EVALUATION OF THE IRON, ANTIOXIDANT AND DIETARY STATUS OF IRON SUPPLEMENTED BREASTFED HEALTHY INFANTS

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Evaluation of the
Iron, Antioxidant and Dietary Status
of
Iron Supplemented
Breastfed Healthy Infants

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Abstract

It is known that 10 – 28% of breast fed infants will develop iron deficiency (ID; serum ferritin ≤ 12 μg/L or Hgb ≤ 110 g/L + Hct ≤ 33% or MCV ≤ 75 fL) by 6 months of age. Iron deficiency anaemia will effect 10 – 15% of the exclusively breast fed population by 6-months of age (IDA; Hgb ≤ 110 g/L + serum ferritin ≤ 12μg/L or Hct ≤ 33% and MCV ≤ 75 fL). ID severe enough to cause anaemia has been proven to be a factor in delayed cognitive and psychomotor development. We initiated a randomized double blind clinical trial to investigate the effects of supplementing breast fed infants with iron and its effects on the incidence of ID and IDA (7.5 mg $FeSO_4$/day). Supplementation was between 1 to 6-month, to a maximum of 150 days. Further investigation into whether the supplement had any adverse effects on the antioxidant status, potential for oxidative damage and mineral absorption of the infants was also carried out. Our findings have shown that ID can affect as high as 55% of infants ($p<0.02$). Iron supplementation, 7.5 mg $FeSO_4$/day, is high enough to demonstrate a difference in the haemoglobin concentrations (124+/-9.13 g/L vs. 116+/-6.68 g/L), hematocrit (0.352+/-0.029 vs. 0.333+/-0.019) and MCV (81.17+/-4.13 fL vs. 77.22+/-3.89 fL) compared to infants whom are not supplemented ($p<0.05$). While plasma ferritin concentrations did not differ between the two groups the infants receiving placebo had a significantly higher rate of decline in ferritin stores between 1 and 3.5 months than did infants who were supplemented (-82+/-54 μg/L vs. -139+/-84 μg/L). This level of iron supplementation does not appear to affect the antioxidant response of erythrocyte superoxide dismutase or catalase nor does it change the total antioxidant power of the infant plasma (FRAP). Plasma mineral concentrations were normal for all infants but plasma zinc concentrations

significantly increased between the 3.5 and 6-month blood samples ($p<0.05$), with not enough data to explain. Positive correlations exist between the infant 1-month haemoglobin and 3.5 and 6-month plasma ferritin concentrations, which may prove useful as a predictor of iron stores for infants who are exclusively breast fed. Breast milk iron concentrations were measured at 1 and 3.5-months (0.47+/-0.14 mg/L to 0.76+/-0.40 mg/L at 1-month; 0.29+/-0.13 mg/L to 0.78+/-0.36 mg/L at 3.5-months) by three separate techniques, direct GFAAS, methods of addition GFAAS and FAAS. While all three methods produced values that are in the reported range for human breast milk iron concentrations the direct GFAAS seems to have been validated by reports in literature and the methods of addition GFAAS as a simple, sensitive and reliable technique. Maternal haemoglobin at delivery was also examined as a predictor of breast milk concentrations and infant haemoglobin at 1-month but no relationships were seen between these variables. In all it appears that iron supplementation (7.5 mg Fe/Day) will improve the iron status of exclusively breast-fed infants without risk of impaired mineral absorption or oxidative damage. The iron status of exclusively breast-fed infants appears to be poor. There is a need for better screening or supplementation of these infants in order to prevent this deficiency.

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Abbreviations

Atomic Absorption Spectrophotometry	AAS
Bayley Scales of Infant Development	BSID
Catalase	Cat
Central Nervous System	CNS
Complete Blood Count	CBC
Ferric Reducing Ability of Plasma	FRAP
Flame Atomic Absorption Spectrophotometry	FAAS
Graphite Furnace Atomic Absorption Spectrophotometry	GFAAS
Haemoglobin	Hgb
Hematocrit	Hct
Infant Behaviour Record	IBR
Iron Deficiency	ID
Iron Deficiency Anaemia	IDA
Mean Corpuscular Volume	MCV
Mental Development Index	MDI
Methanesulfinic Acid Assay	MAA
Plasma Ferritin	Frt
Psychomotor Development Index	PDI
Red Cell Distribution Width	RDW
Superoxide Dismutase	SOD
World Health Organization	WHO

Chapter 1: Literature Review

1.1 Iron Deficiency

Iron deficiency (ID) is a lack of or shortage of iron in the body usually caused by a nutritional deficiency. This is the pool of iron the body draws upon for metabolic processes such as the production of haem for red blood cells, enzymes and co-factors as well as fatty acids and other lipids. Once iron stores are depleted there is an appreciable decline in each of these processes as the different systems begin to compete for iron. The anaemia that may result is a clinical manifestation of prolonged iron deficiency. The progression from ID to IDA in the infant occurs rapidly due to the increased metabolic iron requirement for proper growth and the increasing size of the infant. Iron is required for maintaining existing cells and for cell proliferation throughout this period of rapid growth. Symptoms of iron deficiency anaemia (IDA) include pallor, decreased vitality, dizziness and digestive problems. Iron deficiency during periods of infantile growth can cause damage or developmental set backs beyond those manifested in adults. Proper neurological and visual development requires adequate iron for proper oxygenation of tissue and for the production of long chain fatty acids (Larkin and Rao, 1990). As well, the infant has a rapidly expanding blood volume requiring an abundance of iron for the production of haem and red blood cells. Infants are therefore forced to draw heavily upon the stores laid down in utero depleting them by 6-months of age. Four – six month old infants can cope with this stress provided they are weaned with foods containing iron but not all exclusively breast fed infants are able to manage in this manner (Dewey et al, 1998). For 10 – 20% of this exclusively breastfed population, IDA is inevitable and will potentially be accompanied by other undesirable consequences. Further

discussion of the criteria of ID and IDA will follow. Generally, ID it is characterized by low iron stores, diagnosed using a complete blood count (CBC) which reports the number of blood cells as well as the relative size and amounts of constituents such as haemoglobin, demonstrating low haemoglobin and low serum ferritin usually quantified by ELISA tests.

1.1.1 World Wide Perspective on Iron Deficiency

Today ID is one of the world's most common nutritional deficiencies and is regarded as one of the major health problems worldwide despite near elimination of the conditions in the developed world (DeMaeyer et al, 1985; WHO, 2001). The major causes of iron deficiency and the resulting anaemia worldwide are inadequate dietary sources, low bioavailability and parasitic infection (WHO, 2001). While Canada and the rest of the developed world have a far lower incidence of iron deficiency anaemia compared with less developed countries, it is still the leading cause of anaemia in all age groupings (WHO, 2001). Specific sub-populations in our country suffer from iron deficiency anaemia. Women of menstruating age, the elderly and the malnourished commonly suffer from this disorder but breast fed infants appear to be at the greatest risk of developing iron deficiency. Supplemental iron, both medicinal and dietary, is the most common practice used to avoid and treat the condition in all groups affected.

1.1.2 Iron Status of Infants Through the First Year of Life

Some investigators believe that the endowment of iron and status of the infant at birth is enough to carry them through the first six months with the introduction of solid foods providing adequate sources of iron for the first year of life. Other investigators believe that not all exclusively breastfed infants are protected from IDA with the iron stores laid down at birth. Regardless of the

arguments, data over the past number of years, demonstrates a clear picture for infants with regard to iron status.

Between 20 – 25% of infants worldwide suffer from iron deficiency anaemia and an even higher number suffer from iron deficiency without anaemia (DeMaeyer et al, 1985; WHO, 2001). Therefore it may be taken for granted that breastfed infants are receiving adequate amounts of iron from dietary sources. Several studies worldwide have demonstrated the incidence of ID and IDA in the infant population of the developed world (Figure 1.1). While not all studies were performed on exclusively breastfed infants, those that were demonstrated an incidence ranging between 10 – 28% (figure 1.1) including 10 – 15% of exclusively breastfed Canadian infants suffering from iron deficiency anaemia within the first year of life (Innis et al, 1997; Friel et al, 1997). Iron deficiency was seen in otherwise healthy infants and would most likely not have been diagnosed so early without the scheduled screening provided in these studies. Differing criteria from study to study suggest a need to define exactly what criteria are used to determine the iron status of infants.

1.1.3 What Constitutes Iron Deficiency and Iron Deficiency Anaemia

A clear definition of ID and IDA depends on the circumstances under which the disorder is being investigated. Clinically, IDA can be detected from a complete blood count. Haemoglobin, hematocrit and mean corpuscular volume are the measures used to identify IDA. For Canadian infants the clinical anaemia cut-off values for haemoglobin is a value less than 110 g/L, a hematocrit is less than 33% and a mean corpuscular volume less than 75 fL. The WHO has established the cut-off values for haemoglobin and hematocrit at 110 g/L and 33%, respectively as the worldwide standard for identifying IDA (WHO, 2001). These haematological cut-offs are accurate for identifying IDA when other types of anaemia are ruled out.

Other types of anaemia have to be ruled out when making the diagnosis of IDA. A factor to consider in anaemia of subjects from African descent is the sickle cell trait. The anaemia resulting from the thalassemia trait is most common in subjects of Mediterranean descent. As well recent infections an infant may have had need to be considered, as they too will produce anomalous haematological results. As well microcytic anaemia, vitamin B_{12} (pernicious anaemia) folate deficiency and lead poisoning have to be ruled out for the diagnosis of IDA. Sickle cell is characterized by the crescent shaped red blood cell, infection is characterized by an abnormal haemoglobin value but normal MCV and RDW values while thalassemia is characterized by low MCV and normal RDW (Bogen et al, 2001). Microcytic anaemia is defined by a MCV less than 70 fl with no other abnormal indices. While clinicians use these definitions to confirm the diagnosis of the condition, these criteria are not the only biochemical aspects used by researchers investigating IDA. There are a variety of measures commonly used by researchers to determine whether subjects are iron deficient, anaemic or both.

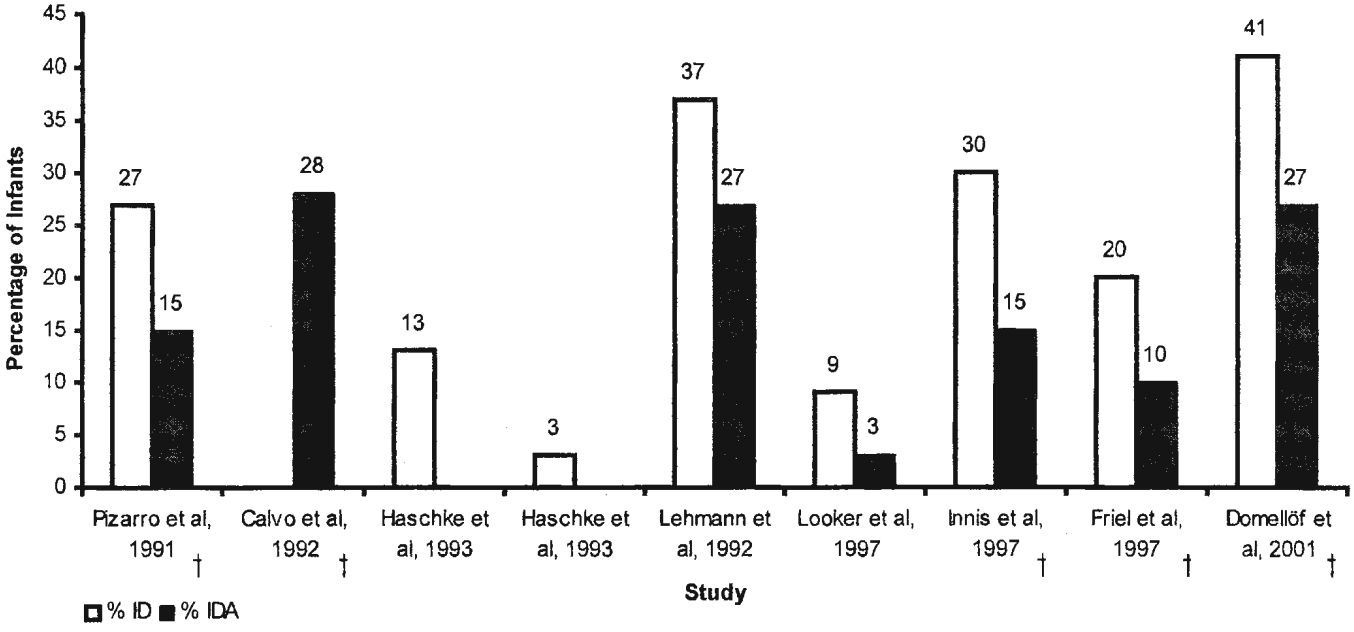
Other researchers use a variety of methods to characterize subject iron status when investigating iron deficiency. Researchers, like clinicians, use the haemoglobin, hematocrit, MCV and other measures determined by a CBC when diagnosing ID and IDA. However, with the advent of the immunoassay there are commercially available kits to elucidate nearly all human proteins and receptors. Serum or plasma ferritin and transferrin receptors are commonly used when defining iron storage status (Sweet et al, 2001; Domellöf et al, 2001; Guldholt et al, 1991). Serum ferritin is the main storage iron storage protein in the human body. Ferritin stores iron in an inactive form and releases when signalled by cells throughout the body. Serum ferritin less then 12 μg/ml is accepted as being characteristic of server iron deficiency with less then 20 μg/ml is

borderline iron deficient (Glassman, 1997). Serum transferrin and transferrin receptor are also useful measures of iron status. Serum transferrin less then 16% saturation is characteristic of severe anaemia and transferrin receptor will be high in cases of iron deficiency but the absolute values are not clearly defined due to differences in methods and lack of a useful standard (WHO, 2001). Erythrocyte protoporphyrin (EPP) has also been used as an indicator of iron status (Domellöf et al, 2001). EPP will increase, as the production of haem is limited and will correlate to decreasing ferritin stores. EPP will also increase in cases of haemolytic anaemia and lead poisoning, therefore should not be used in areas with high prevalence of these conditions.

Classification of iron status using criteria other than the more common, such as Hgb and other haematological values, make it difficult to compare results. The haemoglobin cut-off value varies between 95 - 110 g/L, serum ferritin between 10 – 12 μg/L, hematocrit between 30 – 33%, MCV between 70 – 75 fL, erythrocyte protoporphyrin usually >120 mg/L, transferrin saturation between 10 - 16% or lower and serum transferrin receptor usually >4.4 mg/L (Kim et al, 1996; Domellöf et al, 2001; Lönnerdal et al, 1994; Arvas et al, 2000; Willows et al, 2000; Morley and Lucas, 1999; Male et al, 2001; Pizarro et al, 1991; Harding et al, 2001). Not all researchers use all the indices listed above and not all use them consistently. Upon careful review it appears that the most widely used indicators iron status of infants would be the Hgb, MCV and plasma ferritin. These indicators are useful both to the clinician and the researcher as they are easily obtainable and comparable. The other indices (hematocrit, erythrocyte protoporphyrin, transferrin saturation and serum transferrin receptor) help when no clear determination can be drawn from the other indices if they are available.

Figure 1.1 Summary of findings on the prevalence of iron deficiency and iron deficiency anaemia within the infant population. † Indicates prevalence of iron deficiency and iron deficiency anaemia within exclusively breast-fed infant subjects.

Prevalence of Iron Deficiency and Iron Deficiency Anaemia Among Infants



1.1.4 Infant Iron Requirements and Absorption in the First Year of Life

Samuel Fomon (1993) estimates to prevent the depletion of iron stores at birth, the infant must absorb 0.55 – 0.75 mg iron/day from dietary sources. This amount will allow the infant to meet its developmental needs in the first year of life. This estimate is based on the allocation of iron at birth (birth weight of 2.5 – 3.5 kg) and the desired allocation for a 12-month old infant weighing 10.0 – 10.5 kg. While this is not a challenge for infants consuming iron-fortified formulas (12mg iron/L), the exclusively breastfed infant cannot achieve this goal consuming breast milk alone.

Iron concentrations for human breast milk are reported to be between 0.27 – 0.90 mg/L (Casey, 1996). The concentration of iron in colostrum is higher than in mature milk but most milk consumed by infants is mature milk. Infants would be required to consume at least one litre per day of human milk and have an absorption efficiency of 100% in order to meet their estimated needs. The actual amount of iron that an infant in the first 6 months will obtain daily is more likely to be in the range of 0.09 – 0.23 mg, assuming an absorption efficiency of 50% and a consumption of 650 mL of milk daily (Fomon, 1993; Casey, 1996). There is confusion regarding the actual absorption efficiency of infants with regard to iron from breast milk. McMillan and associates (1977) estimated absorption to be at 50% based on adult models. Saarinen and Siimes (1979) reported an efficiency of 70% for breast milk and 10% for formula. Until recently, 50% absorption of iron from human breast milk has since been assumed to be the standard absorption level accepted by most investigators in the field (Garry et al, 1981; McMillan et al, 1977).

More recently it has been reported that the absorption of iron from dietary breast milk is between 12% and 20% (Davidsson et al, 1993; Abrams et al, 1996, Domellöf et al, 2002). Newer

methodologies and refined techniques in measuring iron turnover in infants have lead to a more conservative view as to the amount absorbed from human breast milk. Iron absorption is not constant and different factors will affect the amount of iron absorbed. For example, the presence of lactoferrin appears to lower the mean dietary absorption of iron to 12% from 20% in lactoferrin free milks (Davidsson et al, 1993). In addition, duel-tracer stable isotope experiments demonstrate that supplemental iron is better absorbed during a feed rather than between feeds, 15% versus 11% respectively (Abrams et al, 1996). Most recently Domellöf (2002) and associates have measured the absorption of iron from breast milk in 6-month exclusively breast fed infants who were either supplemented with ferrous sulphate or receiving a placebo. Absorption was found to be 16+/-11% at 6 months, with no differences between the group receiving iron supplements of those receiving the placebo. This study produced data on absorption similar to that found by both Davidsson et al and Abrams et al and demonstrates iron absorption from an exclusive breast milk diet to be low. These methods for the estimation of the absorption of iron from breast milk are limited as there has been no successful method to intrinsically label breast milk and measure absorption (Figueroa-Colon et al, 1989). There may or may not be higher absorption of iron from the bound sources which do not exchange with the added stable or radioactive isotopes of iron used in the previous methods. A fractional absorption of iron between 12 – 20% is a fair estimate based on the currently available values.

