeeding infants, though this is also unlikely to fully explain the high prevalence. Though altitude, given the high hemoglobin requirements it engenders, may be related to the extremely high prevalence of anemia [44], it is unclear whether it would also explain the high prevalence of ID, especially among infants [47]. Other potential explanations include inadequate dietary intake, frequent enteric infections leading to reduced iron

absorption, maternal factors, and sociodemographics [4]. Further research in this cohort will better elucidate the role of these potential risk factors to provide information to improve the timing and content of ID-prevention interventions.

Maternal iron status

Mothers exhibited the opposite pattern from infants; consistent with prior literature [17-19], their iron status improved over time, even after adjusting for inflammation, from a prevalence of 40% ID at 1 month postpartum to a prevalence of 28% at 6 – 8 months postpartum. Although Fer in some other studies decreased or remained the same postpartum, those results are potentially driven by iron supplementation during pregnancy [21, 22, 48], which was adhered to by only 57% of our cohort.

Adjustment for inflammation

Several previous studies of ID and adjustment for inflammation have taken place in African settings where up to 50% of children were inflamed, and found that estimates of ID can vary by 8 – 30 percentage points depending on the exact adjustment method used [27, 28]. Inflammation adjustment is also considered important in recently postpartum women, since delivery is a known inflammatory process that can transiently increase Fer, potentially leading to underestimates of ID if unaccounted for [49]. Even in this relatively low-inflammation population (< 22% of infants), adjustment of iron biomarkers for CRP and AGP meaningfully affected most estimates of iron status in infants and mothers. Ignoring inflammation tended to underestimate prevalence of ID using Fer by approximately 15 percentage points, a meaningful difference for making clinical and

public health decisions, suggesting that inflammation should be accounted for even where not highly prevalent.

Ferritin cut-offs to predict later ID

While Fer cut-offs are well established for infants of at least 6 months of age, no cut-offs are universally accepted for younger infants, and it is believed that iron homeostasis may not be fully developed even at 9 months of age [5]. Nonetheless, it may be useful to identify young infants that should be closely followed for ID. ROC curves suggested that infant Fer could adequately predict ID at subsequent time points, with better prediction in the short term (4 – 6 months) vs. long term (10+ months), and that very high birth iron stores (Fer >160 μg/L) are needed in this population to maintain iron status through breastfeeding and weaning. Although we could find no other studies with ROC analysis of ID in infants, our findings are in line with research by Ziegler et al. in U.S. infants: of infants who developed ID before 6 months of age, the mean Fer at one month of age was 125 μg/L, much lower than the average of 242 μg/L in the full sample [15], again suggesting that birth stores must be high to avoid ID at weaning age.

Strengths and limitations

This study has several strengths. We were able to follow this cohort of Bolivian infants and their mothers over the course of a full year of life, with multiple time points measured. In addition, we used AGP and CRP to adjust iron biomarkers for the effect of inflammation, increasing the validity of our estimates. A sensitivity analysis utilizing externally generated AGP and CRP referents (first deciles from the pooled multi-country

data of the BRINDA project [29]) yielded similar (and in some cases slightly higher) estimates of ID and IDA among both infants and mothers (data not shown), suggesting that the distribution of these Acute Phase Proteins (APP) among our cohort is not meaningfully different from that of young children in a variety of other global settings. Further, our population of healthy, primarily breastfed infants in a setting of routine national supplementation allows us to assess the natural course of iron status in a healthy developing-country population. However, there are also several limitations to this study. Not all mothers agreed to the third blood draw for their infant, so sample size is reduced by over half for 12 – 18-month-old infants. Sample size was also reduced at the second blood draw (6 – 8 months) due to failure of blood draws as well as study drop-out. However, the population with all three blood draws was highly similar to the population without the third blood draw in terms of demographics in addition to prevalence of ID and IDA (data not shown). Overall, there was a low participation rate (35% of eligible mother-infant pairs were enrolled); although exact percentages are not available, major reasons for failure to enroll included a lack of interest (e.g., due to time constraints or refusal of blood draw) and an inability to follow up with interested mothers (faulty contact information or busy phone lines). While this raises the possibility that mothers and infants refusing blood draws (and therefore not enrolled) were somehow different from those enrolled, it is encouraging that the sociodemographics and birth characteristics of enrolled mothers and infants in the present study were very similar to those in a pilot study in the same hospitals but not requiring blood draws (data not shown). It is also unknown whether ID would continue to increase if this cohort were followed past one year of age. Although these results may be generalizable to other high-altitude Andean

populations, they are likely not generalizable to settings with a high prevalence of inflammation or other causes of anemia (such as malaria or HIV). Anemia results may not be generalizable to lower-altitude settings.

Conclusions

The prevalence of ID was high in this cohort of Bolivian infants, developing early by 6 months of age and increasing through 12 – 18 months of age in the setting of a national MNP supplementation program. The high prevalence of ID in young infants is concerning given the potentially severe sequelae of ID, and suggests a need for interventions that can be implemented in pregnant women or young infants, to improve iron status even before infants are weaned. For instance, more emphasis may need to be placed on supplementation of pregnant women (currently provided but inconsistently adhered to) or delayed cord clamping at birth (currently recommended by the Bolivian national standards [Atención Integrada al Continuo del Curso de la Vida] but inconsistently practiced). More up-to-date data is required on national coverage and intake of MNP. This research also suggests that ID prevalence among young infants may be higher than expected in other developing-country settings, indicating a need for additional research into early interventions. Further, this analysis demonstrates that adjustment for the effect of inflammation can have a meaningful impact on prevalence estimates of ID; the correction methods employed here can be applied to other micronutrients where this effect exists (e.g., retinol binding protein, a marker of Vitamin A status) [23].

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Author contributions

PS, JL, CDB, MK, VI, AFdA, PAR, and RMB designed research. PS, JS, AFdA, PAR, RR, VI, and RMB conducted research. RMB analyzed data. RMB, PS, and JS wrote the paper and had primary responsibility for final content. All authors have read and approved the final manuscript.

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Table 3.1. Characteristics of the Study Sample (N = 374)

	Frequency* or Mean (±SD)	Percent
Infant characteristics		
Age (days) at enrollment	33 ± 8	–
Male	201	53.7
C-section	108	28.9
Preterm (< 37 weeks gestational age)	70	19.2
Low birth weight (< 2500g)	24	7.0
Birth interval < 36 months (vs. ≥ 36 mo. or firstborn)	62	17.2
Maternal characteristics		
Primipara	176	47.7
Maternal age (years)	25.5 ± 6.4	–
Maternal BMI	26.4 ± 3.8	–
Sociodemographics		
Maternal Education		
Primary or less	56	15.3
At least some secondary	229	62.4
At least some superior	82	22.3
Other Sociodemographics		
Mother married or cohabitating	324	86.7
Maternal employment	93	25.3
Household size (number of people)	5.0 ± 2.0	–
Household owns refrigerator	94	25.5
Higher quality floor material (hardwood, carpet, or tile vs. cement)	124	33.6
Water piped indoors	140	37.9
Private toilet	209	60.9

*Of infants with plasma available for at least the 1st blood draw (~2 months of age).

Table 3.2. Sample Characteristics by Age and Time Postpartum

	1st blood draw* (range 1 – 5 mo.)		2nd blood draw (range 6 – 10 mo.)		3rd blood draw (range 10 – 19 mo.)	
	Mean (±SD) or Frequency	Percent	Mean (±SD) or Frequency	Percent	Mean (±SD) or Frequency	Percent
N	374	–	315	–	170	–
Infant Age and Nutritional Characteristics						
Age (months)	2.1 ± 0.3	–	6.7 ± 0.9	–	14.2 ± 2.3	–
Exclusively breastfed**	216	58.2	26	8.3	0	0.0
Any breastfeeding	367	98.4	302	95.9	139	81.8
Stunted (LAZ < -2)	77	20.6	48	14.4	35	20.8
Overweight (WLZ > 2)	28	7.5	18	5.8	6	3.6
Inflammation[+]						
Infant	10	2.7	65	20.6	27	15.9
Maternal	130	35.3	55	17.7	N/A	–
Anemia						
Infant[†]	268	71.8	235	74.6	139	82.2
Maternal[‡]	117	31.7	51	16.3	32	19.0

*1 month postpartum for mothers (range 0.5 – 2 mo.). All included infants must have data for at least the 1st blood draw. Maternal N = 369 at 1st blood draw and N = 312 at 2nd blood draw as infant numbers include twin sets. **Exclusive breastfeeding is defined as infant is currently breastfeeding but has not received any non-breast milk liquids or semi-solid foods. N = 371 at 1st blood draw due to missing data. [+]Inflammation: CRP > 5 mg/L AGP > 1 g/L. [†]Hemoglobin < 13.7 g/dL (cut-off adjusted for altitude). N = 169 for last blood draw. [‡]Hemoglobin < 14.5 g/dl (cut-off adjusted for altitude). N = 312 for 2nd blood draw. N = 168 for 3rd blood draw.

Table 3.3. Maternal and Infant Iron Status Markers, Adjusted* and Unadjusted

	1st blood draw+ (range 1 – 5 mo.)		2nd blood draw (range 6 – 10 mo.)		3rd blood draw (range 10 – 19 mo.)	
	Median	**IQR (25%, 75%)**	**Median**	**IQR (25%, 75%)**	**Median**	**IQR (25%, 75%)**
N	374	–	315	–	170	–
Ferritin (µg/L)						
Infant						
Unadjusted	163.4	(113.8, 211.7)	15.9†	(8.0, 36.4)	8.6†‡	(4.4, 16.3)
Adjusted*	156.1	(106.4, 201.6)	10.2†	(5.4, 19.8)	6.3†‡	(3.2, 10.8)
Maternal						
Unadjusted	27.2	(13.1, 47.1)	32.6	(17.7, 46.2)	N/A	–
Adjusted*	18.4	(9.5, 32.3)	24.7†	(14.0, 35.2)	N/A	–
sTFR (mg/L)						
Infant						
Unadjusted	4.1	(3.5, 5.0)	5.3†	(4.4, 6.4)	6.5†‡	(5.2, 8.2)
Adjusted*	3.8	(3.2, 4.6)	5.1†	(4.2, 6.2)	6.5†‡	(5.3, 8.5)
Maternal						
Unadjusted	5.4	(4.2, 6.6)	4.3 †	(3.6, 5.4)	N/A	–
Adjusted*	5.5	(4.3, 6.7)	4.3 †	(3.6, 5.4)	N/A	–
BI (mg/kg)						
Infant						
Unadjusted	11.8	(10.1, 12.9)	2.5†	(-0.4, 5.7)	-0.7†‡	(-3.6, 2.3)
Adjusted*	11.9	(10.2, 13.0)	1.3†	(-1.9, 4.0)	-1.8†‡	(-5.1, 0.9)
Maternal						
Unadjusted	4.2	(1.5, 6.9)	5.7†	(3.3, 7.4)	N/A	–
Adjusted*	2.9	(0.0, 5.5)	4.7†	(2.5, 6.5)	N/A	–

*Linear regression method used, with first decile values as inflammatory marker referents. Infant referents—1st blood draw: CRP = 0.10 mg/L, AGP = 0.18 g/L; 2nd blood draw: CRP = 0.12 mg/L, AGP = 0.29 g/L; 3rd blood draw: CRP = 0.14 mg/L, AGP = 0.31 g/L. Maternal referents—1st blood draw: CRP = 0.44 mg/L, AGP = 0.49 g/L; 2nd blood draw: CRP = 0.32 mg/L, AGP = 0.46 g/L. BI

Table 3.3. Maternal and Infant Iron Status Markers, Adjusted* and Unadjusted

	1st blood draw+ (range 1 – 5 mo.)		2nd blood draw (range 6 – 10 mo.)		3rd blood draw (range 10 – 19 mo.)	
	Median	**IQR (25%, 75%)**	**Median**	**IQR (25%, 75%)**	**Median**	**IQR (25%, 75%)**

adjusted by applying Cook's formula to adjusted Ferritin and sTFR. +1 month postpartum for mothers (range 0.5 – 2 mo.). †Significantly different ($p < 0.0001$) from 2- or 1-month value; Wilcoxon signed-rank test. ‡Significantly different ($p < 0.0001$) from 6 - 8 month value; Wilcoxon signed-rank test.

Table 3.4. Prevalence (%) of Maternal and Infant Iron Deficiency (ID) and Iron Deficiency Anemia (IDA) by Iron Status Marker and Time Point, Adjusted[+] and Unadjusted

	1st blood draw*		2nd blood draw		3rd blood draw	
	ID	IDA	ID	IDA	ID	IDA
N	374	–	315	–	170	–
Ferritin (µg/L)						
Infant						
Unadjusted	0.5	0.5	40.6[‡]	35.2[‡]	64.7[‡,¥]	56.8[‡,¥]
Adjusted[+]	0.5	0.5	55.9[‡]	46.3[‡]	78.2[‡,¥]	66.9[‡,¥]
Maternal						
Unadjusted	27.4	14.9	19.9	10.0	N/A	N/A
Adjusted[+]	40.2	20.1	28.0[‡]	10.9[‡]	N/A	N/A
STFR (mg/L)						
Infant						
Unadjusted	1.3	0.8	8.6	8.3[‡]	24.1[‡,¥]	22.5[‡,¥]
Adjusted[+]	0.8	0.5	7.6[‡]	7.3[‡]	26.5[‡,¥]	24.9[‡,¥]
Maternal						
Unadjusted	11.7	7.3	1.0[‡]	0.6[‡]	N/A	N/A
Adjusted[+]	11.4	7.3	1.0[‡]	0.6[‡]	N/A	N/A
BI (mg/kg)						
Infant						
Unadjusted	0.3	0.3	27.6[‡]	25.7[‡]	54.1[‡,¥]	51.5[‡,¥]
Adjusted[+]	0.3	0.3	39.0[‡]	35.6[‡]	66.5[‡,¥]	60.4[‡,¥]
Maternal						
Unadjusted	16.6	10.1	6.8[‡]	4.2	N/A	N/A
Adjusted[+]	25.0	14.4	10.0[‡]	6.1[‡]	N/A	N/A

Table 3.4. Prevalence (%) of Maternal and Infant Iron Deficiency (ID) and Iron Deficiency Anemia (IDA) by Iron Status Marker and Time Point, Adjusted[+] and Unadjusted

	1st blood draw*		2nd blood draw		3rd blood draw	
	ID	IDA	ID	IDA	ID	IDA

*1 month postpartum for mothers. [+]Linear regression method used, with first decile values as inflammatory marker referents. Infant referents—1st blood draw: CRP = 0.10 mg/L, AGP = 0.18 g/L; 2nd blood draw: CRP = 0.12 mg/L, AGP = 0.29 g/L; 3rd blood draw: CRP = 0.14 mg/L, AGP = 0.31 g/L. Maternal referents—1st blood draw: CRP = 0.44 mg/L, AGP = 0.49 g/L; 2nd blood draw: CRP = 0.32 mg/L, AGP = 0.46 g/L. BI adjusted by applying Cook's formula to adjusted Ferritin and sTFR. [‡]Significantly different ($p < 0.0001$) from 2- or 1-month value; Wilcoxon signed-rank test. [¥]Significantly different ($p < 0.0001$) from 6 - 8 month value; Wilcoxon signed-rank test.

0

Figures and Figure Legends

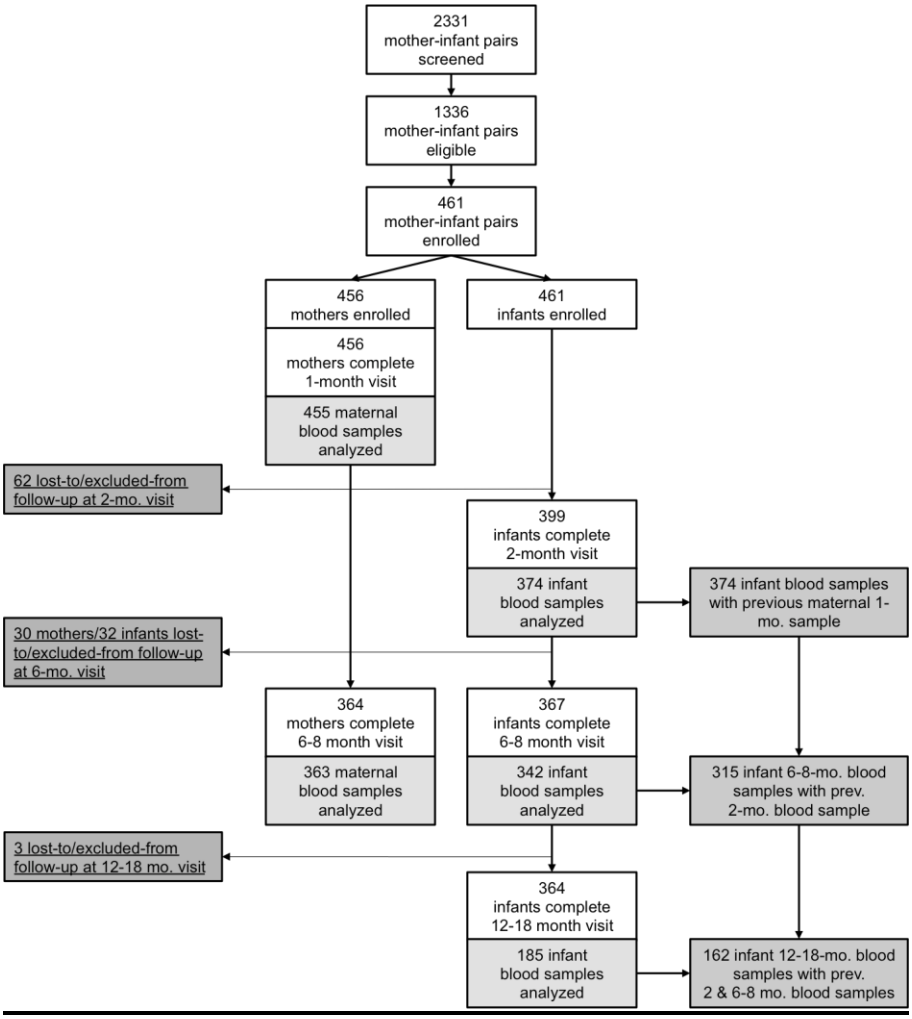
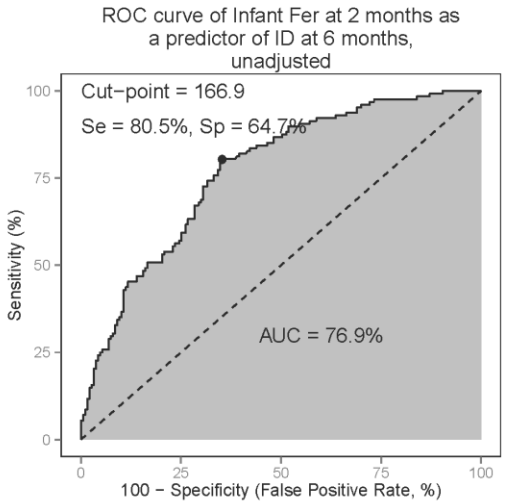
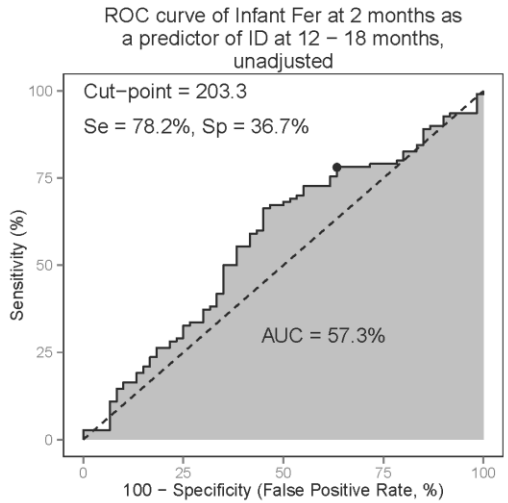
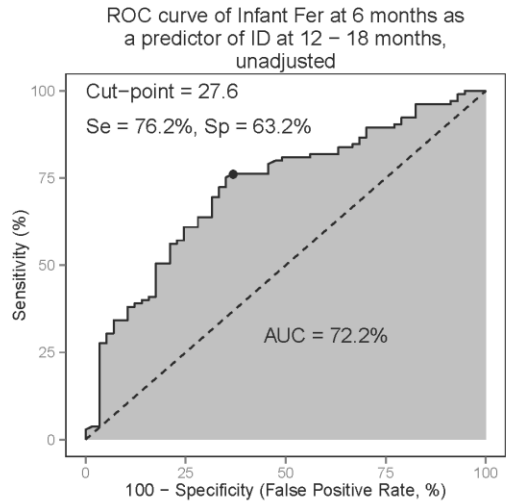


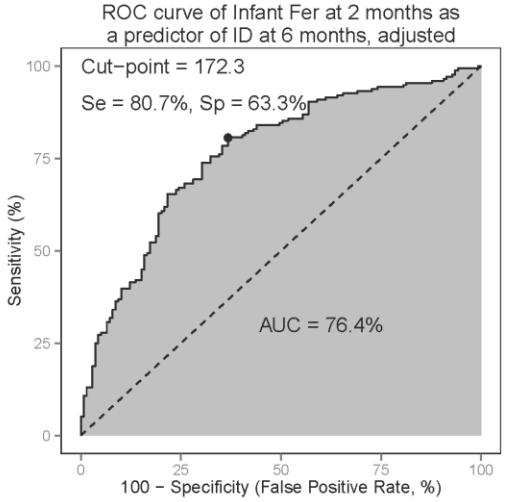
Figure 3.1. Participant Flow.