In light of the latest figures pertaining to iron absorption from human breast milk it is clear that exclusively breastfed infants are at risk of developing iron deficiency during critical periods of growth in the first six months of life. Since many factors affect the absorption efficiency of both supplemental and inherent iron of the infant gastrointestinal tract careful review as to the proper

dosage and timing of supplementation is required. While supplemental iron presents the infant with the risk of oxidative damage, iron deficiency also exposes the infant to other sizable risks. These risks can be potentially damaging to an infants cognitive and psychomotor development with the effects lasting well into childhood (Lozoff et al, 2000).

1.2 Effects of Iron Deficiency on Infant Development

Infants suffering from IDA face challenges along the course of normal development, which may have long reaching effects. Key periods of development are jeopardized when infants have severe nutritional deficiencies. The blood cell production, central nervous system development, enzyme production, and susceptibility to illness and infection, all rely in part on proper iron status.

1.2.1 Iron Containing Enzymes

A number of proteins contain iron as an essential part of their biochemical function. Haem is one such protein, utilizing iron as an O_2 carrier. Haem is required by haemoglobin for transport of O_2 throughout the body. Haem is also a constituent of myoglobin, responsible for O_2 storage/transport in muscle tissue. An iron deficiency will lead to a decrease in oxygen carrying capacity due to the reduction in haem production. This is of particular importance in infants, given the rapid growth and expansion of blood volume during the first year of life. The mitochondrial system of electron transport is dependent on enzymes that contain haem such as cytochrome c oxidase. Proteins involved in oxidative and mitochodrial metabolism also require iron to work properly, i.e. non-haem containing proteins like glucose-6-phosphate dehydrogenase, dioxygenase, succinate dehydrogenase, NADH dehydrogenase and aconitate hydratase (Connors et al, 1996; Bradbury et al, 1997). Iron deficiency anaemia is characterized by decreased vitality,

which can be attributed to both the reduction in oxygen carrying capacity and the competition for iron in metabolic pathways.

1.2.2 Central Nervous System

It should be of particular interest that the normal human brain contains as much iron, on a weight basis, as the liver (Connors et al, 1996). Animal studies have shown that the distribution of iron in the brain, according to region, is relatively even as a function of protein or brain wet weight (Focht et al, 1997). However, brain iron is not evenly distributed among the cell types of the brain and CNS. Oligodendrocytes are the cell type most dependent on iron for proper function (Sanyal et al, 1996; Blissman et al, 1996). Given that the oligodendrocytes have the highest concentration of iron, CNS development and maintenance relies heavily on the presence of iron for myelination and oxidative metabolism (Connors et al, 1995; Bradbury et al, 1997). Iron is required by many enzymes such as glucose-6-phosphate dehydrogenase, dioxygenase, succinate dehydrogenase, NADH dehydrogenase, aconitate hydratase and cytochrome *c* oxidase (Connors et al, 1996; Bradbury et al, 1997). The oligodendrocytes rely on oxidative metabolism for energy production, which is partly responsible for the high iron content of the brain.

The oligodendrocytes are cells that produce myelin for the CNS and again require high iron concentrations to facilitate the production of cholesterol and fatty acids (Larkin and Rao, 1990). Myelin is composed of 80% fatty acid, leading to the variety of enzymes found in oligodendrocytes that catalyze the production of fatty acids – haem containing enzymes (cytochromes) as well as fatty acid desaturases and lipid dehydrogenases and the cholesterol biosynthesis enzymes such as squalene oxidase (Connors et al, 1996; Larkin and Rao, 1990). High levels of ferritin in the

CNS counteract the destructive potential of this high level of iron surrounded by high levels of polyunsaturated fatty acids. The ferritin protein has a ferroxidase activity, which facilitates the oxidation of Fe^{2+} to the less reactive Fe^{3+}. The protein then stores the iron as a ferric-oxyhydroxyphosphate complex (Sanyal et al, 1996).

Myelination has been referred to as a "once and for all" process given that the body does not have the capability for re-myelination after de-myelination has occurred (Larkin and Rao, 1990). While it is difficult to demonstration the effects of iron deficiency in the brain of human neonates, animal studies have shown the profound effects of the disorder during the critical period of myelination. Myelination begins about 10-days after gestation in rats; if an iron insult is inflicted on rats around this age the effects are devastating. A 30% reduction in myelin proteins has been reported as well as decreases to the total brain lipid in iron deficient neonatal rats (Larkin and Rao, 1990). These findings are particularly alarming since there may be a large number of infants that are iron deficient who go undiagnosed until they are severely deficient, displaying clinical signs of anaemia. It is not possible to perform analysis of CNS tissue in iron deficient human neonates. However, the same conclusions regarding impaired myelination can be drawn from rat studies and applied to all mammalian development (Larkin and Rao, 1990).

Altered nervous system responses have been reported in infants diagnosed with iron deficiency anaemia when compared to non-anaemic infants (Roncagliolo et al, 1998). Decreased auditory nerve responses and increased latency times between stimulus and neuron firing have been measured in IDA infants compared to non-anaemic infants and is suspected to be related to level of myelination (Roncagliolo et al, 1998). Like myelination, the brain's neurotransmitters are also affected by iron depletion. Decreases in the learning capacity, functionality of Dopamine D2

receptors and reversals of several circadian cycles (pain thresholds, motor activity etc) are common in iron deficient rats. These rat studies also show that the time required to learn a water maze increases over time as length of iron deficiency insult increases (Yehuda et al, 1990). These findings give weight to the argument that decreased CNS activity is a consequence of iron deficiency in neonates.

1.2.3 Cognitive and Psychomotor Development

Webb and Oski (1973) concluded the iron status of young children affected their performance on developmental tests. Webb and Oski tested school children aged 12 - 14 years using the Iowa Basic Skills testing, and demonstrated that anaemic children scored lower then iron sufficient children. Oski and Honig (1978) evaluated infants with iron deficiency anaemia and assessed the difference between scores when half the group had its iron replenished by medicinal intervention. Infants were tested for behaviour, cognitive and psychomotor development prior to and after iron therapy, using the Bayley Scales of Infant Development. Oski and Honig noted that the infants who were treated with iron and had their status corrected re-tested higher than they originally tested while iron deficient. Oski et al (1983) demonstrated iron deficient infants would score better on a developmental test after only 1 week of iron therapy. Oski and associates credited iron deficiency not the anaemia as causing the developmental impairments since one week of iron therapy is not sufficient to relieve an infant of anaemia (Oski et al, 1983). Oski and associates therefore concluded the iron deficiency rather than anaemia is the cause of the decreases seen in cognition and psychomotor development for iron deficient infants. While the conclusion as to the cause of the developmental differences may be correct it was not the reason

for the increase in test scores seen by Oski et al. A later study by Betsy Lozoff and associates would explain these observations.

Lozoff and associates (1982) examined infants aged 6 – 24 months for impaired mental development as a function of iron status. The results supported those seen by Webb and Oski (1973) and Oski et al (1978) demonstrating infants with low iron status causing anaemia have lower mental test scores than those with normal blood iron status. The study demonstrated that there is an appreciable decline in all infants test scores with age but the decline is far greater in those infants suffering from IDA.

While there is not a wide range of research in the area of iron deficiency and cognitive development there appears to be a clear connection with low iron status and delayed cognitive development. Lozoff and associates (1987) then evaluated the differences between iron sufficient and iron deficient infants and the effects of short-term and long-term treatments on repeat tests of infant mental and psychomotor development. Her group also examined intra-muscular injections of iron and oral drops and their effectiveness after 1 week and 3 months. As seen in previously Lozoff and associates showed that infants with poor blood iron status score significantly lower than iron sufficient infants on indices of mental and behavioural development and have below normal psychomotor scores. No differences in the method of iron treatment (oral drops versus IM injection) were seen with respect to correcting iron status or increasing developmental test scores. Developmental testing after short-term iron treatment resulted in increases in all groups' test scores including the placebo. This increase in all groups demonstrates a test score increase independent of the iron status since the placebo group also increased. This effect has been referred to as the practice effect where familiarity with the test is the most probable reason for an

increase in the test score. This practice effect may have contributed to the increase in test scores seen in Oski et al (1978) study. Long-term treatment of 3 months, returning the blood indices to normal, results in increased mental test scores but the results do not reach the levels achieved by the previously iron sufficient group. This may be an indication that the iron insult has caused a permanent set back in mental development (Lozoff, 1987).

Walter (1983) and his associates investigated the differences between infants suffering from iron deficiency anaemia, infants who were iron deficient and iron sufficient control infants in cognitive and psychomotor development and behaviour as measured by the Bayley Scales of Infant Development (BSID). Walter and associates used children of the same age, socio-economic and initial birth measurements. All children were 15 months of age and were recruited from an on going study on iron-fortified formula. They also examined the effects of short-term iron therapy on the anaemic and non-anaemic children. The iron treatment had significant effects on the anaemic children's Mental Development Index (MDI) and Infant Behaviour Record (IBR) scores while the control group, which also received the iron therapy, remained unchanged. The results showed a significant difference between the anaemic and iron sufficient groups MDI, with the anaemic children scoring lower but in the normal range for American children. The anaemic infants showed a significant difference in the *"general emotional tone"* portion of the IBR – indicating that the anaemic children were unhappier taking the test than the non-anaemic counterparts. They could see no initial difference in the Psychomotor Development Index (PDI) between any of the groups. The group in this study with iron deficiency but no anaemia did not show any significant differences in the test-retest. The authors did note a sub-group within the iron deficient non-anaemic infants who did show a significant increase in MDI after iron therapy. The authors concluded anaemic

children score lower in BSID tests than those children who are iron sufficient. The increases seen in the test scores after the 11-day iron treatment are attributed to increases in attention span and cooperation in the infants not to an effect on the developing brain (Walter, 1983).

Walter and his associates (1989) published a study, which compare iron status to BSID scores as well as the effect if any a short-term iron treatment of 10 days and a long-term iron treatment of 3 months had on the development scores. As shown previously, low haematological iron status resulted in lower BSID scores than infants that had normal haematological status (Oski and Honig, 1978; Walter et al, 1983). The authors conclude that the effect of short-term iron therapy is not the principle cause for the increases in the BSID test but increases are more likely attributable to a "*practice effect*" of taking the tests. Iron therapy continued for 3 months after the initial re-test of BSID. Even after long-term iron therapy and return to normal haematological status the anaemic infants who previously tested lower did not approach the same scores as their iron sufficient counterparts in the control groups. The authors concluded iron deficiency during a critical developmental period may result in permanent impairment or a long-term set back in cognitive and psychomotor development (Walter et al, 1989). The level of iron deficiency that will cause these impairments is not known.

Other researchers have published data, which indicate there are negative and potentially long-term effects of IDA. Idjradinata and Pollitt (1993) concluded that iron deficiency, severe enough to cause anaemia, would result to lower BSID scores for those children compared to the children who have normal iron status. The authors also conclude that long-term treatment is required to correct the differences in test scores (Idjradinata and Pollitt, 1993). Moffatt and

associates (1994) examined mildly anaemic infants and showed that mild anaemic was not enough to bring on the cognitive effects seen by other researchers.

More recently Lozoff and associates (1998) concluded that infants who suffer from IDA and the resulting behavioural changes risk becoming functionally isolated from peers and overly dependent on caregivers. Lozoff and associates observed distinct behavioural differences between infants who were suffering from IDA compared to those who were not anaemic. Correction of the IDA does not always appear to correct all the behavioural differences observed (Lozoff et al, 1998). It has also been suggested that infants suffering from chronic IDA as infants will not perform as well academically or behaviourally as much as 10 years after the IDA insult (Lozoff et al, 2000).

In summary, iron deficiency severe enough to cause anaemia is potentially hazardous to infants within the first year of life. Iron deficient infants suffering from anaemia consistently score lower in mental and physical development tests. The depletion of cellular iron to a point that causes anaemia may cause an insult which requires long term therapy to correct, and may put the child at a great disadvantage both mentally and physically for a term that extends beyond the time frame of the insult.

1.3 Benefits and Risks to Iron Supplementation

Vitamin D is the only nutrient currently recommended for supplementation in infants within the first year of life (Canadian Pediatric Society, 1998). While any wide spread supplementation recommendation has to be debated and the benefits must far outweigh the risks for the target population there does appear to be a case for iron supplementation of exclusively breastfed

infants. The potential risks of iron supplementation are oxidative damage to the gastrointestinal tract, unfavourable microbial colonization of the gut, diarrhoea or constipation, impaired mineral absorption and hereditary hemochromatosis. While these potential risks of supplementation are serious there are also benefits to iron supplementation. The problem of iron deficiency in infants has been described as a "tragedy", given that it is easily identifiable and treatable yet it goes undetected in many infants until it reaches the late stages demonstrating anaemia (Buchanan, 1999). If a portion greater then 5% of a population suffers from a health related problem it rates as a public health concern (WHO, 2001). Since 10 – 28% of exclusively breast fed infants suffer from IDA the problem should rate as a moderate public health concern (WHO, 2001). Yet it is not truly recognized as a major concern for parents who choose to exclusively breast-feed.

It is known that the iron content of human breast milk is low compared to the infants metabolic need and decreases over the course of lactation (Siimes et al, 1979; Fransson et al, 1984; Fomon, 1993; Lönnerdal and Hernell, 1994). Given the metabolic need for iron of infants through the first year of life and the inability to increase breast milk iron concentrations through maternal dietary intervention the argument for supplementation becomes stronger (Lönnerdal, 1986; Arnaud et al, 1993; Fomon, 1993; Zavaleta et al, 1995).

There is hesitation in recommending iron supplementation for infants because infants not requiring additional iron may develop complications. There is a proportion of the population who suffer from hemochromatosis, a metabolic disorder caused by a mutation in the HFE gene that controls iron uptake in the gastrointestinal tract and can lead to dangerous levels of circulating and stored iron (Beutler et al, 2001). This disorder is a rare metabolic disorder but does effect 3% of the population as the homozygous form and 2 – 24% as the heterozygous form (Beutler et al,

2001). Infants with this disorder would be at a higher risk of oxidative damage (liver damage, diabetes mellitus, cardiomyopathy and arthritis) from the increased circulating iron caused by even the mild iron supplementation chosen for the present study (7.5 mg $FeSO_4$/day).

Potential changes to the micorflora of the infant gut have been described as a potential problem with iron supplementation. The intestinal microflora of the infant gut will vary according to geographic location and socio-economic status but may also be altered by dietary factors (Lundquist et al, 1985). Favourable colonization may not occur due to treatment with antibiotics and the presence of high iron in the diet may lead to unfavourable bacterial colonization and diarrhoea (Hall et al, 1989). While there is a difference in the microflora of breastfed infants compared to those that are fed a high iron formula there does not appear to be enough evidence to suggest that the presence of iron or the difference in gastrointestinal microflora is associated with any increase in diarrhoea (Scariati et al, 1997; Orrhage and Nord, 1999).

One risk associated with iron supplementation that has not been clearly elucidated is impairment in the absorption of other minerals. Iron supplementation at a high level has been shown to inhibit zinc absorption in adults nearly 50% (O'Brien et al, 2000). The addition of zinc to the supplementation regime does not counteract the negative effect of iron on zinc absorption (O'Brien et al, 2000). The choice of weaning foods may exacerbate this effect as was shown in a cohort of Swedish infants (Persson et al, 1998). The effects of low iron supplementation on zinc absorption have not been evaluated to the knowledge of the author. Other minerals could potentially be affected by iron supplementation given similar transport pathways in the intestine but these have not been investigated.

A further potential risk of iron supplementation in infants is the possibility of oxidative damage to tissue. Free radical mediated tissue injury is becoming an increasingly widespread concern (Halliwell, 1994). Administering supplemental iron poses some level of risk if the infant's antioxidant capabilities are unable to cope with the added stress. It has been clearly demonstrated that individuals consuming diets high in lipids will produce correspondingly increased levels of free radicals (Erhardt, 1997). Infants who are exclusively breastfed receive the majority of their calories from lipids, as high as 60% putting them at risk for having habitually higher levels of free radicals in the gastrointestinal tract (Fomon, 1993). Animal studies have shown prolonged exposure to high levels of iron will increase lipid peroxidation in the intestinal mucosa and possibly affect cell proliferation (Lund, 1998; Lund, 2001). In adult human studies, it has been shown that supplementary iron at a level of 19 mg per day was enough to cause increased free radical production in the feces of the volunteers (Lund, 1999). There was no indication that the increase in free radical production in the feces had any deleterious effects on the volunteers. No gastrointestinal problems were reported with a low level of iron supplementation in infants in a pilot study at the University of Iowa investigating the iron status with iron supplementation at 7.0 mg iron per day (Ziegler, abstract).

1.4 Focus of Study

Samuel Fomon has been one of the first to recommend iron supplementation to exclusively breastfed infants (Fomon, 2001). IDA challenges between 10 – 28% of breastfed infants but the condition is both avoidable and preventable. It is reasonable to assume that mild iron supplementation between 1 and 6-months of age for healthy, term, exclusively breastfed infants will improve iron status in the first year of life. Mild iron supplementation should not

aggravate normal physiological iron metabolism nor should it pose any gastrointestinal challenge to the infants.

The study presented in this manuscript focused on the haematological benefits of 7.5 mg per day of iron supplementation over the first 6 months of life. This study also assessed the dietary intake of infants during this period - including the iron concentration of human milk along with the antioxidant responses to iron supplementation and a pilot study focusing on the free radical generating potential of infant feces during supplementation. The present study used an *intent to treat protocol*, where all infants enrolled were included in data analysis irrespective of supplement compliance provided that samples were collected at all clinics. This protocol was chosen to ensure results reflected the general population, accounting for the full range of prescription compliance.

The study attempts prevent depletion of iron store in infants between 1 – 6 months of age. The design is unique in this respect as most previous studies of ID and IDA in infants through the first year of life focus on treatment after depletion of stores has occurred. Therefore, the first hypothesis of this study is, mild iron supplementation of exclusively breastfed infants between 1 – 6 months at a level of 7.5 mg iron per day will have an appreciable effect by 6 months on iron status as measured by haemoglobin, hematocrit, MCV and plasma ferritin compared with a control placebo group. The objective of the first hypothesis is to compare the haematological data of the study and placebo groups, identifying any benefits or negative aspects to iron supplementation in this population. Critics of recommending supplements to breastfed infants, even those at risk of developing IDA may suggest that there are potential hazards to providing elemental iron to this population. Iron is a powerful oxidizing agent and has to potential to cause oxidative damage to organisms in high concentrations. To address this concern the level of iron supplementation was

set at half the amount an infant of the same age but fed commercial formulas receive. This level should prove to be safe. The second hypothesis of the study is there will be no negative oxidative responses will result from this level of iron supplementation. To prove this hypothesis the study will assess the antioxidant status of the infants at 1 month for a baseline and again a 3.5 and 6 months using infant plasma FRAP and erythrocyte catalase and SOD as indicators and a pilot study will be conducted to examine the free radical generating capacity of the infants' feces related to iron concentration in feces.