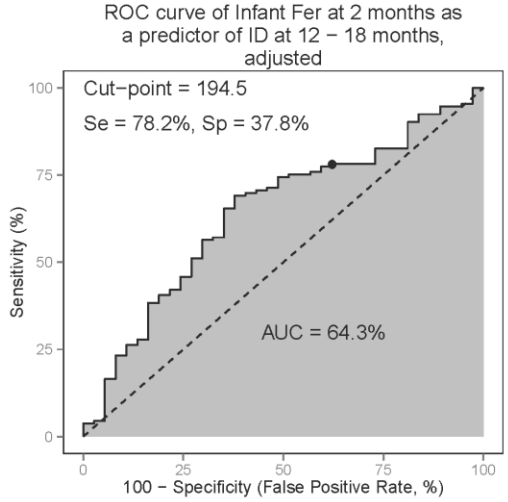
Of 2331 screened mother-infant pairs, 1336 were eligible and 461 enrolled. A total of 374 infants provided samples at 2 months, 342 at 6 – 8 months, and 185 at 12 – 18 months of age. Of 342 infants with samples at 6 – 8 months, 315 had plasma analyzed at 2 months. Of 185 infants with samples at 12 – 18 months, 170 had samples from 2 months, and 162 had samples at 2 and 6 – 8 months.


ROC curve of Infant Fer at 2 months as a predictor of ID at 6 months, unadjusted
Cut−point = 166.9
Se = 80.5%, Sp = 64.7%
AUC = 76.9%
Sensitivity (%)
100 − Specificity (False Positive Rate, %)


ROC curve of Infant Fer at 2 months as a predictor of ID at 12 − 18 months, unadjusted
Cut−point = 203.3
Se = 78.2%, Sp = 36.7%
AUC = 57.3%
Sensitivity (%)
100 − Specificity (False Positive Rate, %)


ROC curve of Infant Fer at 6 months as a predictor of ID at 12 − 18 months, unadjusted
Cut−point = 27.6
Se = 76.2%, Sp = 63.2%
AUC = 72.2%
Sensitivity (%)
100 − Specificity (False Positive Rate, %)


ROC curve of Infant Fer at 2 months as a predictor of ID at 6 months, adjusted
Cut−point = 172.3
Se = 80.7%, Sp = 63.3%
AUC = 76.4%
Sensitivity (%)
100 − Specificity (False Positive Rate, %)


ROC curve of Infant Fer at 2 months as a predictor of ID at 12 − 18 months, adjusted
Cut−point = 194.5
Se = 78.2%, Sp = 37.8%
AUC = 64.3%
Sensitivity (%)
100 − Specificity (False Positive Rate, %)

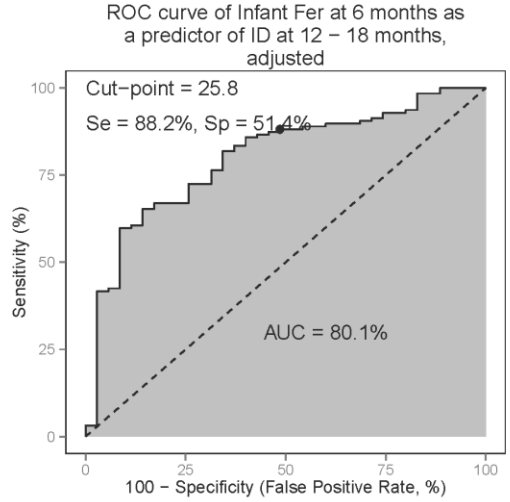

ROC curve of Infant Fer at 6 months as a predictor of ID at 12 − 18 months, adjusted
Cut−point = 25.8
Se = 88.2%, Sp = 51.4%
AUC = 80.1%
Sensitivity (%)
100 − Specificity (False Positive Rate, %)

Figure 3.2. ROC Curves of infant ferritin.

Infant ferritin at 2 months of age is an adequate predictor of infant ID at 6 – 8 months of age, but not of infant ID at 12 – 18 months. Cut-points for Fer at younger ages provide indicated Se and Sp for ID at later ages. The dashed line indicates no predictive value. Abbreviations include ID (iron deficiency), Fer (ferritin), ROC (receiver operating characteristic), Se (sensitivity), Sp (specificity), AUC (area under the curve).

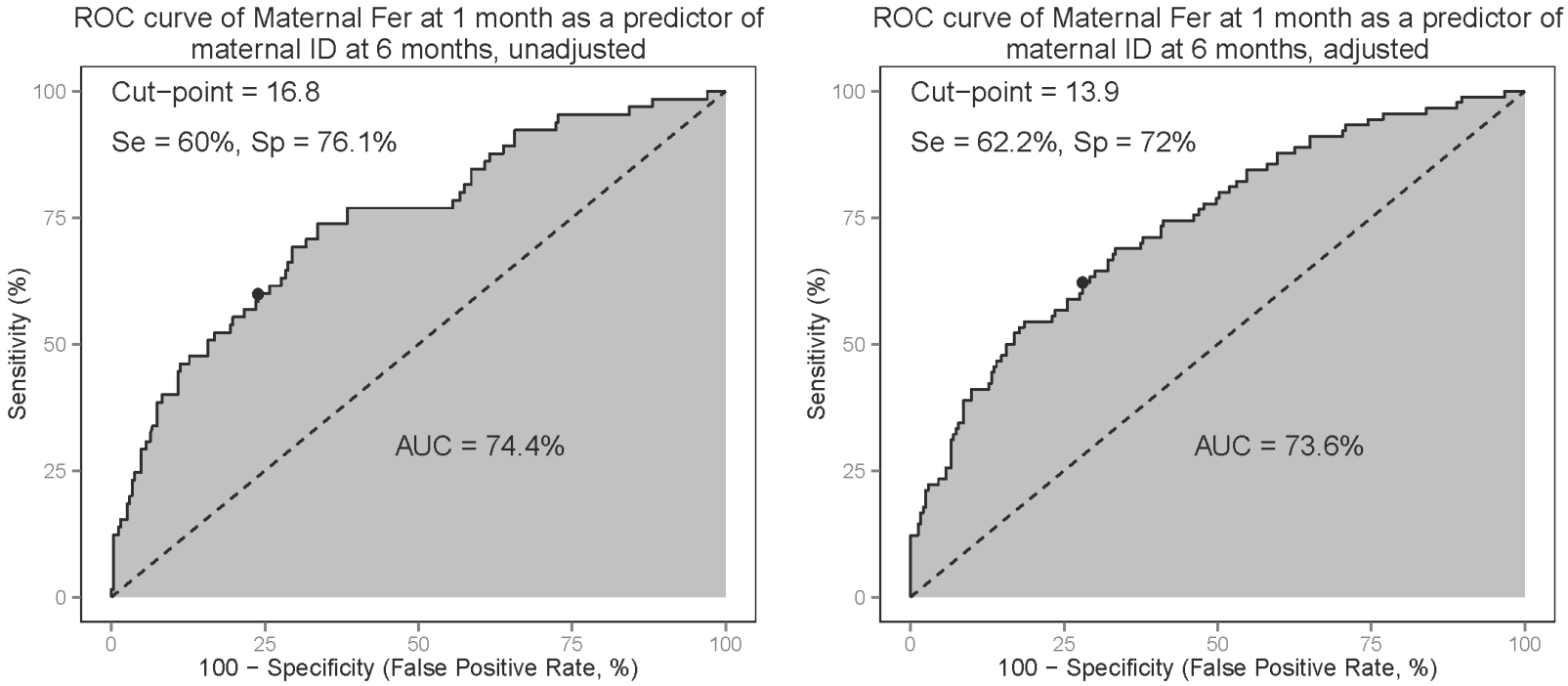


Figure 3.3. ROC Curves of Maternal Ferritin.

Maternal ferritin at 1 month is an adequate predictor of maternal ID at 6 – 8 months postpartum, and prediction is not changed by adjustment for inflammation. Cut-points for Fer at 1 month provide indicated Se and Sp for ID at 6 – 8 months postpartum. The dashed line indicates no predictive value. Abbreviations include ID (iron deficiency), Fer (ferritin), ROC (receiver operating characteristic), Se (sensitivity), Sp (specificity), AUC (area under the curve).

Chapter 4: Impact of Random Error in Effect Estimates Using Inflammation-Corrected Iron Levels

In preparation. Targeted as a letter to the editor or short communication.

Introduction

Micronutrient deficiencies affect millions of women and children every year, and are the subject of numerous research investigations and programs worldwide [1]. Yet the measurement of micronutrient status is in many cases transiently affected by inflammation, the body's response to stress, trauma, or injury [2]. Although numerous methods have been proposed and utilized to correct for this effect, there is a lack of international consensus on which method is best, and the topic remains of considerable interest [3, 4].

One of the more recently developed and recommended methods relies on a linear regression of micronutrient biomarker values on indicators of inflammation, usually C-reactive protein (CRP) and alpha(1)-acid glycoprotein (AGP) [4, 5]. In this method, the nutritional biomarker is regressed on CRP and AGP (with any necessary transformations), and these estimated parameters are then applied within an equation that corrects the observed nutritional biomarker to an estimated counterfactual value under conditions where CRP and AGP are not indicative of inflammation. Although this method is thought to more closely approximate the biological processes, the use of regression introduces a source of random error into the estimated corrected values via the estimated parameters. Therefore, when these estimated corrected values are used in any modeling, e.g., as a predictor, the standard errors of the effect estimate will be artificially small unless the extra source of random error is accounted for.

This analysis aims to demonstrate the potential effect of regression-correction-induced random error on effect estimates, utilizing a test case. The test case in question will be the estimated effect of maternal ferritin (a marker of iron status) one month postpartum on infant ferritin at 6 – 8 months postpartum. Multiple imputation will be used to generate a revised estimate of the mother-infant association as well as its standard deviation. This analysis will provide insight into the benefits and limitations of the regression method of adjustment.

Methods

Data were drawn from the *Nutrición, Inmunología, y Diarrea Infantil* (NIDI) study, the primary aim of which was to assess the effect of nutritional status on response to the rotavirus vaccine. In brief, 461 healthy infants and their mothers were recruited from two hospitals in El Alto, Bolivia, and followed from one month through 6 – 8 months of age. A variety of sociodemographic data was taken at each of 6 visits, and venous blood was collected at a target schedule of 1 and 6 – 8 months postpartum from mothers and 2 and 6 – 8 months of age from infants. Blood was analyzed for ferritin (a marker of iron status), CRP (a marker of acute inflammation), and AGP (a marker of chronic inflammation).

Ferritin for mothers and infants was corrected by first regressing ferritin on CRP and AGP (with log transformations to improve fit):

$$\ln(Ferritin) = \beta_0 + \beta_1 \ln(CRP) + \beta_2 \ln(AGP).$$

Estimated coefficients of AGP and CRP were then used in the adjustment equation:

$$\ln(Fer_{corr}) = Fer_{obs} - \hat{\hat{\beta}}_1\left(\ln(CRP_{obs}) - \ln\left(CRP_{ref}\right)\right) - \hat{\hat{\beta}}_2(\ln(AGP_{obs}) - \ln(AGP_{ref})).$$

As there are no established reference values for normal CRP and AGP in the literature, the first deciles of CRP and AGP in the non-inflamed population were used as the referent values for the reported results. Ferritin was not corrected for AGP or CRP values below the referents.

The effect of maternal ferritin at 1 month on infant ferritin at 6 – 8 months was modeled using linear regression, controlling for infant ferritin at 2 months based on DAG analysis and log transforming the outcome to meet normality assumptions. The multiple imputation was carried out as followed: First, using the variance-covariance structures from the correction equations for maternal and infant ferritin, we randomly generated 10 new sets of parameter estimates. These estimates were then used, one set at a time, to correct the iron biomarker values, thus producing 10 different sets of estimated maternal and infant ferritin. The mother-infant regression equation was then run for each dataset. An overall mean estimated effect was calculated as the arithmetic mean of the effect estimates from the 10 sets, and an overall standard error was calculated according to formulae laid out by Rubin [6]:

i) Variance "within" models: U = $\frac{\sum_{i=1}^{M} Var_i}{M}$, where M is the number of models (sets of estimates) and Var_i is the variance of the parameter estimate in model i for the effect of interest

ii) Variance "between" models: B = $\frac{\sum_{i=1}^{M} \hat{\beta}_i^2}{M-1}$, where $\hat{\beta}$ is the estimated effect of interest in model i

iii) Total variance: T = $U + \left(1 + \frac{1}{M}\right) * B$

iv) The T distribution was used to calculate confidence intervals with degrees of freedom equal to: df = $(M-1) * (1 + \left(\frac{M}{1+M} * \frac{U}{B}\right))^2$

v) The percent change for a 10-unit difference in ferritin was calculated: P = $100 * (\exp\left(10 * E(\beta)\right) - 1)$

Results

This analysis demonstrated a significant effect of infant ferritin at 2 months on infant ferritin at 6 – 8 months of age—for every 10-unit increase in ferritin at 2 months of age, ferritin at 6 – 8 months of age increased by 8% (Table 1). However, the effect of maternal ferritin at 1 month on infant ferritin at 6 – 8 months of age was non-significant. The ratio of the "within" variance to the "between" variance was > 1 in both cases, and very high for the effect of maternal ferritin.

Discussion

In this short report, we investigated the effect of regression-based inflammation correction using the test case of the estimated effect of maternal on infant ferritin. This analysis demonstrated no significant effect of maternal ferritin on infant ferritin at these time points, though early infant ferritin was significantly related to later infant ferritin. Examining the ratio of variance within models to variance between models showed that

the variance within each model outweighed the variance between models, especially for maternal ferritin. This indicates that the random error stemming from regression-based correction of ferritin was small in relation to random error based on population variation. This result is reassuring for the use of regression-based correction to micronutrient biomarkers, as it suggests that this method introduces only minor distortion to the standard errors for effect estimates based on these corrected values. Future research may repeat this simulation in a different population and with a different nutritional biomarker to confirm whether these results can be generalized.

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Table 4.1. Effect of early maternal and infant ferritin on later infant ferritin

	Percent change in Fer at 2nd draw	95% CI	P-value*	Ratio of within to between variances
10-unit increase in maternal Fer at 1st draw	2.3	(-2.3, 7.0)	0.33	58
10-unit increase in infant Fer at 1st draw	7.9	(6.2, 9.7)	< 0.0001	2

*F test with numerator df = 1 and denominator df = df as above. $F = t^2$ and $t = E(\beta) / \text{sqrt}(T)$.

Chapter 5: Effect of Feeding Practices on Infant Iron Status at 6 – 8 Months of Age

In preparation.

Target Journal: Pediatrics

Effect of Infant Feeding Practices on Iron Status in a Cohort of Bolivian Infants

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Short title: Effect of Feeding Practices on Iron Status

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Conflict of Interest: The authors have no conflicts of interest to disclose.

Clinical Trial Registration: Not applicable as this was not a clinical trial.

Abbreviations: ID (iron deficiency), IDA (iron deficiency anemia), Fer (Ferritin), sTFR (soluble transferrin receptor), BI (body iron), Hb (hemoglobin), APR (Acute Phase Response), CRP (C-reactive protein), AGP (alpha(1)-acid glycoprotein), MNP (multiple micronutrient powder)

The findings and conclusions in this article are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

What's known on this subject

Infant birth iron stores are rapidly depleted over the first 4 – 6 months of age. Due to the relatively low iron content of breast milk, exclusively breastfed infants may be at higher risk of iron deficiency.

What this study adds

This study suggests that even healthy infants are at high risk of iron deficiency, and that an increased length of exclusive breastfeeding is associated with poorer iron status. However, this may not outweigh benefits of 6 months of exclusive breastfeeding.

Contributors' statements:

Ms. Burke contributed to the design and execution of the study, cleaned and analyzed the data, and drafted the manuscript. Drs. Leon, Suchdev, and Rebolledo, and Ms. Fabiszewski de Aceituno designed and conceptualized the study, oversaw research, and reviewed the final manuscript as submitted. Drs. Revollo and Iñiguez contributed to the design and conceptualization of the study, provided critical input and oversight of field work, and reviewed the final manuscript as submitted. Drs. Klein and Drews-Botsch contributed to the study design, critically reviewed the manuscript, and approved the manuscript as submitted.

All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

The findings and conclusions in this article are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

Abstract

Objective

Iron deficiency (ID) is one of the most common micronutrient deficiencies worldwide, and can have severe consequences in infants. Though exclusive breastfeeding (EBF) is recommended for 6 months, breast milk has a relatively low iron content. The present study seeks to estimate the impact of feeding practices on iron status at 6 – 8 months of age among a cohort of infants in Bolivia.

Methods

Mother-infant pairs were recruited from 2 hospitals in El Alto, Bolivia, and followed from one through 6 – 8 months of age. Singleton infants born > 34 weeks gestational age and completing required blood draw visits were eligible for inclusion (N = 292). ID and anemia were defined using standard cut-offs; IDA was defined as ID plus anemia. The effect of length of EBF (infant received only breast milk with no other liquids or solids), and the age of introduction of complementary feeding (CF) in EBF infants, was assessed for ID, IDA, and anemia (logistic regression) and ferritin (Fer) and hemoglobin (Hb, linear regression).

Results

EBF of 4 months and above was significantly associated with ID, but not with IDA or anemia. Among infants EBF to 4 months, the age of introduction of CF was not significantly associated with any outcome. Continuous Fer and Hb were significantly lower with increasing months of EBF and age at first CF.

Conclusion

Results suggest a relationship between prolonged EBF and ID, but do not support changes to current recommendations. More research is needed in diverse populations, including exploration of early interventions.

Introduction

Iron deficiency (ID) is one of the most common micronutrient deficiencies, affecting an estimated 40% of children under 5 and 38% of pregnant women globally [1]. In continued conditions of iron imbalance, ID can progress into iron deficiency anemia (IDA); both ID and IDA are associated with severe consequences in young children, including potentially irreversible deficits in behavior and cognitive development [2, 3].

Infants have high iron needs due to their rapid growth [4, 5]. Traditionally, young infants are thought to be protected from ID via their birth iron stores, which are largely accumulated during the last trimester of gestation and slowly depleted through the first 4 – 6 months of life [6, 7]. Yet ID has been identified even in very young populations of healthy infants [8-12], raising questions about optimal infant and young child feeding and supplementation practices[13]. The World Health Organization (WHO) recommends exclusive breastfeeding (EBF; defined as no foods or liquids other than breast milk and vitamins or minerals) for infants up to 6 months of age, due to the excellent nutritional content as well as the demonstrated immunological benefits of breast milk [14, 15]. Nonetheless, although the iron in breast milk is highly bioavailable, it is present in only small amount s[8, 13, 16], prompting discussion as to whether EBF should be recommended, instead of to 6 months of age, only to 4 months of age (as in earlier recommendations) [15, 17]. In multiple studies, the length of EBF or predominant breastfeeding (PRBF) has been associated with poorer iron status [18-22], while in other

studies, EBF has not been associated with iron status [23, 24], or has been inconsistently associated with markers of iron status [25, 26].

Infant feeding practices are highly variable across cultures as well as ages, and represent a wide range of iron contents. While breast milk is low in iron, infant formula is typically high in iron (though the iron is less bioavailable than breast milk iron) [27]. Cow's milk, often used as a breast milk supplement or replacement in some cultures, has been found to be negatively associated with infant iron status [21, 28, 29], potentially through negative impacts on iron absorption as well as increased intestinal bleeding [30]. Typical complementary foods in many countries, especially initial weaning foods, have a poor iron content unless they are fortified [31]. For this reason, multiple micronutrient powders (MNPs) are recommended in many settings to be used with complementary foods as a home fortificant [32].

Few studies capture the wide variety of infant diet, and much of the existing literature on the impact of infant feeding on iron status employs cross-sectional designs, limiting the ability to understand longitudinal patterns. Further, very few studies account for the effects of inflammation, which can transiently increase ferritin, the most sensitive marker of iron status (and the marker recommended by WHO and the Centers for Disease Control and Prevention [CDC]) [33, 34]. This is particularly important given the known protective effects of breastfeeding on vulnerability to diarrheal illnesses and other childhood infections, which may also cause inflammation [15].

In the present study, we aim to assess the effect of recommended breastfeeding practices on iron status among a cohort of healthy infants followed from birth through the first 6 – 8 months of life, while adjusting for the effect of inflammation on iron status markers. Specifically, we assess the effect of EBF and the effect of timing of introduction of semi-solid foods, on iron deficiency (ID), anemia, and iron deficiency anemia (IDA) at 6 – 8 months of age. This study, in El Alto, Bolivia, a high-altitude setting where iron needs may be higher [35], will help to provide information on the impact of recommended feeding practices on iron status, in a developing country with a high burden of malnutrition [36].