Central to the study is the argument of whether or not human breast milk provides adequate amount of iron for the infant during the first 6 months of life. There has been a wide range of breast milk iron concentrations published over the last 4 decades. If the calculations of iron requirement of Fomon (1993) are valid some of these published ranges of iron concentration appear to leave the infant short of the required intakes until solid, iron rich foods are introduced to the diet. The last hypothesis of the study is the iron concentration of human breast milk is actually at the lower end of the published range and method sensitivity will play a part in the reported value. To investigate this possibility we determined the concentration of iron in human breast milk from a sub-set of mothers enrolled in the study, comparing common methods for the determination of iron in biological fluids.

This study will contribute to the area of infant iron nutrition by providing answers to some key questions currently in the literature on this subject. If mild supplementation in deed does prevent the depletion of iron stores of infants', ages 1 – 6 months, then we need to develop a tool to distinguish those infants who will develop IDA from those who will not. It is not a goal of this study to recommend wide spread iron supplementation of all breastfed infants but rather to

determine if IDA can be prevented during the critical periods of infant growth that occurs in the first 6 months of life. If no adverse gastrointestinal side effects or change in antioxidant status it should be accepted that treatment at this concentration of iron is safe. While an infant may not need 7.5 mg/day to prevent IDA or depletion of iron stores, the study should provide a clear starting point to determining the amount required. The results from the breast milk analysis will answer the question of whether or not infants (from birth to 6 months) are meeting their metabolic requirement for iron. Together with the data on the effectiveness of iron supplementation and its safety the breast milk iron analysis will help to confirm if there is a proportion of the breastfed population that requires iron supplementation from birth until the appropriate iron rich solid foods are introduced. This study examines IDA from a unique perspective; preventative opposed to reactive, as has been investigated in past studies on the subject. The data and analysis from this study will make a significant contribution to the area of infant iron nutrition.

Chapter 2: Methods and Materials

2.1 Study Summary

This analysis was designated to be an intermediate biochemical analysis of a larger study investigating the haematological and cognitive benefits of supplementing exclusively breast-fed infants from 1 month to 6 months of life. Figure 2.1 illustrates the overall study design and summarizes the key factors of the original study design. Figure 2.2 illustrates the aspects of this intermediate biochemical analysis.

2.2 Subject Selection

Subjects were recruited by our research nurse in the days following birth once suitable candidates were identified by the medical staff at the Grace General Obstetrics Unit and Obstetrics/Gynaecology Unit of the Health Sciences Centre. All subjects were healthy, term, Caucasian and exclusively breast-fed infants. Exclusive breast feeding for the purposes of this analysis and the larger study is defined as > 90% of all nutrients an infant receives from breast feeding. Parents were asked to attempt exclusive breast feeding until after the 3.5 month clinic and if possible until after the 6-month clinic. All parents were asked to comply with the recommendations of the Canadian Paediatric Society (1998) for nutrition in healthy term infants. All parents were informed verbally and in writing as to the study protocol.

For the original study, design there was a requirement for 220 total infants, which would allow for 100 infants per group assuming 10% attrition. This number of infants would be required to demonstrate a 10% difference between the groups using the BSID psychological tests. When the intermediate analysis was undertaken, 20 infants per study group (supplemented and placebo) had been seen at the 6-month clinic. Not all infants were able to provide enough blood sample for

all analysis. It was decided that due to funding and timing restraints the analysis would be carried out at this point with this number of subjects despite the fact there may not be enough subjects to detect differences with clinically significant power. The number of subjects used for each test or assay is presented in the results section along with the corresponding observed power using this sample.

2.3 Dietary Records

Dietary records were supplied to the parents several days before each clinic with instructions to record all food intake and amounts for study subjects. These records were collected by clinic staff and entered into a nutrient database. Average amounts of breast milk consumed at 1 and 3.5 month was assumed to be 750 ml/day and 650 ml/day at 6 months (Heinig, 1993, Dewey, 2001).

2.4 Fecal Samples

Fecal samples from infants were collected by parents (using acid washed plastic disposable spoons and acid washed plastic bags provided by the investigators) and brought to healthy baby clinics. Samples were frozen at -20 degrees Celsius until analysis. Some parents produced frozen samples while others brought fresh feces to clinics. The investigators have considered this and realize that fecal analysis is limited to aspects which would be unaffected by this handling. Analysis was limited to fecal iron content and MSA production. Both were a function of iron content and our interpretation is limited to this respect.

Figure 2.1 Complete study summary flow chart.

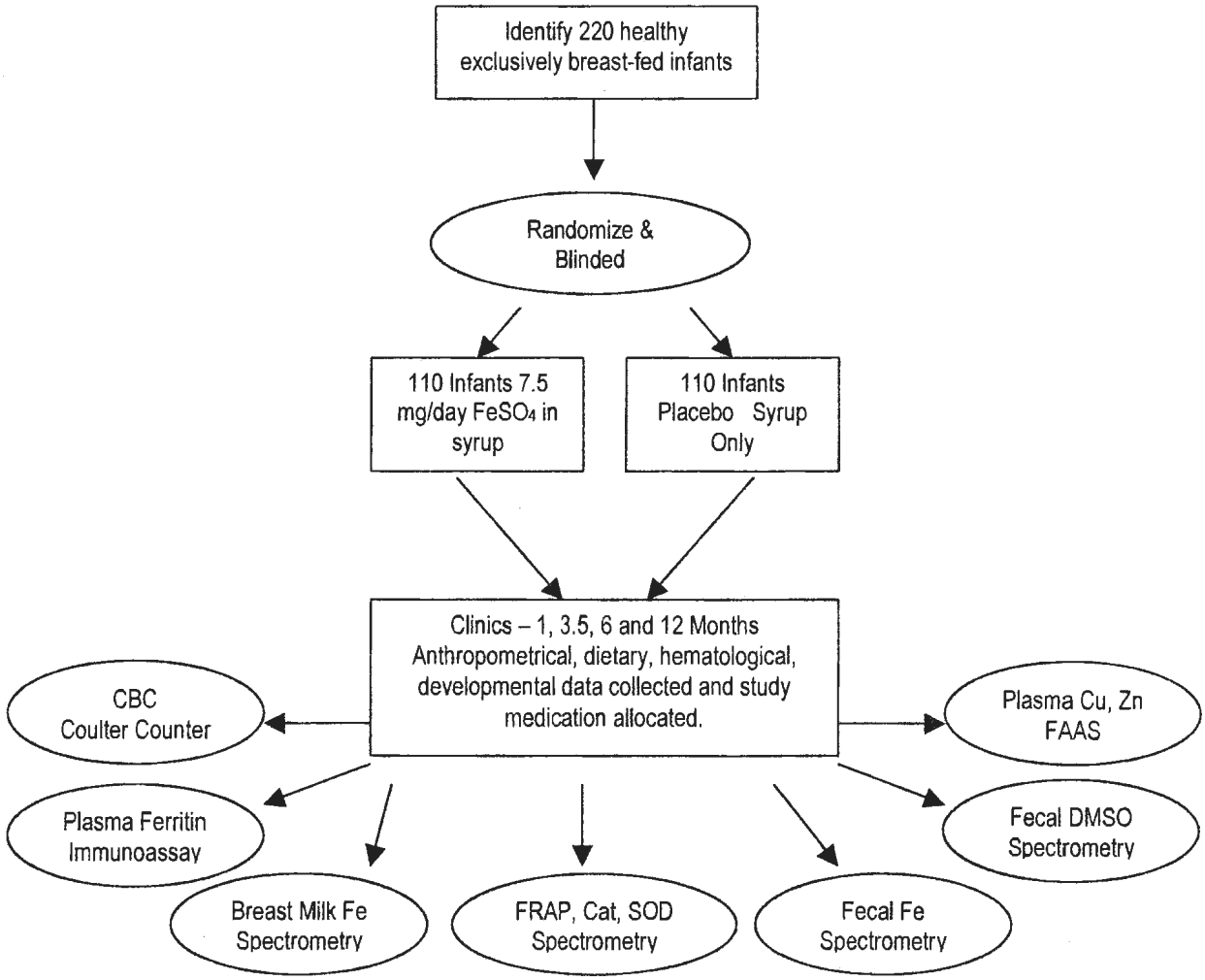
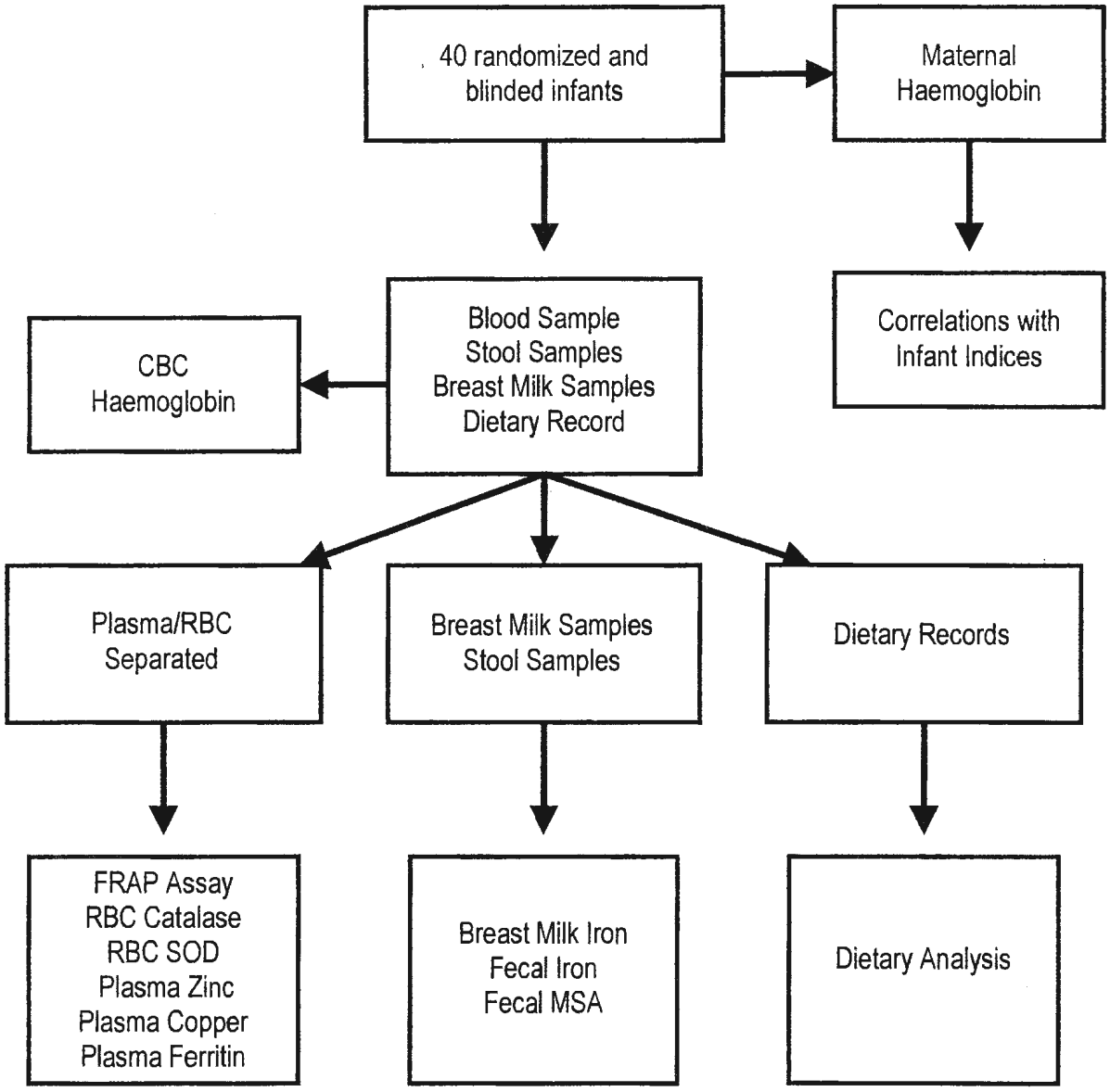

Identify 220 healthy exclusively breast-fed infants
Randomize & Blinded
110 Infants 7.5 mg/day $FeSO_4$ in syrup
110 Infants Placebo Syrup Only
Clinics – 1, 3.5, 6 and 12 Months Anthropometrical, dietary, hematological, developmental data collected and study medication allocated.
CBC Coulter Counter
Plasma Cu, Zn FAAS
Plasma Ferritin Immunoassay
Fecal DMSO Spectrometry
Breast Milk Fe Spectrometry
FRAP, Cat, SOD Spectrometry
Fecal Fe Spectrometry

Figure 2.2 Intermediate biochemical analysis flow chart.


40 randomized and blinded infants
Maternal Haemoglobin
Blood Sample
Stool Samples
Breast Milk Samples
Dietary Record
CBC
Haemoglobin
Correlations with Infant Indices
Plasma/RBC Separated
Breast Milk Samples
Stool Samples
Dietary Records
FRAP Assay
RBC Catalase
RBC SOD
Plasma Zinc
Plasma Copper
Plasma Ferritin
Breast Milk Iron
Fecal Iron
Fecal MSA
Dietary Analysis

2.5 Blood Sampling and Processing

2.5.1 Blood Sampling

Blood was drawn by technician using one of two methods. A butterfly catheter and 3 ml syringe was used to draw blood from the back of the hand in infants 1 month of age and transferred to a Becton Dickenson sodium heparin sprayed vacutainers tube. Infants 3.5 months of age and older had blood draw by brachial venipuncture using the previously described vacutainers. After CBC and haemoglobin determination samples were aliquoted to acid washed 1.8 ml Eppendorf tubes and were then centrifuged at 5000 rpm for 10 minutes to separate the sample into fractions. The plasma layer was removed and aliquoted into 100 μl capped tubes. The remaining red cell layer was washed 3 times by adding approximately an equal amount of isotonic saline, inverted and centrifuged at 5000 rpm. Washed, packed red blood cells were aliquoted into acid washed 100 μl capped tubes or 1.8 ml Eppendorf tubes for later analysis. All blood samples were stored under nitrogen at –70 degrees Celsius until analyzed.

2.5.2 Haemoglobin, Hematocrit and Mean Corpuscular Volume Determination

Forty-four μL of whole blood was used for the determination Hgb, Hct and MCV by automated Coulter Counter Model Sx. Hgb was determined by lysing the cells in a portion of the aliquot to release the haemoglobin into a dilute Drabkin's reagent (1:500) and measuring spectrophotometrically the absorbance at 540 nm. The Coulter principle is the basis for cell counting and sizing in the Coulter Counter. Measurable changes in resistance correspond to cell size and numbers when nonconductive particles (cells) are passed through a precisely controlled aperture in an electrolytic solution. The method is the standard in cell counting and sizing protocols. Hct (packed cell volume; pcv) was determined by calculations based on the total cell count of the sample. MCV is automatically determined by the following equation:

$$MCV = \frac{PCV}{RBC} X\ 10$$

2.5.3 Plasma Ferritin Determination

Plasma ferritin was determined using the IM_x System microparticle enzyme immunoassay (Abbott Diagnostics, Chicago, IL; #2219-20). Solid phase rabbit polyclonal antibodies for human ferritin and soluble phase mouse monoclonal antibodies for human ferritin were used in this sandwich assay. The resulting florescence was measured on a micro plate reader according to the standards contained in kit.

2.5.4 Criteria for Iron Status

Iron status was measured using the criteria outlined in Table 2.1. These criteria were determined by examining those used by other researchers and the reported normal values for infants at these ages (Domellöf et al, 2001; Moe, 1965; Persson et al, 2001; Pizarro et al, 1991; Arvas et al, 2000; WHO, 2001). In summary, an infant was regarded as iron sufficient if the haemoglobin was greater than 110 g/L and plasma ferritin was greater than 12 μg/L. Iron deficiency without anaemia had two classifications: 1) all haematological parameters normal with plasma ferritin less than of equal to 12 μg/L; 2) plasma ferritin greater than 12 μg/L but haemoglobin less than or equal to 110 g/L with one of either hematocrit or MCV below the cut-off value for that parameter. Iron deficiency anaemia was classified by two sets of criteria also: 1) haemoglobin and plasma ferritin less then the specified cut-off values; 2) normal plasma ferritin but haemoglobin, hematocrit and MCV below the cut-off values. Incidences of each ID and IDA was recorded at each clinic time.

2.6 Breast Milk Collection and Analysis

2.6.1 Breast Milk Collection and Storage

Mothers in the study were asked to provide a sample of milk for mineral analysis if possible at each clinic. Each mother expressed the milk manually and collected it in 10 ml acid washed polypropylene capped test tubes. The test tubes were transported on ice to the laboratory where they were aliquoted to 1.5 ml acid washed Eppendorf tubes and stored at –20 degrees Celsius until analyzed.

2.6.2 Breast Milk Iron by Diluted Direct Graphite Furnace Atomic Absorption Spectophotometry

The direct analysis was carried out in a Perkin-Elmer 2380 Atomic Absorption Spectrophotometer with a Perkin-Elmer Graphite Furnace and Perkin-Elmer HGA-300 Programmer according to Liang and associates (1989) with modifications for breast milk. Frozen samples were prepared by placing in a 37 degrees Celsius water bath and allowed to come to temperature. The samples were then taken from the water bath and diluted 20X using 1% HNO_3 to eliminate the matrix effects and obtain an iron concentration that fell along the range of standards used (0.10 – 0.60 μg/ml). Ten micro litres of sample was then injected into the GFAAS in triplicate and was preceded and followed by blanks of 1% HNO_3. Certified material, *AQCS A-11 trace elements in milk powder* (IAEA, Austria), was prepared at a theoretical concentration of 0.47 μg Fe/ml with ultra pure water and treated in the same fashion as the breast milk samples to ensure accuracy. The A-11 iron concentration was determined to be 0.40 μg Fe/ml.