Methods

Study population and design

Data for the present study were drawn from the *Nutrición, Inmunología, y Diarrea Infantil* (NIDI) study, the primary aim of which was to assess the effect of nutritional status on response to the rotavirus vaccine (Rotarix®). In brief, 461 healthy infants (2 – 4 weeks of age) and their mothers were recruited from 2 hospitals in El Alto, Bolivia (altitude 4000m), during well-child or vaccination visits. This is a primarily urban and largely indigenous population; while most have access to sanitation and improved water, socioeconomic resources are typically low [37]. Exclusion criteria included infant illness at recruitment, infants suspected to have immunodeficiency (e.g., HIV), congenital malformations, and maternal inability to speak and understand Spanish or Aymara. Recruitment took place from May 2013 through March 2014, and infant-mother dyads were followed through 6 – 10 months of age, with final data collected in March of 2015.

Hospital visits occurred at target dates of 1, 2, 3, 4, and 6 – 8 months of age (this last visit corresponding to 60 days after the second dose of the Rotarix vaccine). Two blood draws were performed, at target ages of 2 and 6 – 8 months. For the present study, iron status was assessed using the second blood draw, as this corresponds to the age at which iron stores begin to show depletion; further, only 2 infants were ID at 2 months (Burke). For the present study, only singleton infants and infants born at > 34 weeks gestational age, with non-missing data on outcomes and covariates, and who were not ID at 2 months (N = 270) were eligible for analyses, given that multiples and early preterm infants may have different feeding practices [38] and may be more vulnerable to ID compared to term, singleton infants [5].

Ethical approval

The protocol and instruments for this study were approved by the Emory University IRB (IRB00056127) and the Bolivian "Comité de Etica de la Investigación" (Research Ethics Committee). Mothers provided written informed consent in Spanish or Aymara.

Laboratory analysis and definitions of iron status

Venous blood was collected (1mL) from mothers and infants using zinc-free syringes and tubes. Hemoglobin (Hb) was measured at point-of-care using a HemoCue® Hb system. Plasma was analyzed by sandwich ELISA for C-Reactive Protein (CRP; a marker of inflammation; limit of detection [LOD]: 0.5 mg/L) and alpha(1)-acid-glycoprotein (AGP; a marker of inflammation; LOD: 0.1 g/L) [39]. Hb was adjusted for the high altitude (3500 – 4000m) of El Alto and the surrounding area of La Paz [40]. Anemia in infants

was defined as corrected Hb < 11 g/dL, based on WHO guidelines [41]. Low iron stores were defined as ferritin (Fer) < 12 µg/L [34]. In all cases, Fer was adjusted for the effect of inflammation (CRP and AGP) using a linear regression method, described in detail elsewhere [37, 42]. IDA was defined as ID plus anemia. Mothers and infants were referred for anemia according to Bolivian guidelines (mothers < 13.7 g/dl; infants < 10.9 g/dl; both uncorrected for altitude), and infants were referred for stunting (length-for-age Z score < - 2) or wasting (weight-for-length Z score < - 2) at any visit.

Data collection

Sociodemographic data was collected by trained Bolivian interviewers at the first study visit via questionnaire. Birth weight was corroborated by health card in 60% of cases, but was not significantly different by maternal report. At each visit, interviewers collected data on recent infant morbidities and feeding practices, including whether the infant was breastfed within the last 24 hours, and whether the infant had previously received any non-breast milk liquids (e.g., formula, tea) or semi-solid foods.

Variable definitions and statistical analysis

EBF was defined as infant having received only breast milk up until the current assessment, without any non-breast milk liquids or semi-solid foods; one month of formula feeding was allowed as long as the infant had been concurrently breastfed. Given past and present WHO recommendations on feeding practices [14, 15], EBF was categorized as follows: EBF < 4 months, EBF 4 – 6 months, and EBF > 6 months. Infants who were aged > 5 months at their last recorded EBF visit were coded as EBF to 6

months. Infants who were aged > 3 months at their last recorded EBF visit were coded as EBF to 4 months. For analysis of age of introduction of complementary feeding (CF), the population was restricted to infants EBF to at least 4 months, given WHO recommendations [14, 15], and age was dichotomized as 4 – 5 months at first CF versus ≥ 6 months at first CF. Other variables relating to feeding status were not included as they were considered to be potential intermediates.

Potential covariates were informed based on a conceptual diagram, and selected based on bivariate associations with the outcome and the exposure. The initial models included infant age (dichotomized as > 6 months vs. ≤ 6 months), birth weight, sex, maternal age (dichotomized as < 20 years vs. ≥ 20 years), maternal employment, maternal education, maternal relationship status (single vs. married or cohabiting), number of cell phones in household (dichotomized as > 1 vs. ≤ 1, as a marker of socioeconomic status), and quality of household's roofing materials (dichotomized as high vs. low in accordance with Demographic Health Survey [DHS] standards [36]). Final models were reduced to prioritize parsimony and consistency of covariates across outcomes and exposures, while controlling for confounding (maintaining exposure effect estimates within 10% of the initial fully adjusted models) [43].

Linear regression was used to assess relationships of exposures to continuous Fer and Hb (log-transformed to meet normality assumptions and corrected for inflammation [Fer] or altitude [Hb] as appropriate). Binary logistic regression was used to assess relationships between the exposures and the outcomes. All models were tested for collinearity using

Variance Decomposition Proportions (VDPs) and Condition Indices (CIs) and were found to be acceptable. Wald chi-square tests were used to assess significance, and $P < 0.05$ was considered statistically significant. Effect modification was not assessed. Data were cleaned and analyzed using SAS v9.4 (Cary, NC) and the R Environment for Statistical Computing [44].

Results

Characteristics of the study sample

Out of 451 singletons enrolled in the parent study, 365 completed initial study requirements (first dose of Rotarix® vaccine and blood draw at 2 months of age) and were of eligible gestational age (> 34 weeks). Of these, 291 infants had data for both the first and second blood draws, and were without ID at the initial blood draw. A further 21 were missing data on key exposures or covariates. The study population for the present analysis thus included 270 singleton infants born after 34 weeks gestational age and with complete data (Figure 1).

The median age of infants at the time of assessment was nearly 7 months (SD 1 month). Infants were fairly evenly distributed in terms of gender, nearly one third were born via Caesarian section, and one twentieth were low birth weight (Table 1). Mothers had a mean age of 26 years, one half were first-time mothers, one quarter were employed, and most had at least a secondary education.

EBF, Complementary Feeding, and Iron Status

Although nearly all infants had been breastfed at some point in their lives, only 64% were EBF for 4 months, and 36% EBF for 6 months of age; mean length of EBF was 3 months (Table 1). At the time of the blood draw, 83% of infants had received some semi-solid food. Nearly 20% of infants had taken Chispitas (multiple micronutrient powder [MNP] supplements, Table 1). Low iron status was common: 56% of infants were ID, 76% were anemic, and 46% were IDA (Table 1); 61% of anemic infants were also ID (data not shown).

Associations of feeding practices with iron status indicators

Effect of length of EBF with continuous outcomes. Given WHO recommended practices[14], we assessed the impact of EBF categorized as < 4 months, 4 – 6 months, and > 6 months. Adjusted linear regression models demonstrated significant relationships: both Fer and Hb decreased as the number of months of EBF increased, though the effect on Fer was much larger (Fer decreased by 22% for infants EBF 4 – 6 as compared to < 4 months, while Hb decreased only by 3% for the same comparison [Table 4]).

Effect of length of EBF on dichotomized outcomes. Given WHO recommended practices[14], we assessed the impact of EBF categorized as < 4 months, 4 – 6 months, and > 6 months. Longer EBF was significantly associated with ID, but not with IDA or anemia in this population, although IDA results were suggestive of a relationship. Odds of all outcomes were also significantly increased among lower-birth weight infants and those of male sex (vs. female sex). Odds of ID and IDA were significantly lower among

infants whose mothers were employed as well as among infants whose mothers had completed a university education. Older infants had significantly higher odds of IDA and anemia. (Table 2.)

Age at which complementary food (CF) was introduced. To gain more insight into benefits and drawbacks of EBF to 4 as compared to 6 months [14, 15, 17], we also assessed, for infants EBF to 4 months, the age of complementary food introduction (dichotomized as 4 – 5 vs. ≥ 6 months). Among infants EBF at least 4 months, the age at which CF was introduced was not significantly associated with ID, IDA, or anemia (Table 3). Patterns of associations between covariates and iron status were again similar to models assessing exclusive breastfeeding.

Discussion

Iron deficiency, anemia and iron deficiency anemia were common among this cohort of primarily breastfed, healthy Bolivian infants. Analyses demonstrated a significant association between continuous Fer and months of EBF, as well as between ID and length of EBF. In infants EBF to 4 months, there was a non-significant increase in odds of ID in infants introduced to CF at 6 months and beyond. Results for IDA demonstrated similar patterns to ID analyses, but were attenuated and non-significant. Although there was a small significant association between length of EBF and Hb, there was not a significant association between length of EBF and anemia.

While our findings suggest a potential relationship between feeding practices—particularly the duration of exclusive breastfeeding—and iron status, they do not clearly support any change in current recommendations of 6 months of EBF. The association of continuous Fer with months of EBF is consistent with several other studies in diverse settings (two RCTs—in Honduras and Iceland—as well as a cohort study in Mexico) [18, 19, 23, 45] in addition to biological understanding that breast milk is comparatively low in iron versus formula or complementary foods [8, 13, 16]. The lack of significant associations between length of EBF and IDA in our study may reflect a lack of power (a post-hoc power calculation for the effect of EBF to ≥ 4 months on IDA showed < 40% power to detect an OR of 1.5), or it may reflect the influence of anemia (less associated with EBF) on the development of IDA. Sensitivity analyses excluding infants with prolonged EBF (> 6 months), and grouping infants with EBF 6 – 7 months revealed similar patterns (data not shown). Although descriptive analyses suggested a potential interaction between categorized birth weight and EBF, where infants of lower birth weight were more vulnerable to the effects of prolonged EBF (similar to results by Dewey et al. [45]), the interaction terms were not significant (data not shown).

The results of the present study also do not support any change in recommended feeding practices for the prevention of anemia. While continuous Hb was significantly associated with the number of months of EBF, there was no significant relationship of categorized feeding practices to anemia. The finding of a significant relationship between feeding practices and Hb is similar to findings in an RCT of Honduran infants [45] as well as a cohort study of Mexican infants [19]; both populations had a high prevalence of

breastfeeding, similar to our population, although the prevalence of anemia was much lower in the Mexican infants [19] as compared to the Honduran infants [45] or to our own population (Table 1). Two cohort studies—one in Bangladesh [23] and one in Iceland [18]—found no significant associations of Hb with feeding practices; however, it is worth noting that the prevalence of LBW was extremely high in the Bangladeshi infants (30%) [45], while the Icelandic infants had much higher birth weight as well as Hb levels [18], potentially limiting our ability to compare results to these studies.

The present study has several strengths. A primary strength is the sophisticated adjustment for the effect of inflammation on iron biomarkers, using two markers of inflammation to capture varying stages of the APR. The vast majority of previous studies, if they accounted for inflammation at all, have only done so by excluding infants with high CRP [18, 23, 45]. In a sensitivity analysis using uncorrected Fer to define ID and IDA, results were very similar except for an attenuated OR for the effect of the age of CF introduction among infants EBF to 4 months (results in the Appendix, Tables A1 – A3). In another sensitivity analysis using multiply imputed corrected Fer (as in Chapter 4), results were very similar for the linear model (results in the Appendix, Table A4). Another strength is the longitudinal design, which enabled us to follow infants almost from birth, and frequent collection of detailed data on feeding practices. Further, our population of healthy, primarily breastfed infants in a developing country allows us to assess the effect of recommended feeding practices on iron status in a low-resource population. This is also one of few studies to assess ID, IDA, and anemia, as well as continuous Fer and Hb, in the same population of infants. However, the study also has

some limitations. Although data on feeding practices was collected at each visit, there was a gap of at least 2 months between the last and the penultimate visit, introducing the possibility of misclassification. However, it is reassuring that these visits corresponded to roughly 4 – 5 and 6 – 8 months of age, meaning that the vast majority of infants would already have completed the ages corresponding to our EBF cut-offs of < 4, 4 – 6, and > 6 months. Further, the number of months of EBF was not related to the time between these two visits, and all models controlled for age at blood draw (which was related to time between visits), again mitigating the possibility of differential misclassification. Our results, which suggest a relationship between feeding practices and continuous iron markers, but not dichotomized iron status, also raise the possibility that currently recommended cut-offs for ID, IDA, and anemia are not appropriate for infants in this age group (6 – 8 months). However, our study was not designed to assess response to supplementation. Although there was also a low participation rate (mainly due to lack of interest or refusal of blood draw), characteristics of enrolled mothers and infants in the present study were very similar to those in a pilot study by our same group in the same hospitals but not requiring blood draws (data not shown). Although these results may be generalizable to other developing country and high-altitude Andean populations, they may not generalize to settings with a high prevalence of other causes of anemia (such as malaria or HIV). Anemia results may not be generalizable to lower-altitude settings.

Conclusion

While this study suggested a relationship between duration of EBF and iron status, the results do not support any changes to current recommendations of 6 months of EBF, as

odds of ID, IDA, and anemia at 6 – 8 months were not significantly different among infants EBF to 4 months as compared to 6 months or beyond. More research in diverse populations, while controlling for the effect of inflammation, would help to contextualize these results. Nonetheless, the high prevalence of ID, IDA, and anemia, as well as the relationship of iron status to birth weight and feeding practices, suggest a need for additional research to assess the role of early iron supplementation and other preventive interventions in lower birth-weight and other vulnerable populations.

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Tables

Table 5.1: Characteristics of the Study Sample, El Alto, Bolivia (N = 270)

	Frequency or Mean (±SD)	Percent
Infant Characteristics		
Age (months) at blood draw	6.7 ± 0.9	–
Male	142	52.6
C-section	81	30.0
Late preterm (34 - 37 weeks gestational age)	35	13.0
Low birth weight (< 2500g)	15	5.6
Inflammation (elevated CRP or AGP)*	55	20.4
Maternal Characteristics		
Maternal age (years)	26.0 ± 6.5	–
Primipara	127	47.0
Maternal employment	73	27.0
Maternal Education		
Completed university	31	11.5
Completed secondary only	142	52.6
Completed primary only	70	25.9
Did not complete primary	27	10.0
Infant Feeding and Supplementation		
Ever breastfed	269	99.6
Number of months of exclusive breastfeeding [+]	3.1 ± 2.3	–
Exclusively breastfed [+] until 6 months	96	35.6
Exclusively breastfed [+] until 4 months	173	64.1
Ever received formula	138	53.9
Received semi-solid foods at blood draw	223	82.6
Has taken Chispitas[£]	50	18.6
Iron Status Indicators		
Iron deficiency (ID) **	151	55.9
Iron deficiency anemia (IDA) [†]	125	46.3
Anemia [‡]	204	75.6

*Defined as AGP > 1 g/L or CRP > 5 mg/L. **Defined as inflammation-corrected ferritin < 12µg/L, see Methods. [†]Defined as iron deficiency plus anemia. [‡]Defined as altitude-corrected hemoglobin < 11g/dL, see Methods. [+]Defined as infant received no semi-solid foods or non-breast milk liquids until reaching 6 months of age. One month of formula allowed as long as it was accompanied by breast milk. 21 infants were exclusively breastfed beyond 6 months of age. [£]Multiple micronutrient powder (MNP) sachets.

Table 5.2: Association of Exclusive Breastfeeding to 6 months with Iron Deficiency (ID), Iron Deficiency Anemia (IDA), and Anemia* (N = 270)

	ID			IDA			Anemia		
	OR	**95% CI**	**P value****	**OR**	**95% CI**	**P value****	**OR**	**95% CI**	**P value****
Infant exclusively breastfed† for < 4 months	1.00	–	0.008	1.00	–	0.30	1.00	–	0.86
Infant exclusively breastfed† until 4 – 6 months of age	2.02	(1.17, 3.54)	–	1.34	(0.77, 2.34)	–	1.19	(0.63, 2.23)	–
Infant exclusively breastfed† beyond 6 months of age	4.28	(1.39, 15.66)	–	2.17	(0.76, 6.59)	–	1.11	(0.34, 4.38)	–
Infant over 6 months old at blood draw	1.52	(0.90, 2.58)	0.12	1.83	(1.09, 3.11)	0.023	2.39	(1.30, 4.57)	0.006
Male sex	1.84	(1.08, 3.16)	0.026	2.49	(1.46, 4.31)	0.0009	2.77	(1.51, 5.19)	0.001
500g increase in birth weight	0.59	(0.45, 0.78)	0.0002	0.57	(0.43, 0.75)	0.0002	0.61	(0.46, 0.83)	0.0002
Maternal employment	0.51	(0.28, 0.92)	0.025	0.52	(0.28, 0.94)	0.034	0.67	(0.35, 1.30)	0.22

*Iron Deficiency defined as inflammation-corrected ferritin < 12µg/L, see Methods. Anemia defined as altitude-corrected hemoglobin < 11g/dL, see Methods. Iron Deficiency Anemia defined as Iron Deficiency plus Anemia. **Wald Chi-Square tests. †Defined as infant received no semi-solid foods or non-breast milk liquids until reaching 6 months of age. One month of formula allowed as long as it was accompanied by breast milk.

Table 5.3: Association of Age of Introduction of Complementary Foods with Iron Deficiency (ID), Iron Deficiency Anemia (IDA), and Anemia*, Among Infants Exclusively Breastfed† until 4 Months of Age (N = 173)

	ID			IDA			Anemia		
	OR	**95% CI**	**P value****	**OR**	**95% CI**	**P value****	**OR**	**95% CI**	**P value****
Semi-solid food introduced at ≥ 6 months of age	1.67	(0.53, 5.32)	0.38	1.17	(0.37, 3.91)	0.79	0.60	(0.12, 2.22)	0.48
Infant over 6 months old at blood draw	1.82	(0.93, 3.68)	0.087	2.06	(1.06, 4.07)	0.035	1.75	(0.81, 3.98)	0.17
Male sex	1.39	(0.71, 2.74)	0.34	2.18	(1.12, 4.35)	0.024	3.06	(1.44, 6.81)	0.005
500g increase in birth weight	0.53	(0.37, 0.77)	0.0007	0.46	(0.31, 0.68)	< 0.0001	0.54	(0.36, 0.80)	0.002
Maternal employment	0.45	(0.21, 0.94)	0.034	0.48	(0.22, 1.02)	0.060	0.60	(0.26, 1.40)	0.23

*Iron Deficiency defined as inflammation-corrected ferritin < 12µg/L, see Methods. Anemia defined as altitude-corrected hemoglobin < 11g/dL, see Methods. Iron Deficiency Anemia defined as Iron Deficiency plus Anemia. **Wald Chi-Square tests. †Defined as infant received no semi-solid foods or non-breast milk liquids until reaching 4 months of age. One month of formula allowed as long as it was accompanied by breast milk.

Table 5.4: Association of Length of Exclusive Breastfeeding with Ferritin and Hemoglobin* (N = 270)

	Fer			Hb		
	Percent Change	**CI**	**P value****	**Percent Change**	**CI**	**P value****
Infant exclusively breastfed† for < 4 months	0.0	–	0.006	0.0	–	0.021
Infant exclusively breastfed† for 4 - 6 months	-22.3	(-37.3, -3.7)	–	-3.1	(-5.6, -0.7)	–
Infant exclusively breastfed† for > 6 months	-44.2	(-62.5, -16.7)	–	-4.7	(-9.1, -0.1)	–
Infant over 6 months old at blood draw	-22.3	(-30.5, -13.1)	< 0.0001	-1.6	(-2.9, -0.3)	0.017
Male sex	43.3	(17.0, 75.5)	0.0006	4.1	(1.6, 6.6)	0.001
500g increase in birth weight	25.6	(13.7, 38.8)	< 0.0001	2.9	(1.7, 4.1)	< 0.0001
Maternal employment	32.9	(5.9, 66.8)	0.015	1.7	(-1.0, 4.4)	0.23

*Fer and Hb log-transformed to meet normality assumptions. **Wald Chi-Square tests. †Defined as infant received no semi-solid foods or non-breast milk liquids until reaching 4 months of age. One month of formula allowed as long as it was accompanied by breast milk.