Table 2.1 Study Criteria for Iron Status

Table 2.1

Haematological Index	Iron Sufficient	Iron Deficient		Iron Deficiency Anaemia		
Haemoglobin Count	> 110 g/L	> 110 g/L	≤ 110 g/L plus either one	≤ 110 g/L		≤ 110 g/L plus
Hematocrit	> 0.330	> 0.330	or < 0.330 or	> 0.330	or	< 0.330 and
MCV	> 75 fL	> 75 fL	< 75 fL when	> 75 fL		< 75 fL when
Serum Ferritin	> 12 μg/L	≤ 12 μg/L	> 12 μg/L	≤ 12 μg/L		> 12 μg/L

2.6.3 Breast Milk Iron by Method of Addition Graphite Furnace Atomic Absorption Spectrophotometry

Frozen samples were placed in a 37 degrees Celsius water bath and allowed to reach that temperature. Fifty micro litres of breast milk were aliquoted into each of three acid washed Eppendorf tubes and diluted 20X with 1% HNO_3, 10 ng Fe/ml in 1% HNO_3 and 20 ng Fe/ml in 1% HNO_3. The samples were then injected in triplicate and absorbance obtained. Blanks and certified controls were injected after 5 – 8 samples to ensure accuracy. Certified material, *AQCS A-11 trace elements in milk powder* (IAEA, Austria), was prepared at a theoretical concentration of 0.47 μg Fe/ml with ultra pure water and treated in the same fashion as the breast milk samples to ensure accuracy. The A-11 iron concentration was determined to be 0.40 μg Fe/ml. Results were analyzed graphically for the concentrations of iron in the diluted samples and then corrected by multiplying by the dilution factor.

2.6.4 Breast Milk Iron by Flame Atomic Absorption Spectophotometry

The Perkin-Elmer FAAS (flame atomic absorption spectrophotometer) was optimized and set to measure concentration of iron using wavelength λ= 248.3 nm and internally set references. The spectrophotometer was set to calculate the concentration based on the mean concentration of a triplicate set of samples. The standard of 5 ppm Fe was made in the same manner already stated. The samples were prepared for the flame analysis in a 5-day digestion at 70 degrees Celsius in concentrated ultra pure nitric acid. Five hundred micro litres of human breast milk was place in a Teflon tube with 1000 μl of ultra pure HNO_3. The samples were placed on a hotplate and "cooked" for 5 days at 70 degrees Celsius. When the digestion was complete, heating the Teflon tubes to 150 degrees Celsius rapidly evaporated off the acid. One thousand microlitres of ultra pure H_2O was added to wash the samples. The samples were heated until they were dry.

The dried samples were brought up to a final volume of 1000 µl with 0.2 N ultra pure HNO_3 (Alkanani et al, 1994). The samples were aspirated into the FAAS and concentrations were calculated. Certified material, *AQCS A-11 trace elements in milk powder* (IAEA, Austria), was prepared at a theoretical concentration of 0.47 µg Fe/ml with ultra pure water and treated in the same fashion as the breast milk samples to ensure accuracy. The A-11 iron concentration was determined to be 0.44 µg Fe/ml.

2.7 Assays for Oxidative Status

2.7.1 Ferric Reducing Ability of Plasma Assay (FRAP Assay)

Aim: To assess the ability of biological samples to reduce ferric iron to the ferrous state using this assay as a measure of the total antioxidant ability of the sample.

Principle: Low pH and the presence of any reductant with a redox potential favourable for the reduction of Fe^{III}-TPTZ to Fe^{II}-TPTZ (easily reduced oxidant) will produces an intense blue colour change. The Fe^{II}-TPTZ colour complex can be measured by spectrophotometer at a maximal wavelength of 593 nm. The Δ absorbance over 6 minutes at room temperature can be compared to standards of ferrous iron or pure antioxidants.

Method: 0.3 M acetic acid buffer (pH 3.6) $C_2H_3NaO_2 \bullet 3\ H_2O$) was mixed with 10 mM 2, 4, 6-tripyridyl-s-triazine (TPTZ) in 40 mM HCl and 20 mM ferric chloride ($FeCl_2 \bullet 6\ H_2O$) in a 10:1:1 ratio respectively (reaction solution). Fifty microlitres of infant plasma were added to 1.5 ml of reaction solution and allowed to react for 6 minutes at room temperature. Absorbance of the resulting blue solution was measured spectrophotometrically at a wavelength of 593nm for maximal absorption. Once an average absorbance was attained, the FRAP value could be calculated using the following formula and the results from a standard curve of ascorbic acid between 100 µM – 1000 µM (Benzie and Strain, 1996). The FRAP value of the standard is

determined by its stoichiometric relationship to TPTZ. For example any species of standard that can donate one electron will have a FRAP value equal to its molar concentration (100 μM $Fe^{II}SO_4$ = 100 μM FRAP value). If the standard can donate more than one electron the FRAP value will be increased by that stoichiometric factor (100 μM ascorbic acid = 200 μM FRAP) (Benzie and Strain, 1996).

Calculations:

Equation

$$\frac{\text{6 minute } \Delta A_{593nm} \text{ Sample}}{\text{6 minute } \Delta A_{593nm} \text{ Standard}} \quad \text{X FRAP value of Standard (μM)} \quad = \text{FRAP value of Sample (μM)}$$

2.7.2 Superoxide Dismutase Activity Assay

Aim: To determine the superoxide dismutase activity from red blood cells of infants receiving either supplemental iron or a placebo at 1, 3.5 and 6 months of age.

Principle: SOD activity is measured by the ability of the enzyme to prevent the oxidation of an indicator substance (cytochrome c) by catalyzing the following reaction:

$$2O_2^{\bullet-} + 2H^+ \longrightarrow H_2O_2 + O_2 \qquad \text{Reaction 2.1}$$

In the case of this assay the SOD will inhibit the $O_2^{\bullet-}$ reduction of cytochrome c by removing it from the medium (reaction 2.2):

$$Cyt(Fe^{III}) + O_2^{\bullet-} \longrightarrow Cyt(Fe^{II}) + O_2 \qquad \text{Reaction 2.2}$$

The assay is carried out at pH 10 to increase the sensitivity and remove the interference usually seen at pH 7.8.

Method: The method is a variation of those summarized by Fridovich (1985) for the quantification of SOD by the xanthine/xanthine oxidase/cytochrome c methods. This procedure is carried out at pH 10.0 as opposed to physiologic pH to increase the sensitivity of the assay.

Enzyme was extracted by adding 100 μl packed read blood cells to 1 ml of cold distilled H_2O and 400 μl of cold extraction solution (3:1 ethanol – chloroform v/v). Suspension was vortexed for 20 – 30 seconds and centrifuged at 14000 rpm for 5 –10 minutes. Supernant was removed and diluted 1:30 v/v with distilled H_2O and kept in ice or frozen at – 20 degrees Celsius until analysis. Xanthine oxidase working reagent was prepared by mixing 80 μl of xanthine oxidase (Sigma Chemicals) to 2.0 ml of sodium carbonate buffer and kept in ice until analysis was carried out. Cytochrome c working reagent is prepared by adding 2.0 ml of ferricytochrome c solution (0.06192g ferricytochrome c in 10ml sodium carbonate buffer) to 5 ml of xanthine solution (0.01521g xanthine in 100 ml sodium carbonate buffer) and kept at 30 degrees Celsius during assay. SOD activity is measured spectrophotometrically at 405 nm by adding 100 μl of sample to 2.0 ml of cytochrome c working reagent in a cuvette and mixed with 60 μl of xanthine oxidase, followed on a chart recorder. All samples were assayed in triplicate. Hgb concentrations of samples determined as per section 2.4.2.

Calculations: SOD activity is determined from standard curve of 0.25 – 10 units SOD/ml of bovine serum (Sigma Chemical) and expressed as units SOD per mg Hgb. One unit of SOD will inhibit the rate of reduction of cytochrome c by 50% in xanthine/xanthine oxidase system under these reaction conditions and volumes.

2.7.3 Assay of Catalase Activity

Aim: To determine the specific activity of red blood cell catalase from infants receiving either iron supplements or placebo at age 1, 3.5 and 6 months of age based on an adaptation of the method outlined by Beers and Sizer (1952).

Principle: The specific activity is expressed as μmol H_2O_2 consumed/min/mg of total protein at 25 degrees Celsius. RBC catalase is measured by spectrophotometer and the reaction followed by chart recorder as it decomposes excess H_2O_2.

Method: Ten microlitres of packed red cells were diluted with 20 ml of phosphate buffer (5 mmol; pH 7.0) in 50 ml falcon tubes. The spectrophotometer was set to measure absorbance at wavelength 240 nm. Absorbance was measured in quartz cuvettes against a reference cuvette containing 1 ml phosphate buffer and 2 ml of sample. One millilitre of H_2O_2 was added to the cuvette containing 2 ml sample, quickly mixed by stirrer and placed in the spectrophotometer. Absorbance was measured on a chart recorder (1 volt; chart speed = 1 mm/second over the course of 1 minute). All samples were measured in triplicate when possible.

Calculation: Specific activity of catalase at 25 degrees Celsius is defined as μmol of H_2O_2 consumed per minute per mg of protein in sample. The following calculation is used to determine the specific activity of catalase once protein concentration is estimated.

$$\text{SA (units/mg protein)} = \frac{\Delta A_{240nm}^{\text{min-1}} \times 1000}{43.6 \times \frac{(\text{mg protein} \times 2/3)}{\text{ml reaction solution}}}$$

2.7.4 Protein Estimation by Lowry Assay Method

Aim: To determine the concentration (mg/ml) of protein in packed red cell working sample of the specific activity of catalase assay using Folin-Ciocalteu reagent according to Lowry et al (1951).

Method: Two solutions were prepared as the working reagents for this assay. Solution 1 contained 6% sodium carbonate (w/v), 4% sodium tartrate (w/v) and 2% copper sulphate (w/v) mixed 48:1:1 respectively. The Folin-Ciocalteu reagent was diluted 2:1 (v/v) with distilled H_2O.

Bovine serum albumin (Sigma Chemicals) of concentration 1 – 100 μg was used as standard for this assay. Reference blanks are prepared by adding 900 μl of distilled H2O to 100 μl of 1 M NaOH to borosilicate glass tubes. Samples were prepared by adding 600 μl distilled H2O, 300 μl of diluted packed red cells and 100 μl of 1 M NaOH borosilicate glass tubes. Spectrophotometer was set to measure absorbance at 750 nm. The remainder of the procedure was carried out under timed conditions for accuracy of estimation. At exactly 10-second intervals, 1 ml of Solution 1 was added to each test tube and allowed to stand for exactly 10 minutes at room temperature. At the end of ten minutes, 100 μl of Solution 2 was added at 10-second intervals. Samples were left to stand for 30 minutes at room temperature and read spectrophotometrically at 750nm. All tubes were read within 10 minutes.

Calculations: Protein was determined from standard curve.

2.7.5 Methanesulfinic Acid Assay

Aim: To compare free radical production in stool samples of infants receiving either supplemental iron or a placebo.

Principle: Methanesulfinic acid is the end-product used in this colorimetric assay as an indication of hydroxyl radical production. The assay was used to identify differences between treatment groups, not to quantify absolute levels of hydroxyl radical production.

Method: Due to the variable moisture content of infant stool (solid stool to diarrhoea), samples were thawed, dried at 30 degrees Celsius and 87% moisture was added to achieve consistent level of moisture. The assay was carried out by our modifications of the methods of Lund et al (1999) and Babbs and Gale (1987). Samples were weighed and homogenized, added to 50 ml falcon tubes and mixed with an equal amount (w/w) of distilled H_2O, then frozen until batch

analysis. One to two grams of the fecal samples (if available) were incubated overnight (18+ hours) in Tris buffered saline (pH 7.0) containing 5% dimethyl sulfoxide (0.7 mol/L), glucose (0.1% w/v) and Na_2EDTA (50 mmol/L) at 37 degrees Celsius. After incubation, samples were centrifuged at 900 X g for 10 minutes and supernant removed. Proteins were removed from supernate by lowering the pH to 1.0 using an appropriate amount of 12 M HCl, allowed to stand for 10 minutes and centrifuged at 900 X g after which the pH of the supernatent was returned to 7.0 using an appropriate amount of NaOH. Supernant was stored at –20 degrees Celsius until batch analysis was completed. Standards of methanesulfinic acid were prepared (10 – 75 mM) using incubation solution as solvent and treated in the same fashion as samples from this point forward. Two-millilitre aliquots of sample were mixed well with H_2SO_4 (200µl; 1 M) followed by 4 ml of 1-butanol. Sample was vortexed and the upper phase was transferred to another borosilicate tube and mixed well with 2 ml sodium acetate buffer (0.5M; pH 5.0). Sample was centrifuged at 500 X g for 3 minutes and 1.8 ml of lower phase was transferred to a new borosilicate tube. Two hundred microlitres of HCl was added to lower pH (pH 2.5) and 200 µl of Fast blue BB Salt (0.03 mM) was added, mixed and left in the dark for 10 minutes to allow for colour development. Aliquots of toluene/1-butanol (1.5-ml; 3:1) was added to each tube and mixed for 120 seconds. Samples were centrifuged (600 x g; 3 min) upper phase was removed and washed with 1-butanol saturated H_2O. One millilitre of pyridine was added to the samples to stabilize the colour until they could be read on the spectrophotometer. Samples were then read on a Spectrum 3000 spectrophotometer at wavelength 420 nm for maximal absorption.

Calculation: Linear equation produced from standards was used to determine the amount of methanesulfinic acid produced in fecal samples. Results are expressed as mmol/g wet weight feces.

2.8 Iron Content of Infant Fecal Samples

2.8.1 Fecal Iron by Flame Atomic Absorption Spectrophotometry

Aim: To determine the amount of iron present in fecal samples of subjects receiving iron supplements or placebo.

Principle: Fecal samples were ashed and total iron present in feces was determined by flame atomic absorption spectrophotometry. Results are an indication of total iron loss due to fecal excretion including iron from dietary sources and occult iron losses.

Method: Approximately 0.3 g of stool was transferred to a pre-weighed 10 ml beaker and wet weight of sample was recorded. Samples were dried at 65 degrees Celsius until a consistent weight was achieved. Beakers containing dried sample were transferred to a muffle furnace and dry ashed at 450 degrees Celsius for 48 hours. After ashing was complete, samples were allowed to cool to room temperature for 1 hour. Samples appeared as a white-grey powder. Concentrated HNO_3 (5 – 7 drops from a pasture pipette) was added to the ashed samples and left to stand for 30 minutes. HNO_3 (5 ml; 1 M) was added to the beakers containing the samples and shaken lightly the ash dissolved. Contents were decanted into a 10 ml volumetric flask and the beaker was rinsed with small amounts of 1 M HNO_3 bringing the volume up to the 10 ml mark. Two hundred microlitres of the solution were pipetted to acid washed Eppendorf tubes and brought up to 1 ml with ultra-pure water. Samples were run on the FAAS and compared with a standard curve previously prepared (1 – 4 ppm or 1.0 – 4.0 μg/ml). The Perkin-Elmer FAAS (flame atomic absorption spectrophotometer) was optimized and set to measure concentration of iron using wavelength λ= 248.3 nm and internally set references. The spectrophotometer was set to calculate the concentration based on the mean concentration of a triplicate set of samples. The samples were then aspirated into the FAAS and concentrations were calculated. *NBS Trace*

Elements In Water was used as a certified value for accuracy and was within 10% of reported value for iron.

Calculation: Results are expressed in mg of iron per gram of dry feces. The following calculations are used to convert the concentrations determined by FAAS to these units.

- **FAAS Conc** x 5 = **CONC** (removes dilution factor)
- **CONC** x 10 = **Fe MASS** (amount of iron in specific amount of ash)
- [1/dry weigh of feces] x **Fe MASS** = Amount of Fe per gram of dry gram of infant feces

2.9 Plasma Copper and Zinc by Flame Atomic Absorption Spectrophotometer

2.9.1 Plasma Copper and Plasma Zinc by discrete Nebulization with FAAS

Aim: To determine the plasma copper and zinc concentrations of infants receiving either iron supplements or a placebo at 1, 3.5 and 6 months of age.

Principle: The use of discrete nebulization FAAS is preferred for an accurate determination of the plasma copper and zinc concentrations in infant plasma samples given the low blood volume obtained from infants in human investigation studies. Significantly, lower volumes of plasma are required for this determination as compared to conventional procedures for the determination of copper and zinc in biological samples.

Method: The method used for copper determination was first described by Makino and Takahara (1991). The Perkin-Elmer 2380 Atomic Absorption Spectrophotometer was set to the following specifications: copper hollow cathode lamp; zinc hollow cathode lamp; slit width 0.7mm; wavelength set to 324.8 nm (Cu) or 213.9 (Zn) for maximal absorption. All samples were analyzed in triplicate when possible. One hundred microlitres of 0.1 N HCl was added to 1.8 ml Eppendorf tubes followed by the addition of 10μl (Cu) or 20μl (Zn) of plasma. Tubes were vortexed for 3 – 5

seconds. Spectrophotometer was calibrated to determine concentrations between 0.2 μg/ml to 4.0 μg/ml for copper analysis and 0.2 μg/ml to 2.0 μg/ml for zinc. Appropriate reference check and certified standards were used to ensure accuracy. Samples were then aspirated slowly through a 70mm Teflon capillary tube. Concentrations were determined directly from the linear equation of the calibration standards.

2.10 Statistical Analysis

Continuous variables were analyzed by repeated measures ANOVA, using Bonferroni p-value adjustment and Levene's test of homogeneity of variance applied. Nonparametric data regarding the incidence of ID and IDA was analyzed by Chi Square. Differences within groups at different time points was determined by Bonferroni step wise comparisons when data was analyzed by treatment group. Pearson correlations and linear regression was used to determine relationships between several possible predictors of haematological predictors of iron stores. Paired t-test was used for analysis of the breast milk iron concentrations by method and independent t test was used to compare groups at 1 – 3.5 months. The level of significance chosen throughout the analysis was $p < 0.05$. Statistical power varied between variables measured however for haematological parameters a sample size of 20 is adequate to detect a 10% difference between groups with a predictive power of 80%.

Chapter 3: Results

3.1 Subject Anthropometric Data

No abnormalities were detected in the growth of the infant subjects in this study. All infants were compared to the normal healthy growth charts for infants. Head circumference, weight and length were normal for all infants irrespective of treatment group. Table 3.1 summarizes the weight, body length and head circumference of the infants separated by treatment group and age.