Figures and Figure Legends

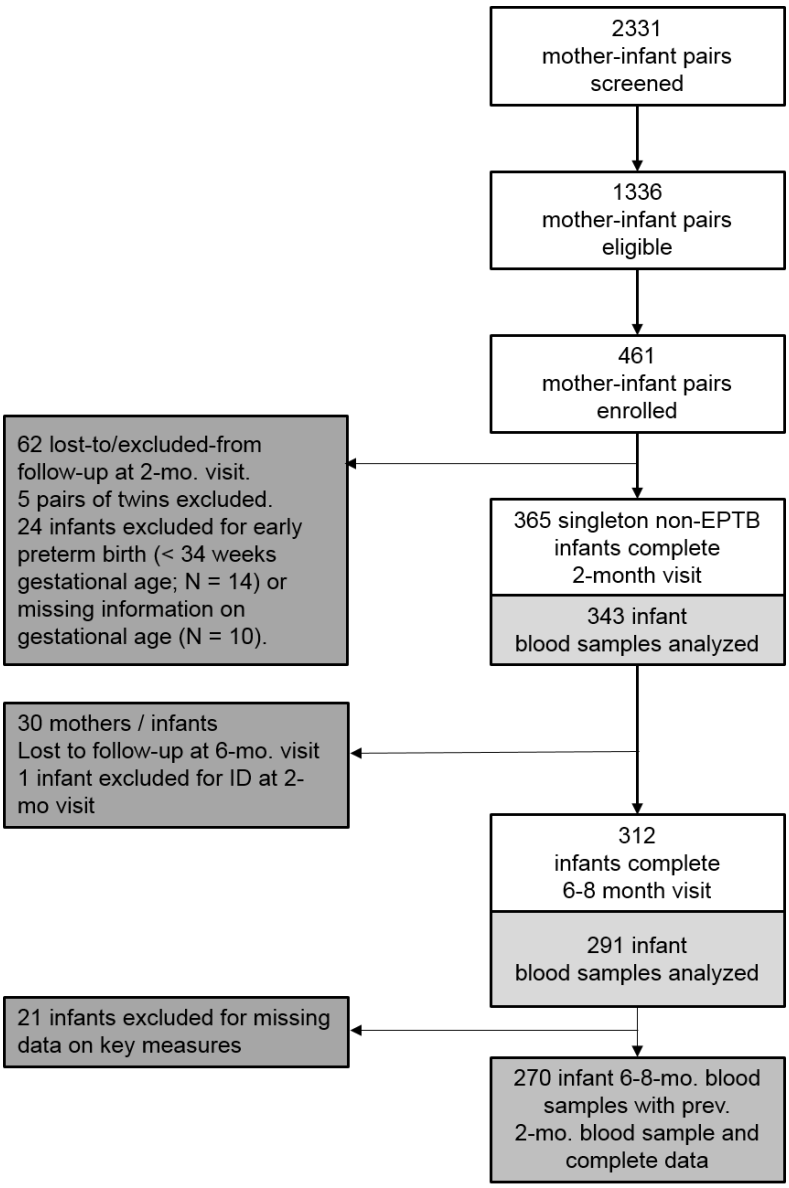

Figure 5.1: Participant Flow.

Of 2331 screened mother-infant pairs, 1336 were eligible for the parent study and 461 enrolled. A total of 343 singleton, non-early preterm infants provided samples at 2 months, with 291 giving samples at 6 – 8 months, and 270 having complete data.

Chapter 6: Conclusions and Future Directions

Summary of Findings

In this dissertation, we examined inflammation and iron deficiency, and the relationship between them, among a cohort of infants in Bolivia, a lower-middle-income country in South America.

While inflammation was significantly increased among older infants and toddlers as compared to young infants, and appeared to be attenuated in breastfeeding infants, it was not clearly explained by any sociodemographic characteristics. This underscores the importance of measuring biochemical markers of inflammation in order to account for its effect on biomarkers of iron, which this work has shown to be meaningful even in infants (Figures A1 – A2).

The prevalence of iron deficiency was extremely high in this infant population (> 50% by 6 months of age), and the estimated prevalence varied meaningfully depending on whether the effect of inflammation was accounted for (Figure A3). When we tested the impact of regression-adjustment of ferritin on the variance of effect estimates using these values, we found that the "between-model" contribution was much smaller than the "within-model" contribution. This is reassuring for using this method.

Our analyses suggested that among this cohort of infants, those who were born of lower birth weight, of male sex, or with non-working mothers, as well as infants with prolonged exclusive breastfeeding, were more vulnerable to development of iron deficiency.

Conclusions and Future Directions

Taken together, these studies suggest that even healthy, breastfed infants are highly vulnerable to early declines in iron status. This decline is perhaps even more striking in this setting of a national multiple micronutrient powder (MNP) supplementation program, and may suggest that additional work is needed to expand this program or improve adherence. Overall, the high prevalence of iron deficiency is concerning given the potential serious consequences of iron deficiency, and may imply a need for earlier interventions to improve the iron status of mothers and their infants. Further, the importance of measuring and accounting for inflammation is underscored.

Future research should continue to assess iron status in infants and young toddlers in developing countries, while accounting for the effect of inflammation. Further, interventions should be tested to improve the iron status not only of infants, but also their mothers. Potential examples include increased emphasis on delayed cord clamping, prenatal iron supplementation, and expansion of multiple micronutrient powder programming. In this population (and most likely in others), qualitative research would also fill an important knowledge gap around barriers and facilitators to micronutrient supplementation adherence as well as recommended infant feeding practices.

Appendix

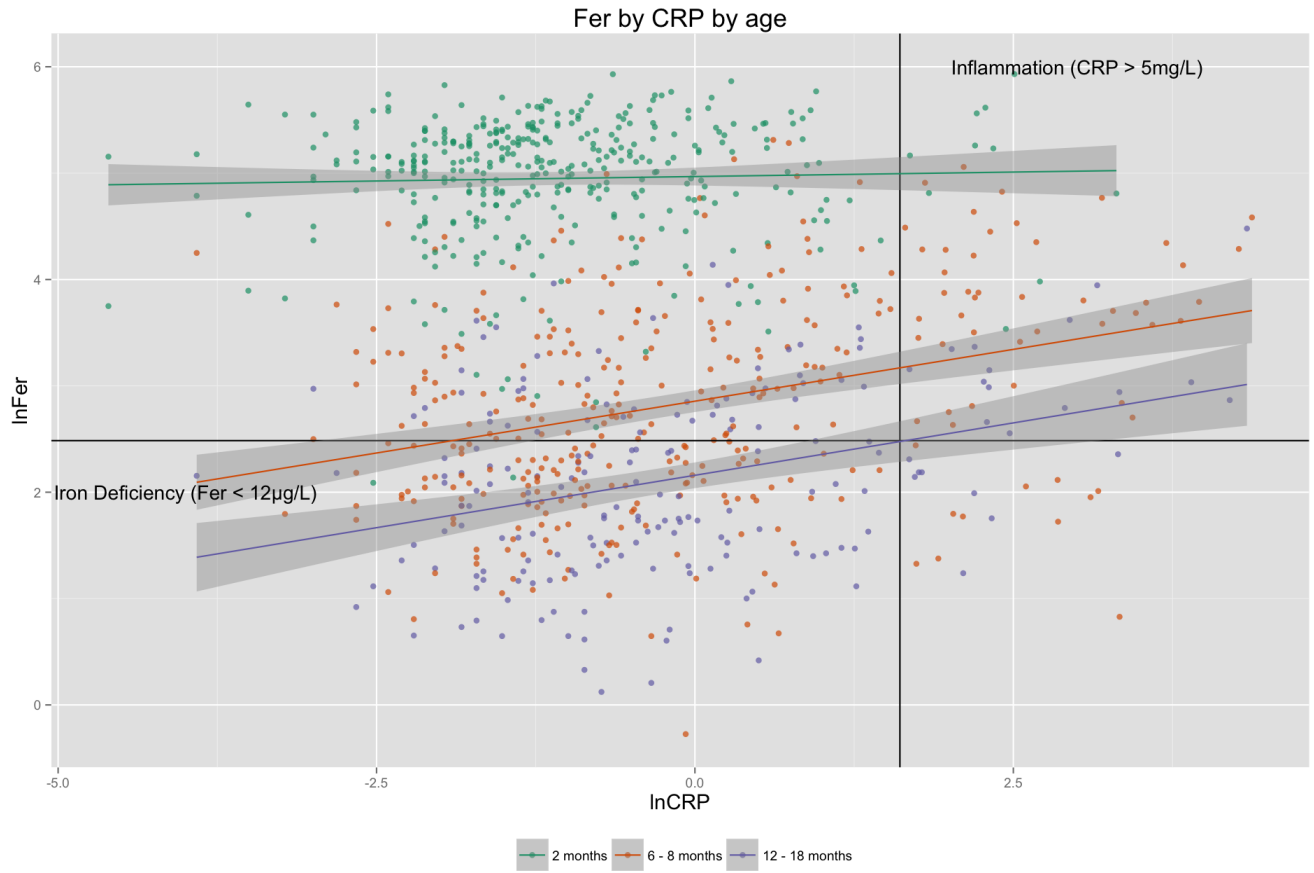

Figure A1. Impact of CRP on Ferritin.

Ferritin rises with increasing CRP, even in infants.

Figure A2. Impact of AGP on Ferritin.

Ferritin rises with increasing AGP, even in infants.

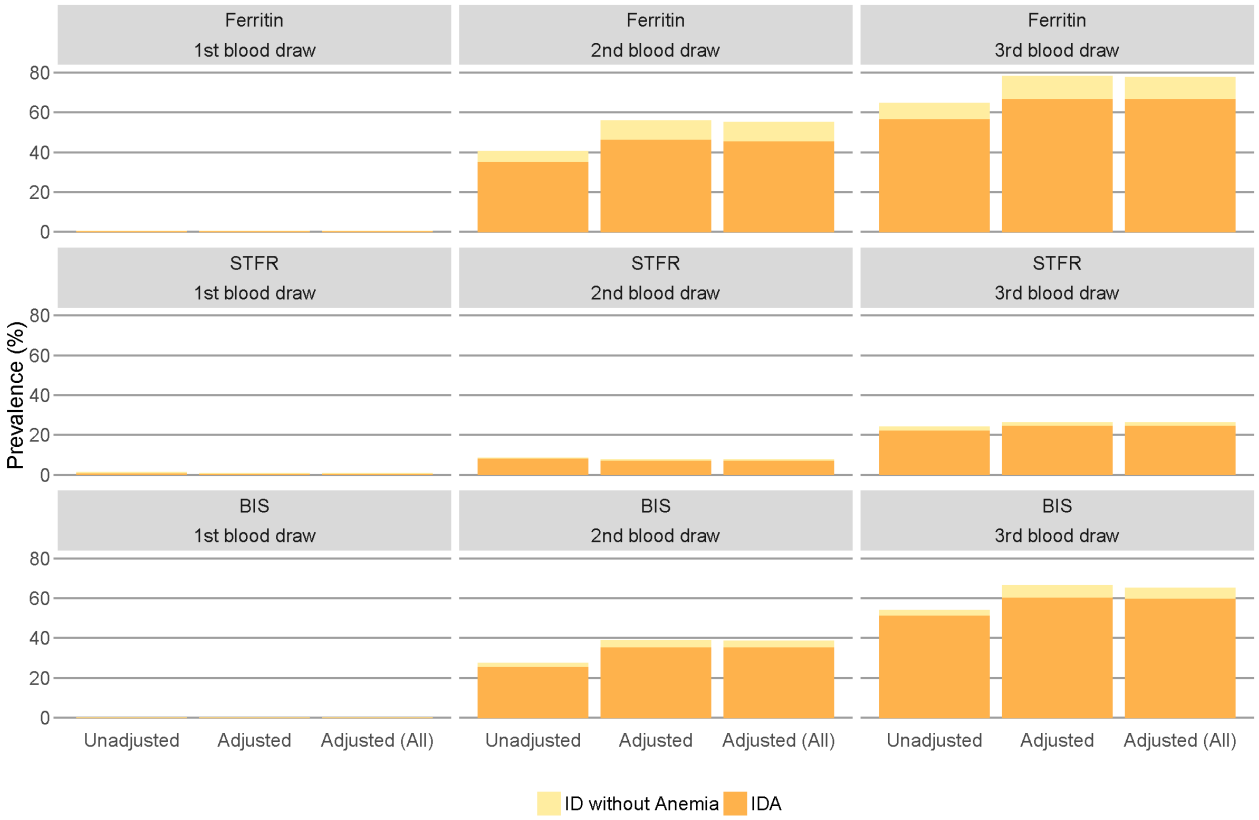

Figure A3. Prevalence of Iron Deficiency and Iron Deficiency Anemia in Infants by Age, Biomarker, and Inflammation-Correction Method.

The prevalence of iron deficiency (ID) and iron deficiency anemia (IDA) among infants varied by age and biomarker. Prevalence estimates were higher for corrected values. Estimates for biomarkers corrected only for participants with elevated acute phase proteins (APP; "Adjusted") were extremely similar to estimates for biomarkers corrected for all participants ("Adjusted (All)").

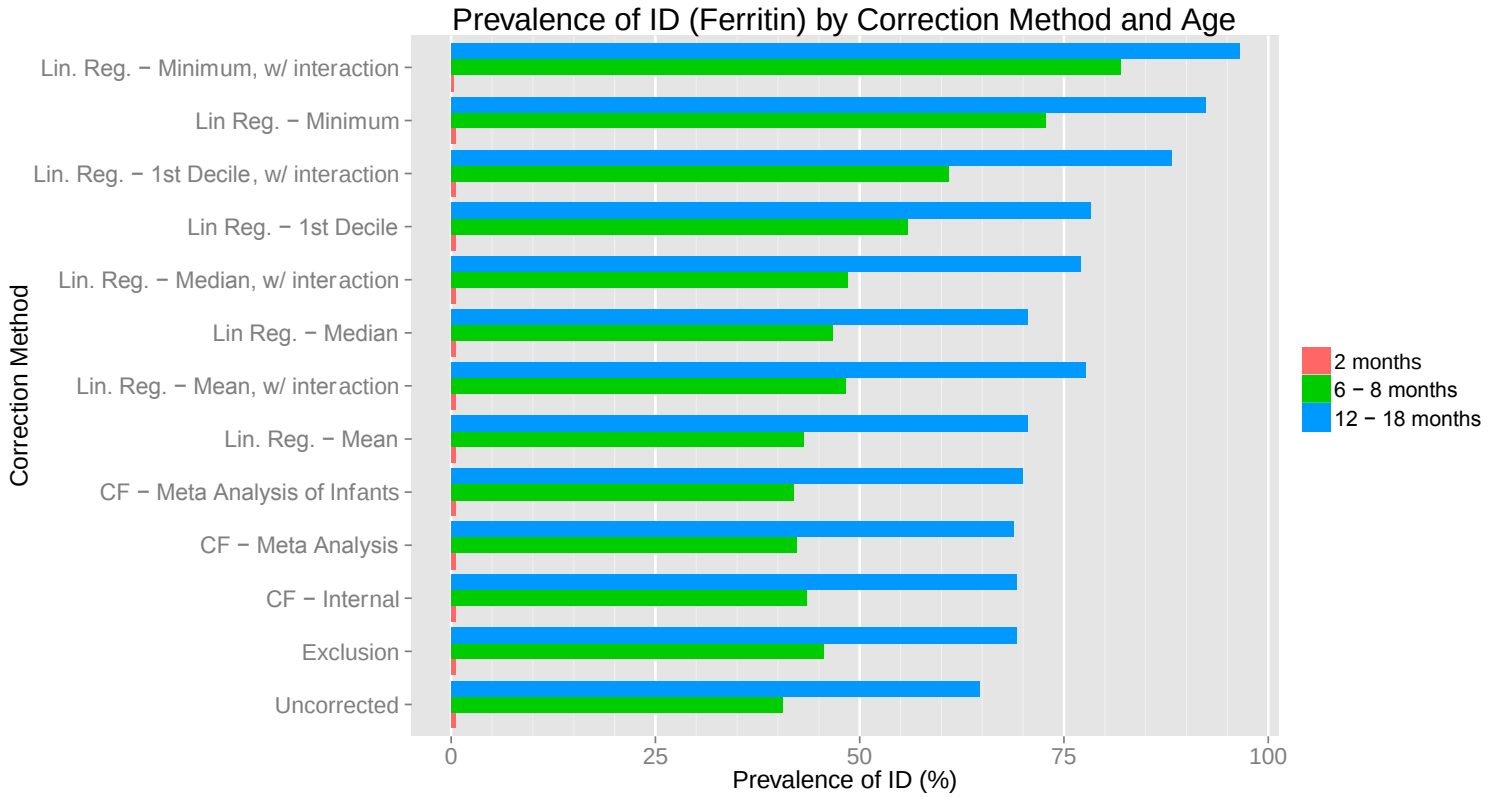

Figure A4. Prevalence of Iron Deficiency in Infants by Age and Inflammation-Correction Method.

The prevalence of low iron stores (Fer < 12µg/L) among infants varied by correction method.

Table A1: Association of Length of Exclusive Breastfeeding with Iron Deficiency (ID) and Iron Deficiency Anemia (IDA)* (N = 270), Uncorrected Ferritin

	ID			IDA		
	OR	**95% CI**	**P value****	**OR**	**95% CI**	**P value****
Infant exclusively breastfed† for < 4 months	1.00	–	0.001	1.00	–	0.053
Infant exclusively breastfed† for 4 - 6 months	2.18	(1.24, 3.94)	–	1.67	(0.93, 3.06)	–
Infant exclusively breastfed† for > 6 months	5.98	(2.06, 19.60)	–	3.24	(1.15, 9.53)	–
Infant over 6 months old at blood draw	1.60	(0.95, 2.74)	0.080	1.96	(1.15, 3.39)	0.015
Male sex	1.80	(1.06, 3.12)	0.032	2.44	(1.40, 4.34)	0.002
500g increase in birth weight	0.61	(0.46, 0.80)	0.0005	0.57	(0.43, 0.77)	0.0005
Maternal employment	0.45	(0.24, 0.83)	0.012	0.50	(0.26, 0.94)	0.036
Mother has completed superior education	0.70	(0.29, 1.62)	0.41	0.67	(0.27, 1.56)	0.36

*Iron Deficiency defined as ferritin < 12µg/L. Anemia defined as altitude-corrected hemoglobin < 11g/dL. Iron Deficiency Anemia defined as Iron Deficiency plus anemia. **Wald Chi-Square tests except for breastfeeding categories, which used likelihood ratio tests. †Defined as infant received no semi-solid foods or non-breast milk liquids aside from vitamins / minerals / medicine. One month of formula allowed as long as it was accompanied by breast milk.

Table A2: Age of Introduction of Complementary Foods with ID, Anemia, and IDA* among infants EBF until at least 4 months (N = 173), Uncorrected Ferritin**

	ID			IDA		
	OR	**95% CI**	**P value†**	**OR**	**95% CI**	**P value†**
Semi-solid food introduced ≥ 6 months of age	1.02	(0.32, 3.36)	0.97	0.91	(0.28, 3.28)	0.88
Infant over 6 months old at blood draw	1.93	(1.01, 3.79)	0.050	2.46	(1.25, 4.96)	0.01
Male sex	1.19	(0.62, 2.30)	0.60	1.92	(0.97, 3.90)	0.066
500g increase in birth weight	0.51	(0.36, 0.74)	0.0004	0.45	(0.30, 0.67)	< 0.0001
Maternal employment	0.38	(0.18, 0.80)	0.013	0.43	(0.18, 0.94)	0.039
Mother has completed superior education	0.97	(0.36, 2.63)	0.96	0.86	(0.29, 2.42)	0.79

* Iron Deficiency defined as ferritin < 12µg/L. Anemia defined as altitude-corrected hemoglobin < 11g/dL. Iron Deficiency Anemia defined as Iron Deficiency plus anemia. **Defined as infant received no semi-solid foods or non-breast milk liquids, aside from vitamins / minerals / medicine until reaching 4 months of age. One month of formula allowed as long as it was accompanied by breast milk. †Wald Chi-Square tests.

Table A3: Association of Length of Exclusive Breastfeeding with Uncorrected Ferritin (N = 270)

	Fer		
	Percent Change	**CI**	**P value****
Infant exclusively breastfed† for < 4 months	0.0	–	0.005
Infant exclusively breastfed† for 4 - 6 months	-25.1	(-40.9, -5.1)	–
Infant exclusively breastfed† for > 6 months	-47.2	(-66.0, -18.0)	–
Infant over 6 months old at blood draw	-21.6	(-30.7, -11.3)	0.0001
Male sex	40.7	(12.5, 75.9)	0.003
500g increase in birth weight	22.7	(9.9, 36.9)	0.0003
Maternal employment	40.9	(9.8, 80.9)	0.008

**Wald Chi-Square tests except for breastfeeding categories, which used likelihood ratio tests. †Defined as infant received no semi-solid foods or non-breast milk liquids aside from vitamins / minerals / medicine. One month of formula allowed as long as it was accompanied by breast milk

Table A4: Association of Length of Exclusive Breastfeeding with Multiply Imputed Ferritin (N = 270)*

	Percent Change	CI	P value**	Ratio of "Within" Variance to "Between" Variance
Infant exclusively breastfed† for < 4 months	0.0	–	–	–
Infant exclusively breastfed† for 4 - 6 months	-22.57	(-37.80, -3.60)	0.022	665
Infant exclusively breastfed† for > 6 months	-48.53	(-65.81, -22.50)	0.001	311

*Multiple imputation conducted as described in Chapter 4. Model controlled for infant age, sex, birth weight, and maternal employment. ** F test with numerator df = 1 and denominator df = df as above. $F = t^2$ and $t = E(\beta) / \text{sqrt}(T)$. †Defined as infant received no semi-solid foods or non-breast milk liquids aside from vitamins / minerals / medicine. One month of formula allowed as long as it was accompanied by breast milk