3.2 Dietary Intake Data and Problems with Study Medication

There were no irregularities found in the three-day dietary records obtained from the subjects' mothers between the two treatment groups. Total energy, protein, carbohydrate and fat were assessed in ensure consistency between infant diets. Dietary iron was assessed to ensure consistency in dietary intakes of iron (supplement not included). Dietary copper and zinc were assessed to compare to plasma levels and ensure consistency between groups. Dietary ascorbic acid was measured to ensure intake in each group was consistent, as ascorbic acid will increase absorption of dietary iron. Dietary ascorbic acid along with selenium were assessed and compared to antioxidant status. The intake of energy, protein, carbohydrate, fat, iron, copper, zinc, selenium and ascorbic acid were consistent between the two groups at 1, 3.5 and 6 months of age when analyzed by repeated measures ANOVA (Appendix A). No difference was observed between

Table 3.1 Anthropometrical Measurements of Two Study Groups Over Duration of Study

Table 3.1

Age	Weight (kg)		Length (cm)		Head Circumference (cm)	
	Supplement	Placebo	Supplement	Placebo	Supplement	Placebo
Birth	3.6 (0.6)	3.7 (0.5)	51.1 (1.8)	50.9 (1.7)	34.9 (1.2)	34.2 (2.7)
1 Month	4.6 (0.7)	4.8 (0.5)	53.9 (5.2)	55.3 (2.6)	37.9 (1.1)	38.4 (1.0)
3.5 Month	6.5 (0.9)	6.8 (0.9)	62.9 (2.1)	63.6 (1.9)	41.4 (0.9)	41.8 (1.1)
6 Month	7.8 (1.0)	8.2 (1.0)	67.9 (2.4)	68.2 (2.3)	43.5 (1.0)	44.2 (1.1)

Values are reported as mean (+/- standard deviation in parenthesis) N = 20 per group. No difference seen between groups at any clinic time as measured by repeated measures ANOVA.

groups in reported problems with taking study medication when analyzed by Chi Square (Appendix B).

3.3 Iron Status Determination from Haematological Data

Based on the definitions of iron deficiency and iron deficiency anaemia described in Chapter 2 (table 2.1) the following haematological data were collected and used to determine the rates of iron deficiency and iron deficiency anaemia found in the subjects by six months.

3.3.1 Haemoglobin, Hematocrit and MCV

Although baseline haemoglobin concentrations for subjects did not differ, by six months of age a difference was seen between the supplemented group and the placebo group when tested by repeated measures ANOVA (2 (groups) x 3 (time periods))($F(1, 38) = 6.89$; $p = 0.01$; figure 3.1) with an observed power of 0.73. The placebo group haemoglobin decreased significantly by 6 months compared to the 1-month baseline blood sample ($F(1, 19) = 5.31$; $p = 0.03$; figure 3.1). Baseline hematocrit (measured as percent red cells per total blood volume) between subjects also differed by six months when tested by repeated measures ANOVA (2 (groups) x 3 (time periods))($F(1, 38) = 5.86$; $p = 0.02$; figure 3.3) with an observed power of 0.66. Mean corpuscular volume (MCV) of all infants dropped over the course of the study. This phenomenon is normal in infant development and was expected. However, a difference was seen in the MCV between the infants receiving supplement and those receiving placebo as early as 3.5 months ($F(1,38) = 5.86$; $p=0.02$; figure 3.3) with an observed power of 0.66.

Figure 3.1 Haemoglobin concentration of infants measured at 1, 3.5 and 6 months of age, receiving either supplemental iron (7.5 mg/day) or placebo. Placebo group declines over 6 months while the supplemented group maintains haemoglobin levels. Mean +/- SEM (error bars) p=0.01 at 6-months; N = 20 per group.

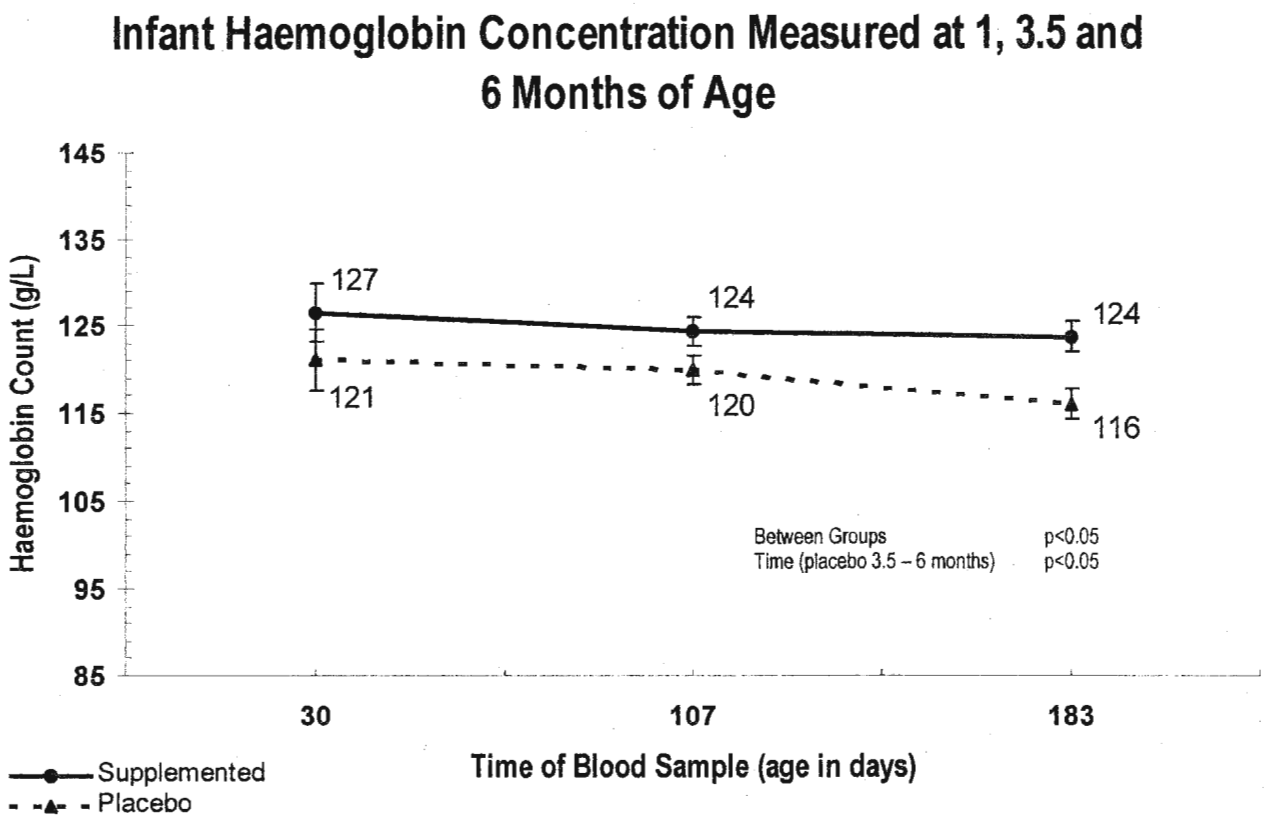


Figure 3.1 Haemoglobin concentration of infants measured at 1, 3.5 and 6 months of age, receiving either supplemental iron (7.5 mg/day) or placebo. Placebo group declines over 6 months while the supplemented group maintains haemoglobin levels. Mean +/- SEM (error bars) p=0.01 at 6-months; N = 20 per group.

Figure 3.2 Hematocrit of infants receiving iron supplements (7.5 mg/day) or a placebo measured at 1, 3.5 and 6 months of age. Mean +/- SEM (error bars) $p<0.02$ at 6 months; N = 20 per group.

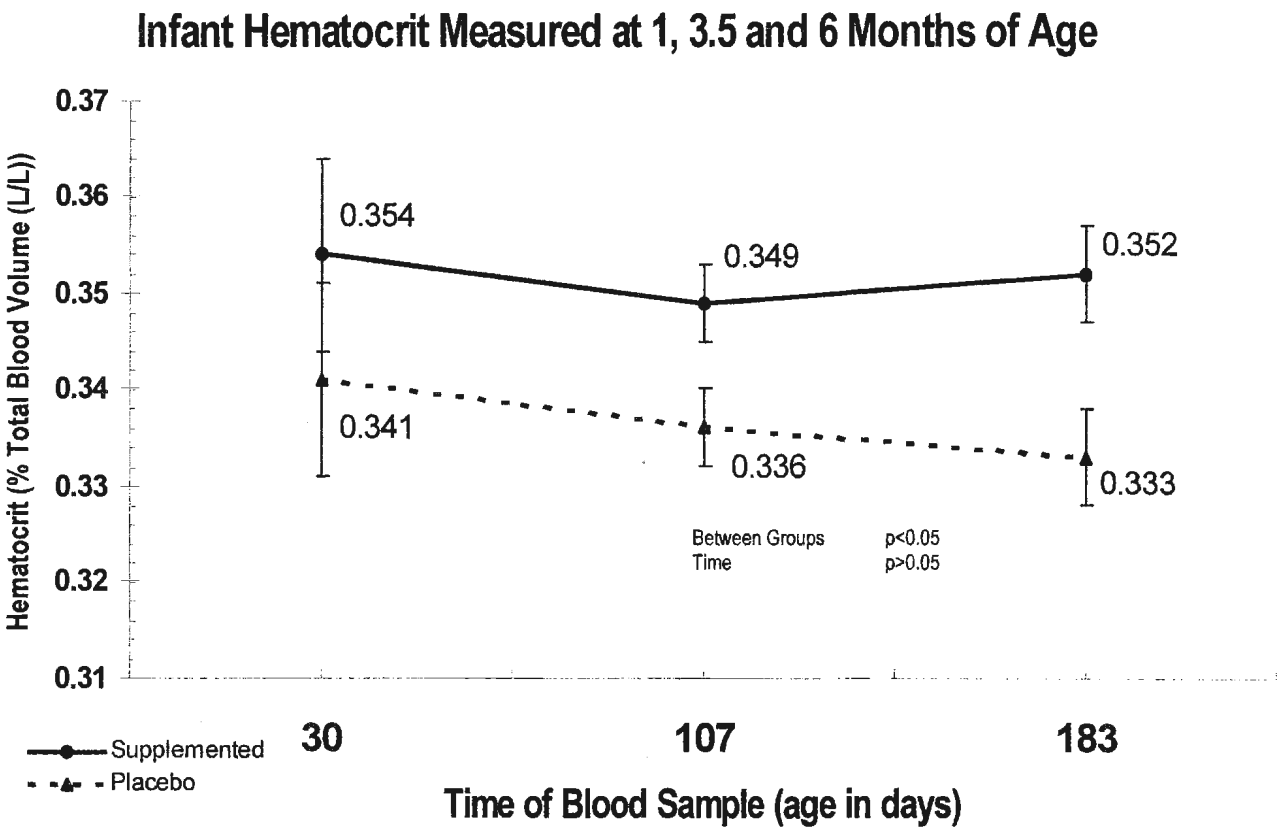


Figure 3.2 Hematocrit of infants receiving iron supplements (7.5 mg/day) or a placebo measured at 1, 3.5 and 6 months of age. Mean +/- SEM (error bars); N = 20 per group.

Figure 3.3 Mean corpuscular volume of infants receiving iron supplements (7.5 mg/day) or a placebo measured at 1, 3.5 and 6 months of age. Both groups decrease over time as is expected for MCV but the supplemented group averages larger red blood cells by 6-months Mean +/- SEM (error bars) $p<0.02$ at 6 months; N = 20 per group.

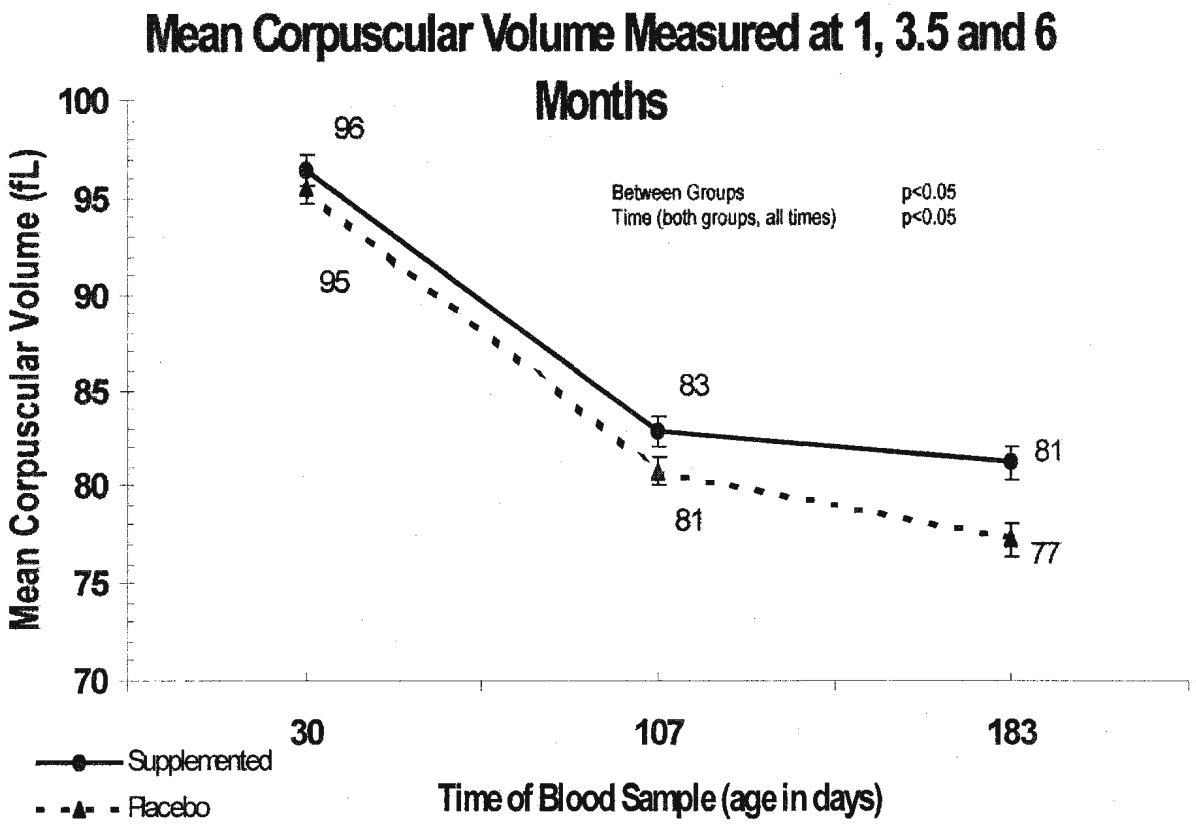


Figure 3.3 Mean corpuscular volume of infants receiving iron supplements (7.5 mg/day) or a placebo measured at 1, 3.5 and 6 months of age. Both groups decrease over time as is expected for MCV but the supplemented group averages larger red blood cells by 6-months Mean +/- SEM (error bars) $p<0.02$ at 6 months; N = 20 per group.

3.3.2 Plasma Ferritin

Plasma ferritin is often used as an indication of the iron stores available in humans. Plasma ferritin did not differ between the treatment and placebo group through the five-month protocol when analyzed by repeated measures ANOVA ($F(1, 33) = 0.05$; $p = 0.85$; figure 3.4). The rate of change in the plasma ferritin did show a significant difference between the baseline ferritin (1-month) and the 3.5-month ferritin ($F(1, 32) = 1.69$; $p = 0.026$; figure 3.5). This decline in iron stores was more rapid in the placebo group over the treatment group indicating the latter group had protection against the drop.

3.3.3 Incidence of Iron Deficency and Iron Deficency Anemia

Based on the data collected from the haematological analysis, all infants were assessed for iron deficiency and iron deficiency anaemia according to the study criteria (table 2.1). The percentage of infants suffering from ID and IDA at each clinic visit is presented in table 3.2. If it is assumed that 20% of infants will suffer from ID and 10% will suffer from IDA these values should have been seen in this study group (Friel et al, 1997; Innis et al, 1997). These levels where chosen as the expected values for the Chi Square analysis. There were more then expected cases of ID (25%) at 1-month in the supplemented group ($X^2 = 5.00$; $p = 0.025$) while the placebo group has an expected number of ID cases (20%). However, by 6 months the placebo group had significantly more cases ID (55%) than expected ($X^2 = 45.00$; $p < 0.0001$). While IDA was seen in 20% of the subjects in the placebo group compared to 10% in the supplemented group, this was not significantly higher then the expected incidence of 10% ($X^2 = 1.053$; $p = 0.305$).

Figure 3.4 Plasma ferritin of infants receiving iron supplements (7.5 mg/day) or a placebo measured at 1, 3.5 and 6 months of age. Mean +/- SD (error bars); N = 16 (supplemented group) and N = 19 (placebo group).

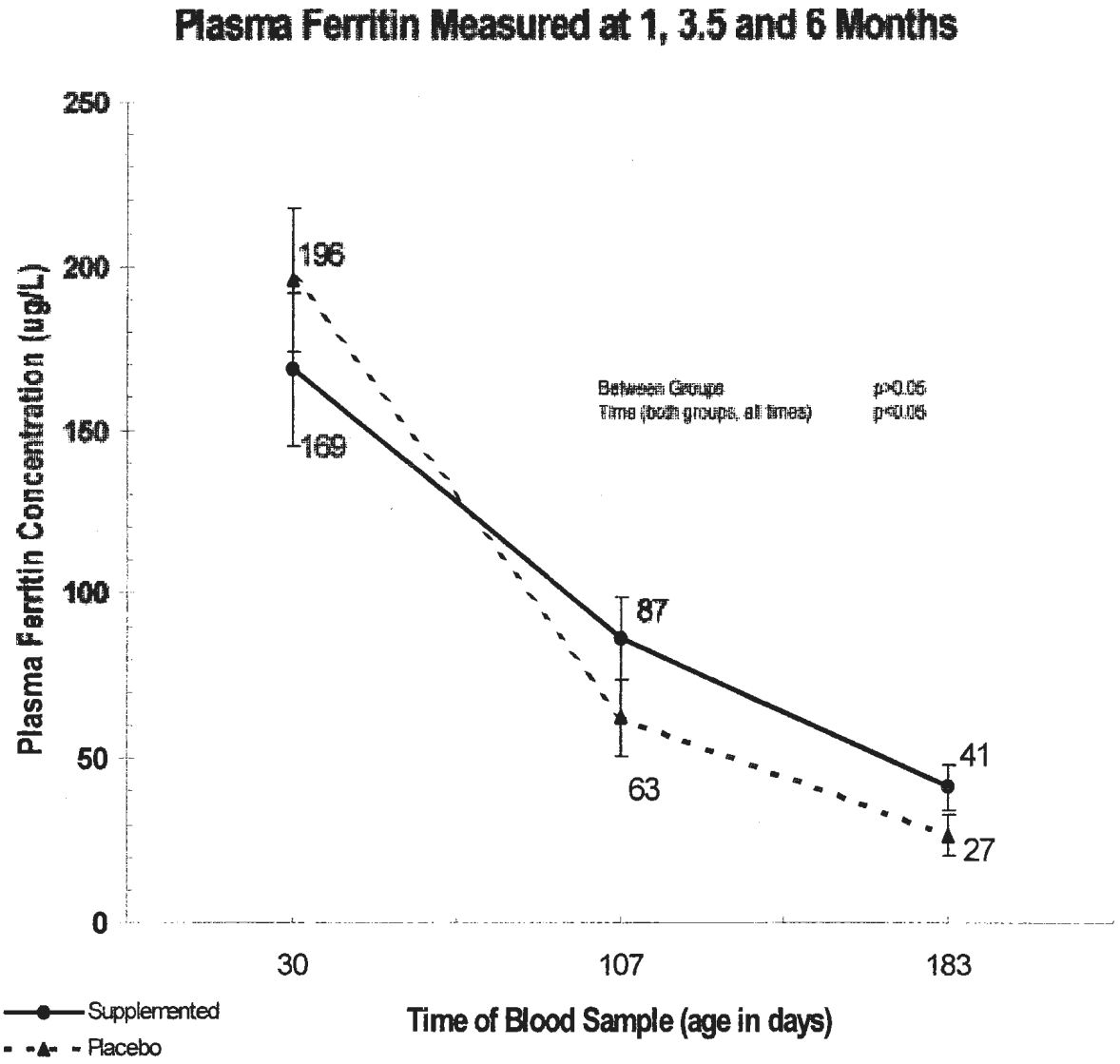


Figure 3.4 Plasma ferritin of infants receiving iron supplements (7.5 mg/day) or a placebo measured at 1, 3.5 and 6 months of age. Mean +/- SEM (error bars); N = 16 (supplemented group) and N = 19 (placebo group).

Figure 3.5 Mean change in plasma ferritin between clinic visits (1, 3.5 and 6-months). Mean +/- SEM (error bars); N = 16 (supplemented group) and N = 19 (placebo group). ‡ Placebo group plasma ferritin declined significantly faster then the supplemented group from 1 to 3.5 months ($F(1, 32) = 1.69$; $p = 0.026$).

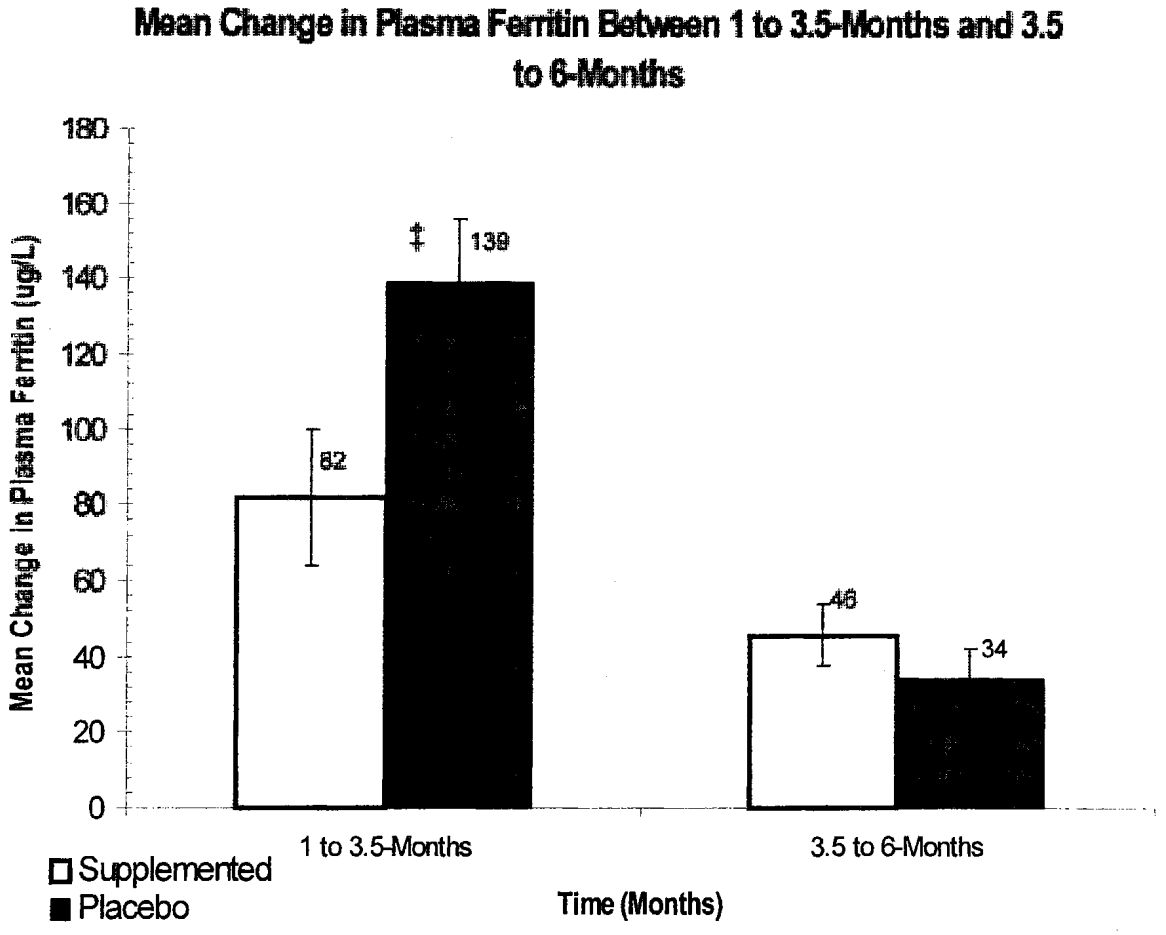


Figure 3.5 Mean change in plasma ferritin between clinic visits (1, 3.5 and 6-months). Mean +/- SEM (error bars); N = 16 (supplemented group) and N = 19 (placebo group). ‡ Placebo group plasma ferritin declined significantly faster then the supplemented group from 1 to 3.5 months ($F(1, 32) = 1.69$; $p = 0.026$).

Table 3.2 Incidence of Iron Deficiency and Iron Deficiency Anaemia Through 6-Months.

Table 3.2

Age	Iron Deficiency		Iron Deficiency Anaemia	
	Supplement	Placebo	Supplement	Placebo
1 Month	25 (5/20)	20 (4/20)	0 (0/20)	0(0/20)
3.5 Month	10 (2/20)	15 (3/20)	0 (0/20)	0 (0/20)
6 Month	20 (4/20)	55† (11/20)	5 (1/20)	10 (2/20)

Values in parenthesis are number of cases from total subjects. † denotes significantly different from expected value ($X^2 = 5.00$; $p = 0.025$).

3.3.4 1-Month Haemoglobin as a Predictor of Infant Plasma Ferritin at 3.5 and 6-Months

The haematological data was examined for relationships that might serve as predictors of infant iron stores. The infants haemoglobin at 1-month appears to be a useful predictor of the infant iron stores as plasma ferritin. The supplemented infants' 1-month haemoglobin showed a correlation with the 3.5-month plasma ferritin but not with 6-month plasma ferritin (Figure 3.6; Pearson correlation coefficient = 0.554; $p < 0.03$; two tailed). The placebo infants' 1-month haemoglobin correlated to both the 3.5-month and 6-month plasma ferritin (Figure 3.7; Pearson correlation coefficient = 0.457 at 3.5-month and Figure 3.8; Pearson correlation coefficient = 0.462 at 6-month; $p < 0.05$; two tailed). The lack of correlation between the supplemented infants' haemoglobin and the 6-month plasma ferritin may be due to the positive effects of the iron supplement on iron status.

3.4 Antioxidant Status of Subjects

3.4.1 Ferric Reducing Antioxidant Power Assay

The FRAP assay was chosen as the measure of total antioxidant capacity for the subjects in the study (Benzie and Strain, 1996). Chosen for its relative simplicity and high reproducibility, the FRAP assay is an indication of the total reducing ability of the infant plasma sample. This measure accounts for enzymatic and non-enzymatic systems present in plasma samples. There were no differences between the infants with respect to treatment protocol ($F(1, 31) = 0.011$; $p = 0.918$; figure 3.9).

Figure 3.6 Supplemented infants 3.5-month plotted against the 1-month haemoglobin. Pearson correlation coefficient = 0.554 at $p < 0.03$ (two tailed) N= 17.

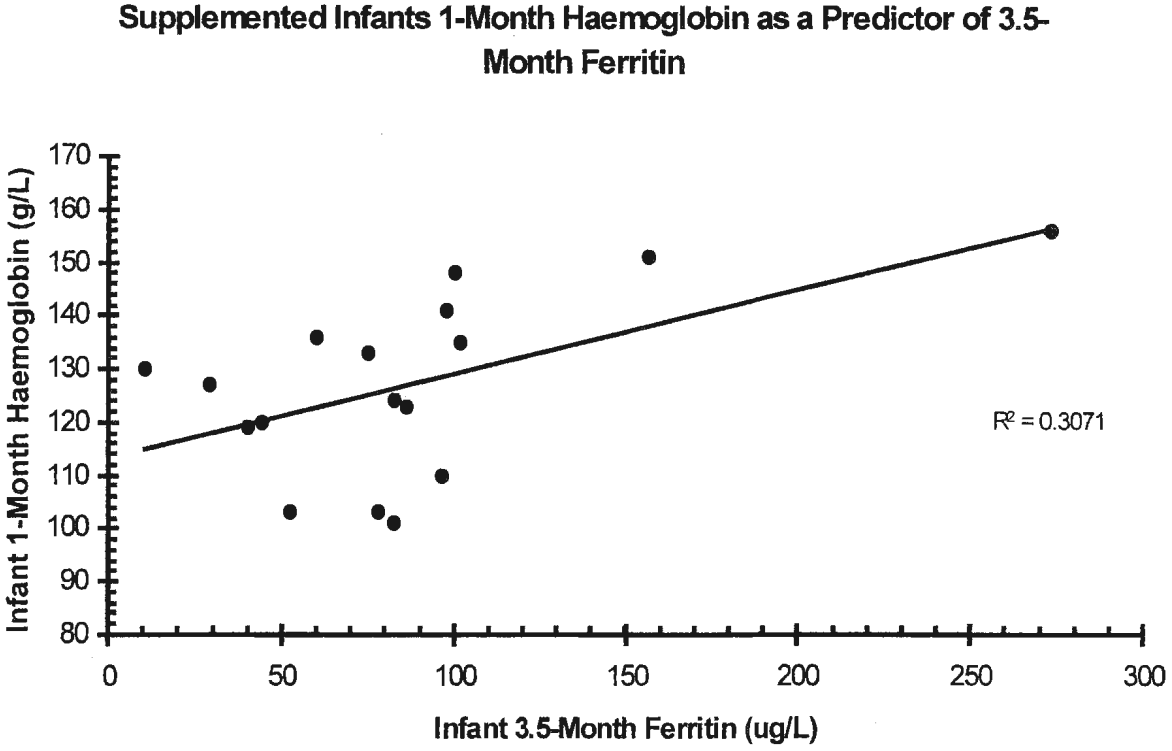


Figure 3.6 Supplemented infants 3.5-month plotted against the 1-month haemoglobin. Pearson correlation coefficient = 0.554 at $p < 0.03$ (two tailed) N= 17.

Figure 3.7 Placebo infants' 3.5-month plotted against the 1-month haemoglobin. Pearson correlation coefficient = 0.457 at $p < 0.05$ (two tailed) N= 19.

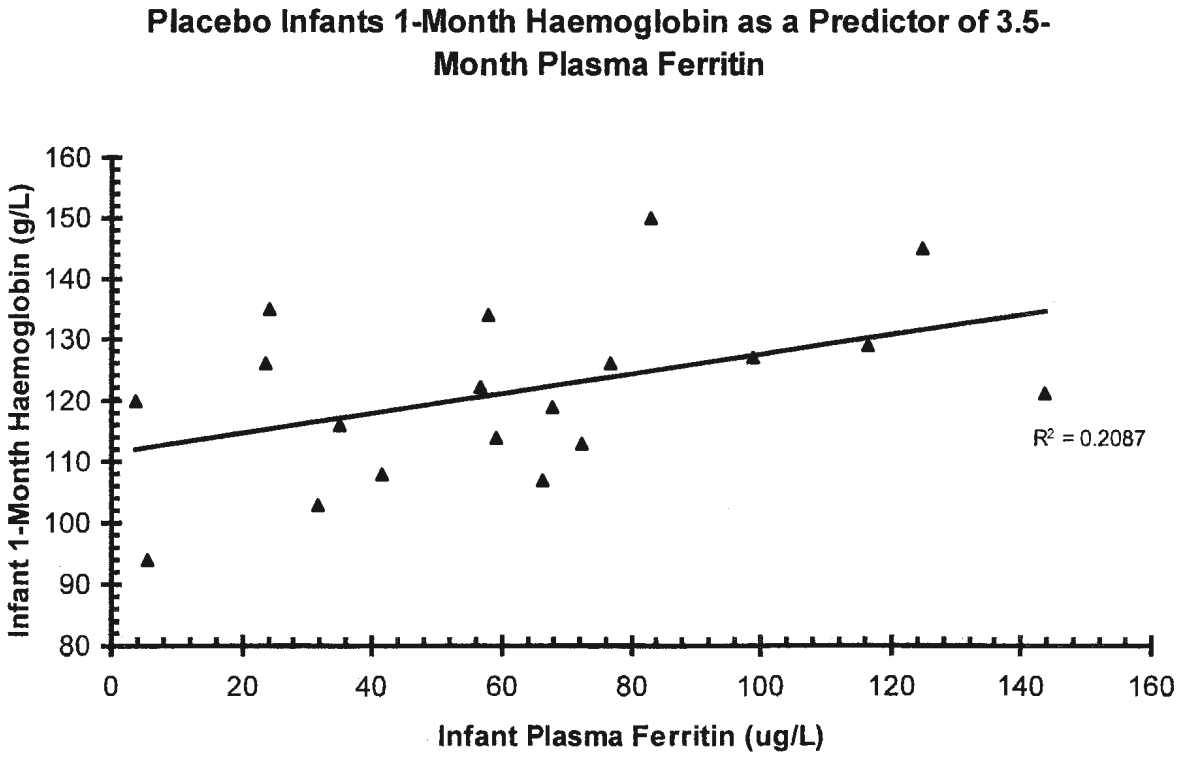


Figure 3.7 Placebo infants' 3.5-month plotted against the 1-month haemoglobin. Pearson correlation coefficient = 0.457 at $p < 0.05$ (two tailed) N= 19.

Figure 3.8 Placebo infants' 1-month haemoglobin plotted against plasma ferritin at 6-months demonstrating a possible predictor of 6-month iron stores. Pearson correlation coefficient = 0.457 at $p < 0.05$ (two tailed) N= 19.

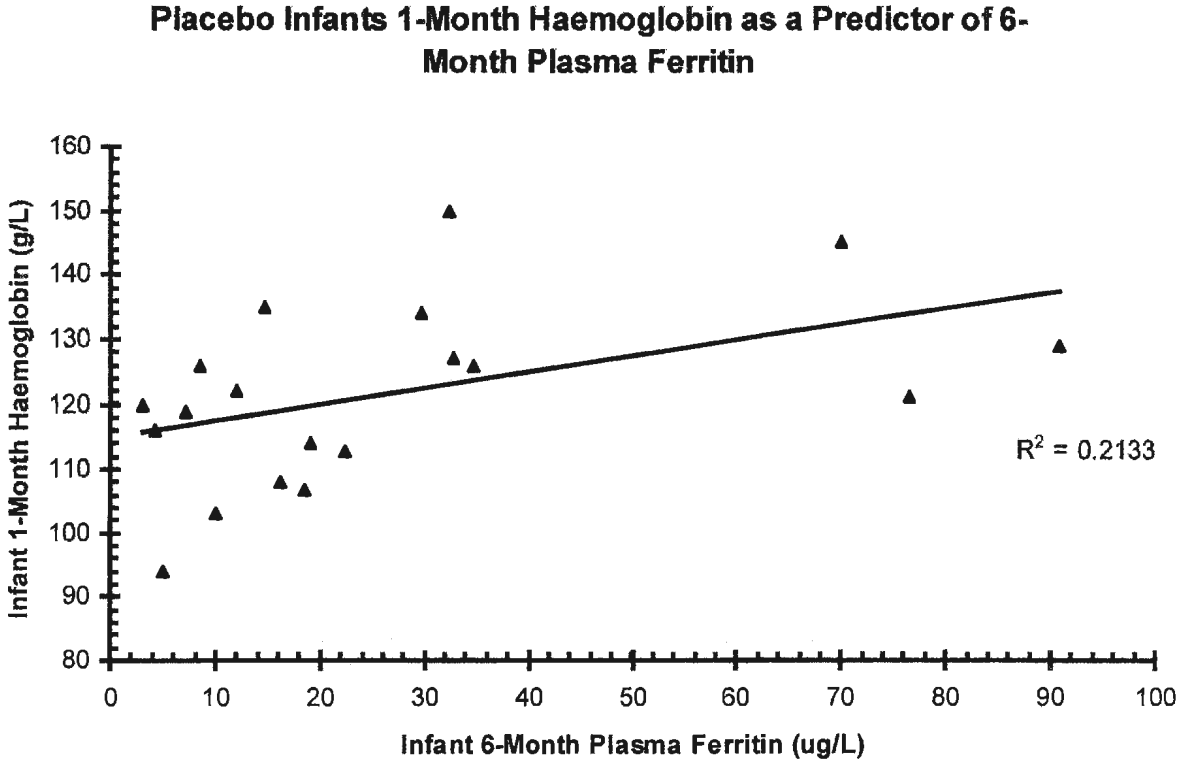


Figure 3.8 Placebo infants' 1-month haemoglobin plotted against plasma ferritin at 6-months demonstrating a possible predictor of 6-month iron stores. Pearson correlation coefficient = 0.457 at $p < 0.05$ (two tailed) N= 19.

3.4.2 Erythrocyte Catalase Specific Activity

The catalase specific activity was measured to determine if the supplemental iron would invoke an increase in the activity of catalase. No difference was seen in the catalase specific activity between the two treatment groups ($F(1, 34) = 0.004$; $p = 0.949$; figure 3.10). These findings are in agreement with the lack of difference in the FRAP values recorded for both groups

3.4.3 Erythrocyte Superoxide Dismutase Activity

The erythrocyte SOD activity was also measured as an indication of any antioxidant response to the supplemental iron. Erythrocyte SOD was also used as an indicator of impaired copper and/or zinc absorption due to the supplemental iron as Cu and Zn deficiencies would be detectable in this enzyme. No difference was seen between either the supplemented group or the placebo group with respect to SOD levels ($F(1, 33) = 0.212$; $p=0.648$; figure 3.11). There was no indication of any impaired copper/zinc absorption as measured by SOD levels given the fairly stable enzyme activity levels seen in the supplemented group (figure 3.11).

3.5 Plasma Mineral Concentration

3.5.1 Plasma Copper Concentration

The plasma copper concentration was measured to insure there was no inhibition of dietary copper absorption in the gastrointestinal tract due to the supplemental iron. No difference was seen between groups in the plasma Cu concentrations at any of the clinic times ($F(1, 27) = 0.041$; $p = 0.842$; figure 3.12). There was a significant increase in the plasma Cu concentrations of both groups over 1 – 3.5 month period ($F(1,15) = 56.6$; $p < 0.0001$; figure 3.12).

Figure 3.9 FRAP value expressed asμmol/L ascorbic acid for of infants receiving iron supplements (7.5 mg/day) or a placebo measured at 1, 3.5 and 6 months of age. Mean +/- SEM (error bars); N = 16 (supplemented group) and N = 17 (placebo group).

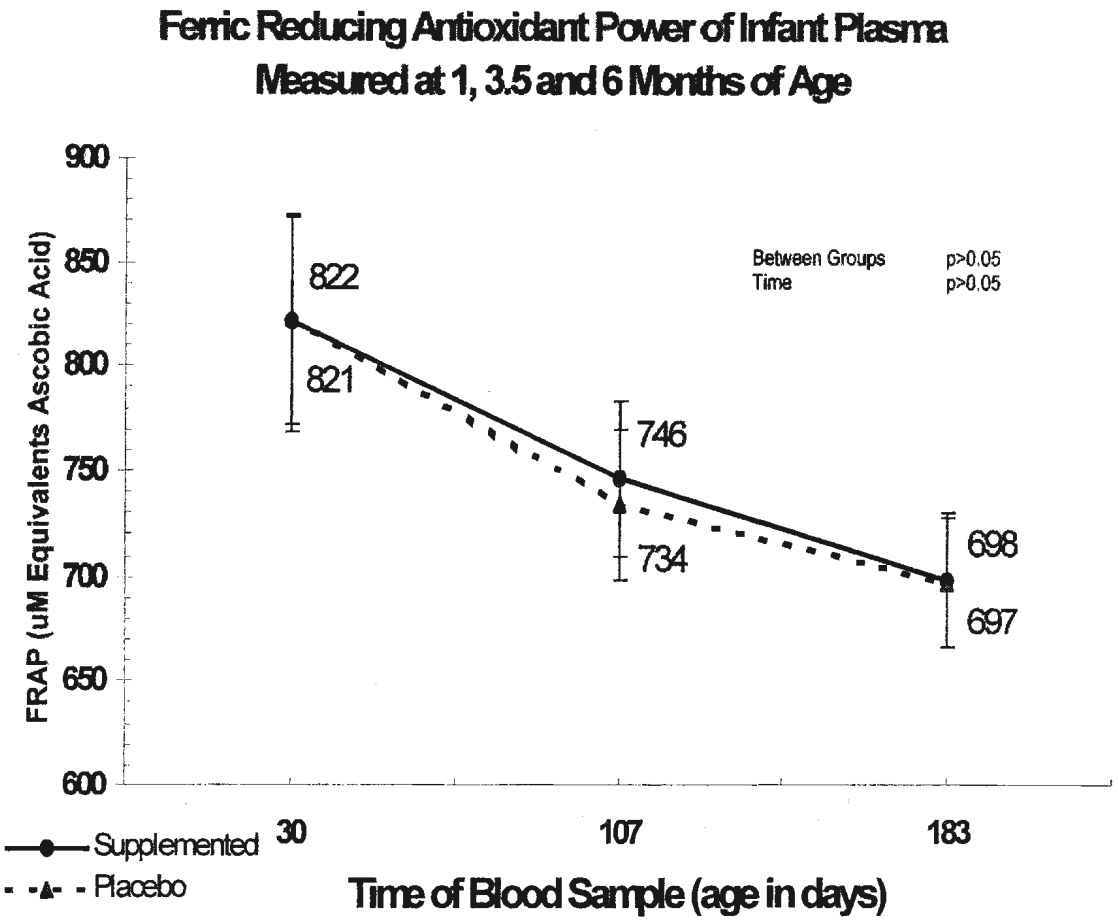


Figure 3.9 FRAP value expressed asμmol/L ascorbic acid for of infants receiving iron supplements (7.5 mg/day) or a placebo measured at 1, 3.5 and 6 months of age. Mean +/- SEM (error bars); N = 16 (supplemented group) and N = 17 (placebo group).

Figure 3.10 Erythrocyte catalase specific activity measured as specific activity per mg of protein for infants receiving iron supplements (7.5 mg/day) or a placebo measured at 1, 3.5 and 6 months of age. Mean +/- SEM (error bars); N = 18 (supplemented group) and N = 19 (placebo group).

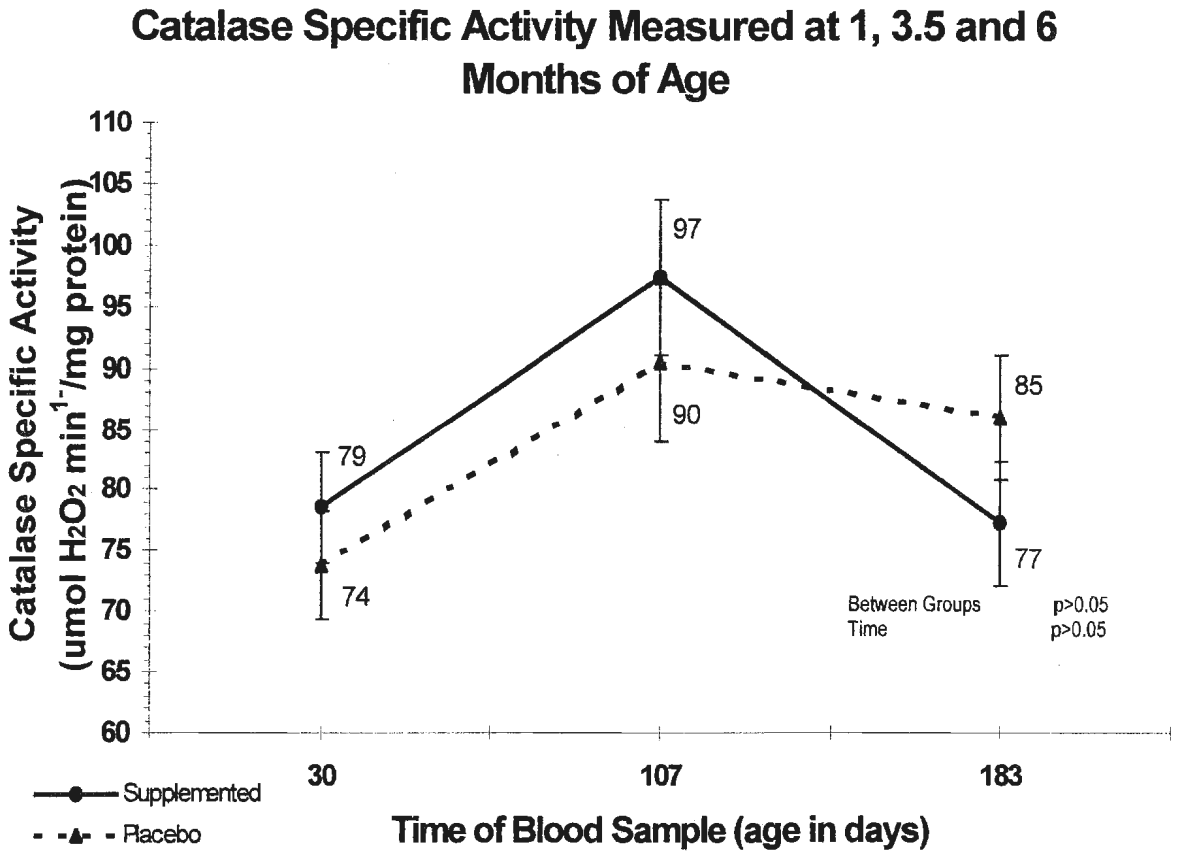


Figure 3.10 Erythrocyte catalase specific activity measured as specific activity per mg of protein for infants receiving iron supplements (7.5 mg/day) or a placebo measured at 1, 3.5 and 6 months of age. Mean +/- SEM (error bars); N = 18 (supplemented group) and N = 19 (placebo group).

Figure 3.11 Erythrocyte superoxide dismutase expressed as units of SOD per mg of haemoglobin for infants receiving iron supplements (7.5 mg/day) or a placebo measured at 1, 3.5 and 6 months of age. Mean +/- SEM (error bars); N = 17 (supplemented group) and N = 19 (placebo group)

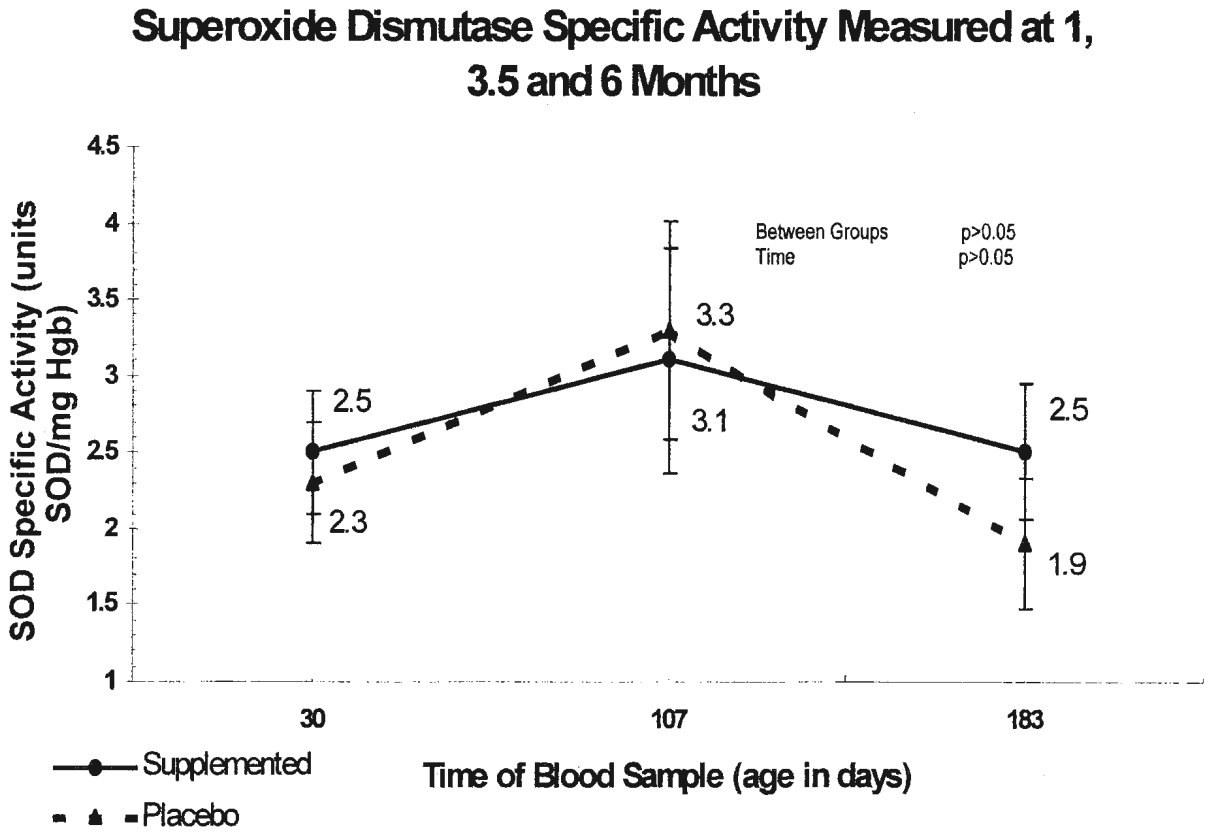


Figure 3.11 Erythrocyte superoxide dismutase expressed as units of SOD per mg of haemoglobin for infants receiving iron supplements (7.5 mg/day) or a placebo measured at 1, 3.5 and 6 months of age. Mean +/- SEM (error bars); N = 17 (supplemented group) and N = 19 (placebo group)

3.5.2 Plasma Zinc Concentration

Plasma zinc concentrations were also monitored to determine if the supplemental iron inhibited the absorption of dietary zinc. Plasma zinc, like plasma copper was determined by atomic absorption spectrophotometry using discrete nebulization. No difference was measurable between the two groups ($F(1, 27) = 0.041$; $p = 0.842$; figure 3.13) but the supplemented group had a significant rise in zinc plasma concentrations between the 3.5 and the 6-month blood samples ($F(1, 16) = 18.8$; $p = 0.01$; figure 3.13). There are several plausible reasons for this increase that are described in the discussion.

3.6 Human Breast Milk Iron Concentrations

Human breast milk iron concentration was measured in both groups to obtain the mean level of iron intake for the study subjects and to compare three common methods used in the literature to determine levels of iron in human breast milk. These concentrations were also compared to the maternal haemoglobin concentration measured in hospital during delivery.

3.6.1 Breast Milk Iron Concentrations

The breast milk iron concentrations were measured at 1 and 3.5 months by direct GFAAS and method of additions GFAAS and by FAAS. The change in iron concentration from 1 to 3.5 months was measured as well as the difference between the three methods at each sample time. A difference was show to exist between the use of direct GFAAS and FAAS at 1-month (paired t-test $p = 0.03$) while both GFAAS methods differed from the FAAS at 3.5-months (paired t-test $p = 0.01$; figure 1). FAAS yielded a consistently higher concentration of iron in both milk samples (1-month

and 3.5-month; figure 1). There was no difference between the two GFAAS methods at either time point but the characteristic decline over lactation was seen between milk samples (figure 1).

3.6.2 Maternal Haemoglobin as Predictor of Breast Milk Iron Concentration

The maternal haemoglobin obtained at the time of delivery was used as an indicator of maternal iron status and compared to the 1-month breast milk iron concentration to determine if any relationship existed (figure 3.17; direct GFAAS). No relationship was seen and in support of previous reports maternal iron status is independent of maternal breast milk iron concentrations.

3.6.3 Maternal Haemoglobin as a Predictor of Infant Haemoglobin at 1 Month

The scatter plot of infant haemoglobin and maternal haemoglobin does not demonstrate any type of relationship that would yield any predictive value for infant haemoglobin. As with the haemoglobin and 1-month breast milk iron concentrations there is no significant relationship (figure 3.18).

Figure 3.12 Plasma copper concentrations are expressed as μg/ml plasma for infants receiving iron supplements (7.5 mg/day) or a placebo measured at 1, 3.5 and 6 months of age. Mean +/- SEM (error bars); N = 16 (supplemented group) and N = 13 (placebo group)

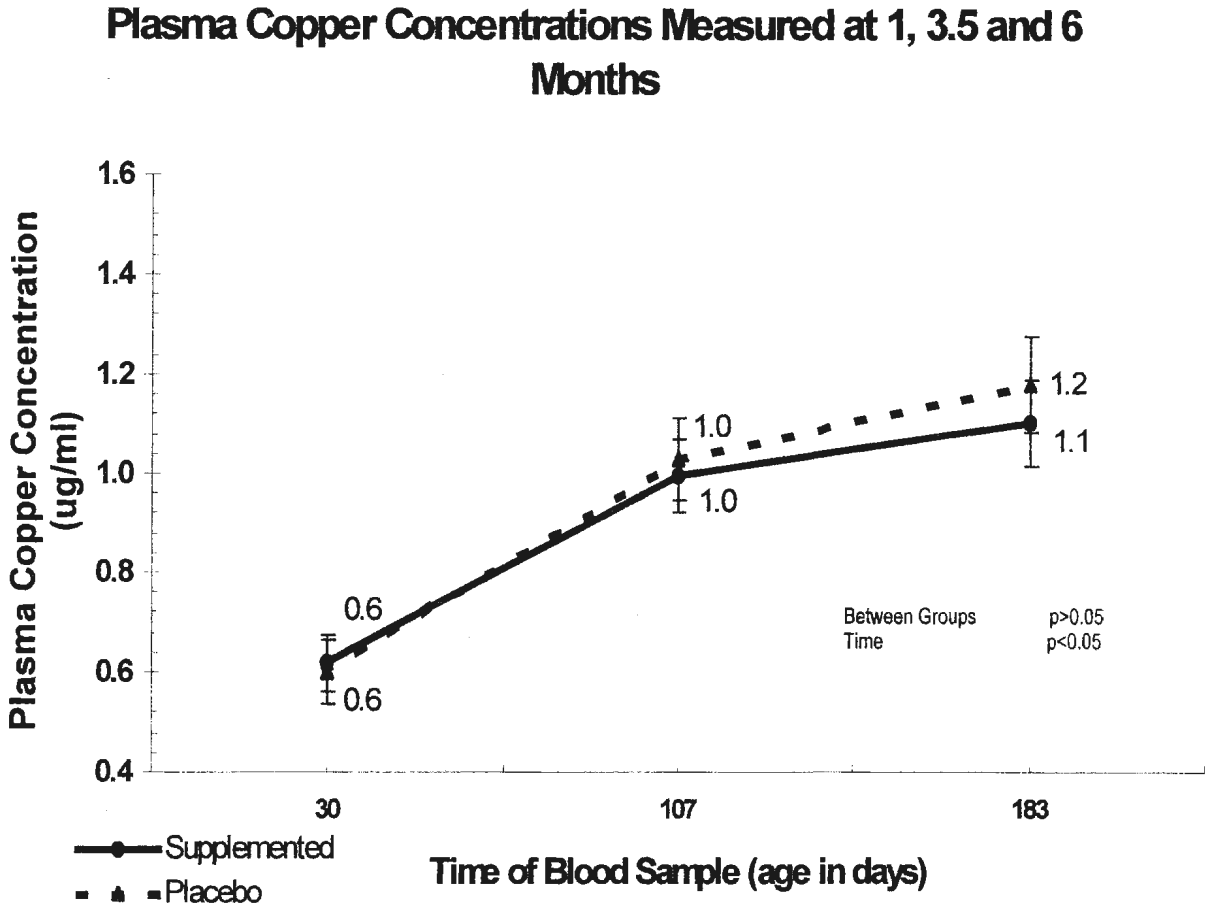


Figure 3.12 Plasma copper concentrations are expressed as µg/ml plasma for infants receiving iron supplements (7.5 mg/day) or a placebo measured at 1, 3.5 and 6 months of age. Mean +/- SEM (error bars); N = 16 (supplemented group) and N = 13 (placebo group)

Figure 3.13 Plasma zinc concentrations are expressed as μg/ml plasma for infants receiving iron supplements (7.5 mg/day) or a placebo measured at 1, 3.5 and 6 months of age. Mean +/- SEM (error bars); N = 18 (supplemented group) and N = 16 (placebo group).

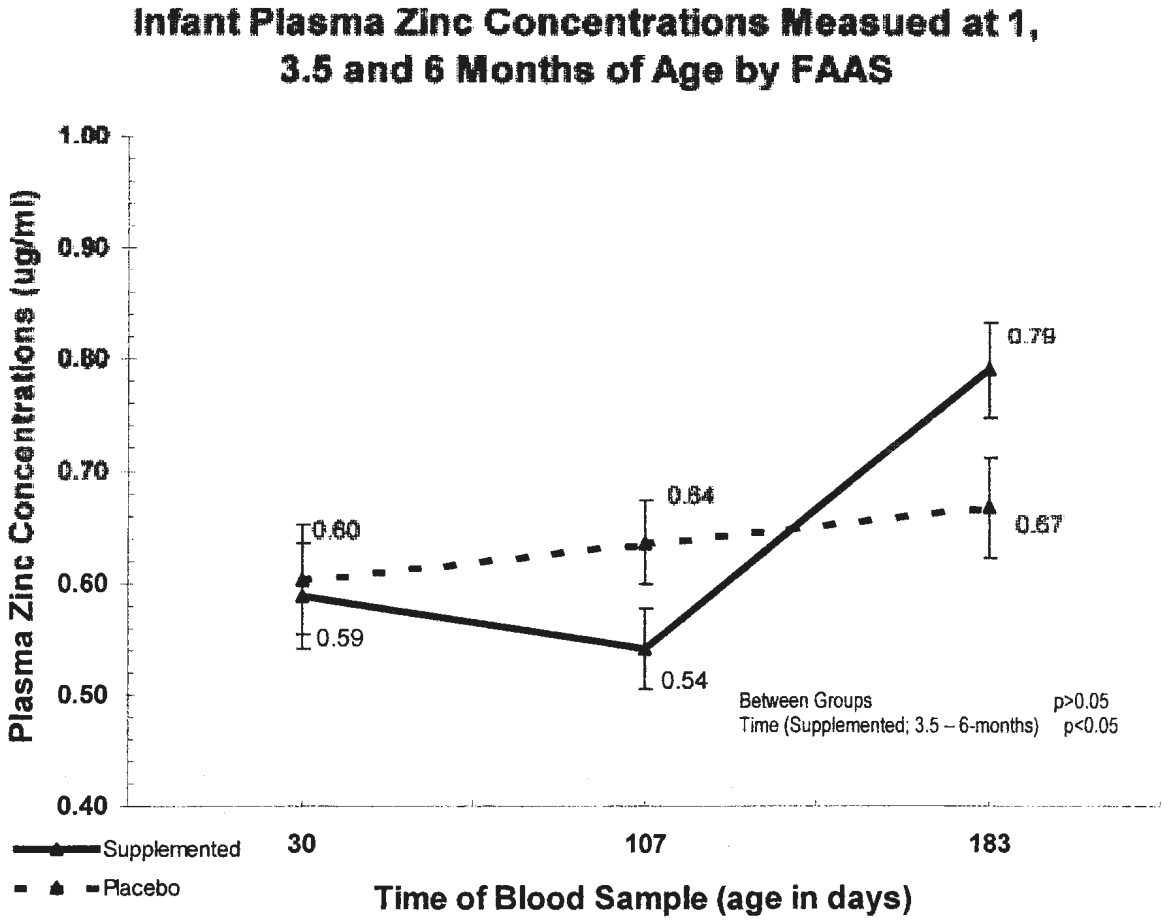


Figure 3.13 Plasma zinc concentrations are expressed as μg/ml plasma for infants receiving iron supplements (7.5 mg/day) or a placebo measured at 1, 3.5 and 6 months of age. Mean +/- SEM (error bars); N = 18 (supplemented group) and N = 16 (placebo group).

Figure 3.14 Iron concentration of human breast milk measured at 1 and 3.5 months by direct GFAAS, methods of addition GFAAS (N = 22 at 1 month and N = 25 at 3.5 months for both direct and methods of addition) and FAAS (N = 10). Concentration is expressed as μg/ml of human breast milk. Mean +/- SD (error bars). † = Difference between method; $p<0.05$. ‡ = Difference between time within same method; $p<0.05$.

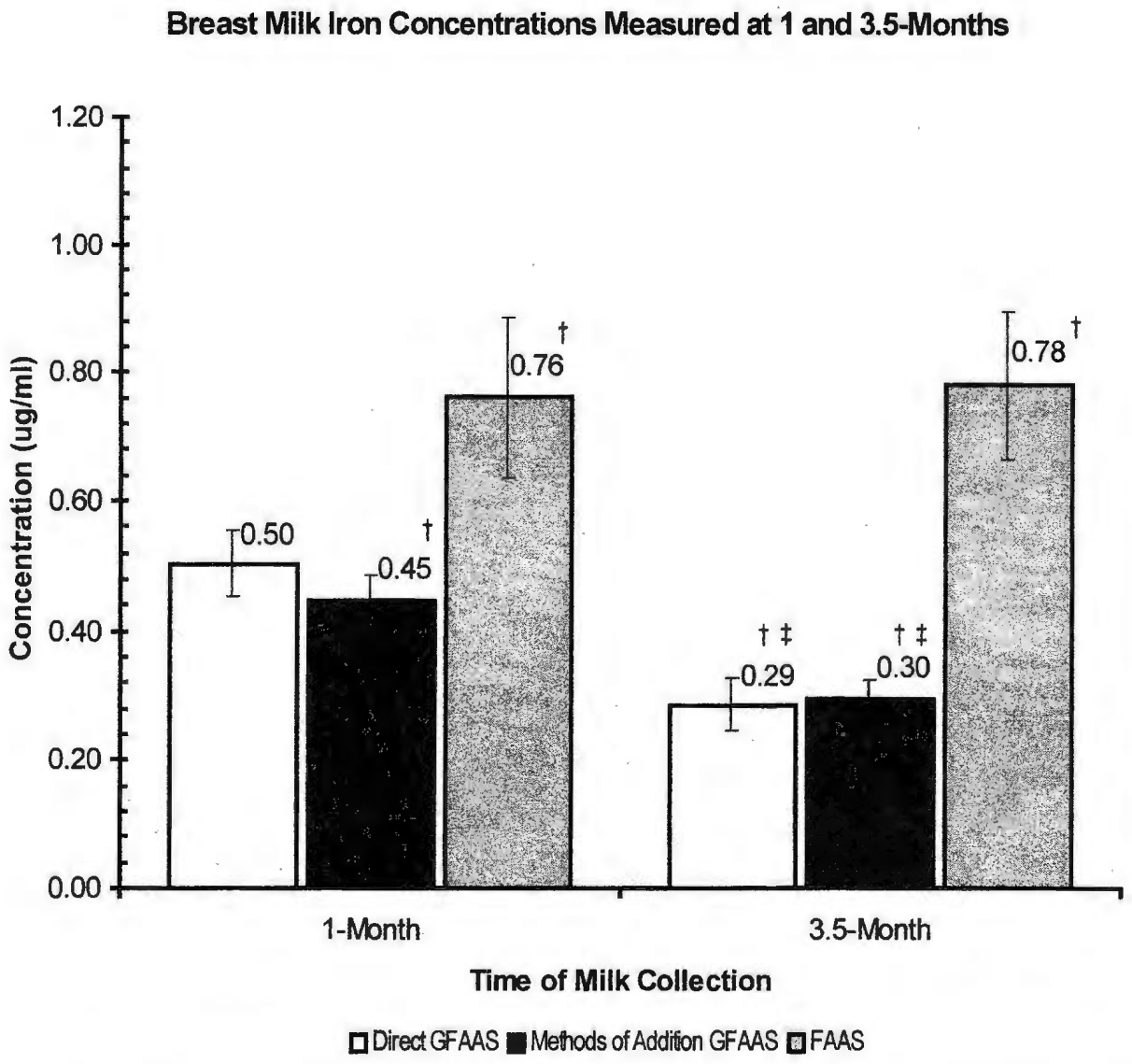


Figure 3.14 Iron concentration of human breast milk measured at 1 and 3.5 months by direct GFAAS, methods of addition GFAAS (N = 22 at 1 month and N = 25 at 3.5 months for both direct and methods of addition) and FAAS (N = 10). Concentration is expressed as µg/ml of human breast milk. Mean +/- SEM (error bars). † = Difference between method. ‡ = Difference between time within same method.

Figure 3.15 Maternal breast milk iron concentrations plotted against maternal haemoglobin at delivery (N = 21).

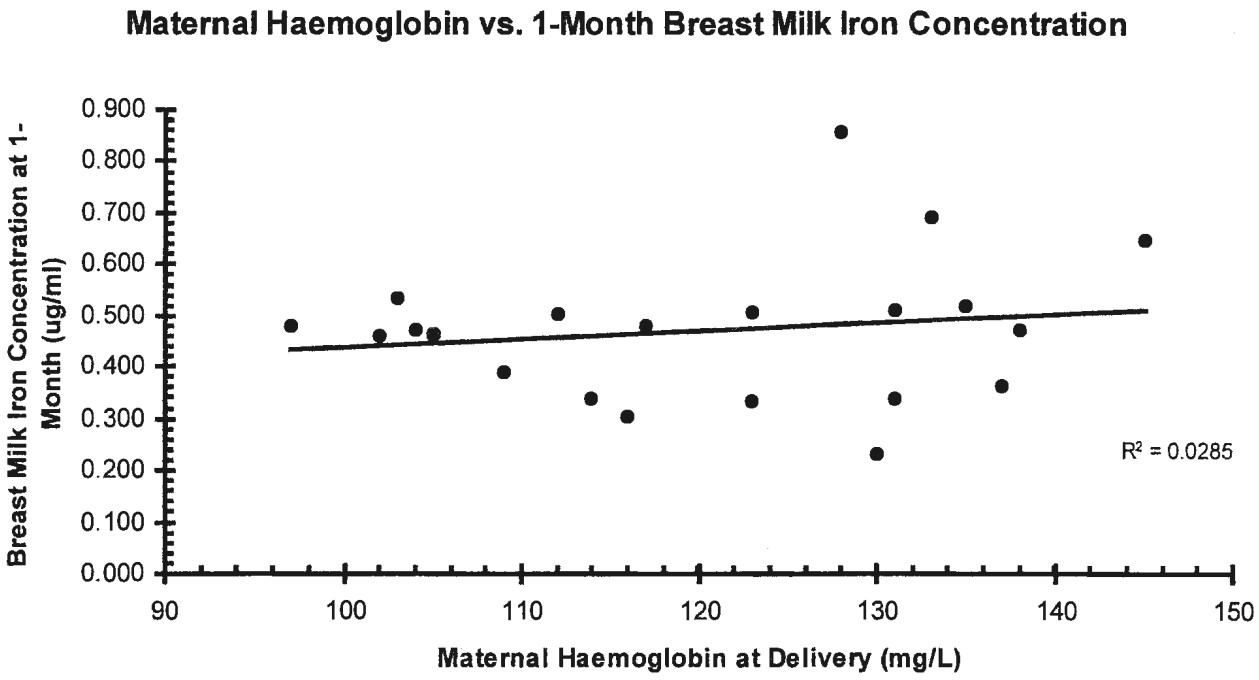


Figure 3.15 Maternal breast milk iron concentrations plotted against maternal haemoglobin at delivery (N = 21).

Figure 3.16 Maternal haemoglobin plotted against infant haemoglobin at 1 mont (N = 39).

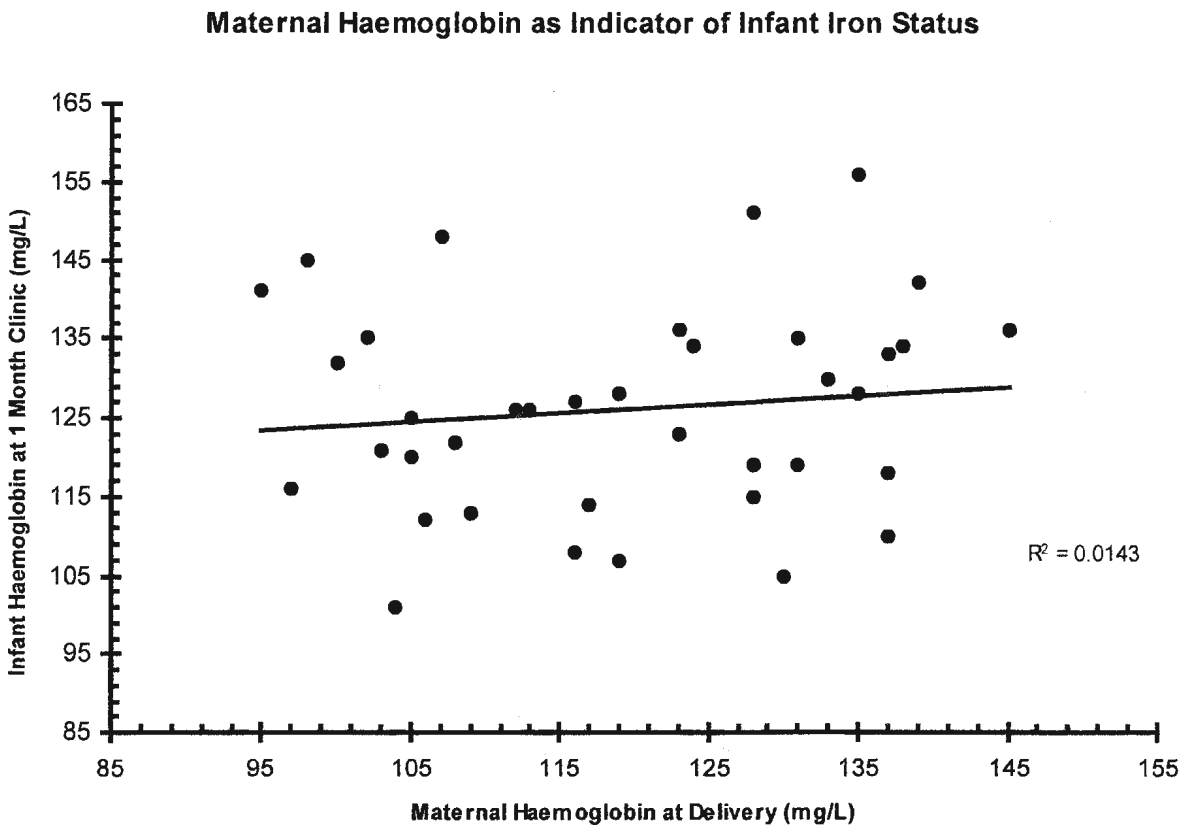


Figure 3.16 Maternal haemoglobin plotted against infant haemoglobin at 1 month (N = 39).

Chapter 4: Discussion

4.1 General Comments

To date there have been no intervention studies investigating the possibility of preventing iron store decline in healthy breast fed infants through iron supplementation. Our study has demonstrated the clear benefits associated with iron supplementation in infants by preventing ID at 6 months of age. While a portion of newborns have adequate iron stores, allowing them to reach 6 months of age before any overt signs of iron deficiency, 55% in the present study did not reach this benchmark age before suffering the insult of iron deficiency. The analysis of iron and haematological status as a function of iron supplementation has shown significant positive increases in Hgb, Hct and cell size (MCV) and lower incidences of ID.

Potential risks associated with iron supplementation include increased oxidative stress and metabolic consequences associated with this stress, gastrointestinal discomfort of the infant from the ferrous sulphate supplement itself and potential negative interactions with the absorption of dietary zinc and copper. Assaying for activities of erythrocyte superoxide dismutase and catalase and measuring the total reducing ability of infant plasma (FRAP assay) between 1 – 6 months of age was used to assess antioxidant status. No differences were observed between the two groups for any of these indicators, indicating no increase in oxidative stress. Parental reporting of problems associated with taking the supplement, such as constipation, showed no difference between treatment groups and could have been explained by numerous other infant related situations causing the same symptoms. There were also no differences observed between

treatment groups with respect to copper or zinc absorption between 1 and 6-months of age regardless of treatment as measured by total plasma copper and zinc concentrations. However, significant increase plasma zinc concentrations between 3.5 months and 6 months was observed, which requires further investigation to assess whether zinc absorption was suppressed as a result of the iron supplement. If this were the case there would be implications for both supplementing breast fed infants and infant formula compositions.

The data was also analyzed for relationships, which may provide a predictive tool to identify infants who are at risk of developing an ID before 6 months of age. While there were no strong correlations relationships were observed between infant 1-month Hgb as a predictor of 3.5 and 6-month plasma ferritin in the placebo group and 1-month Hgb predicting plasma ferritin in 3.5 month supplemented infants. These relationships will need to be further investigated to determine if they are plausible techniques for identifying infants at risk of becoming ID in the first few months of life.

The data presented in this thesis has contributed to several gaps in the current understanding of ID and IDA in breast fed infants less than 6 months of age. The decline of iron stores can be safely prevented by mild iron supplementation. Caution is needed as even mild iron supplementation may have effects on the absorption and status of other minerals, namely zinc. This study also demonstrated 1-month infant iron and haematological status is independent of maternal status at birth. There was also no relationship between breast milk iron concentrations and exclusively breastfed infant iron status. These thoughts are discussed in further detail in the reminder of this chapter.

4.2 Analysis of Findings

4.2.1 Subject Anthropometric, Dietary and Racial Findings

All infants were within the limits of normal growth compared to the standard infant growth charts for length, weight and head circumference (Table 3.1) (Fomon, 1993). All subjects were infants born at the Grace General or Health Sciences Centre Obstetrics Unit. Infants were all healthy at each clinic visit, displaying no signs of infection. The three day dietary records collected on the infants from the mothers did not reveal anything unexpected as all mothers exclusively breastfed at the time of the 1-month clinic with over 95% still exclusively breast feeding at 3.5 months and a combination of breast milk, formula and solid foods by 6-months. There were no differences seen between the groups for the energy, protein, fat or carbohydrate intake (Appendix A). In addition, iron (not including supplement), copper, zinc, selenium and ascorbic acid intake did not differ between groups (Appendix A). With both groups virtually identical with respect to growth, diet and race any difference seen between the groups with respect to haematological data, oxidative responses and stress or plasma mineral concentrations should be due to the treatment medication.

4.2.2 Breast Milk Iron Concentrations

It is known that the levels of many human breast milk constituents do not vary greatly between populations of mothers throughout the world (Casey, 1996). The published values for iron vary as much as 66% depending on the method used. Trugo (1988) and associates reported mature human breast milk containing 0.90 μg/ml iron in Brazil while both Siimes and associates

(1979) and Macy and Kelly (1961) report the value at 0.30 μg/ml iron in Finland and the United States respectively. Fransson and associates (1984) demonstrated that geographic region and socio-economic status has little effect on the concentration of iron in human milk, comparing Swedish women to privileged and underprivileged Ethiopian women. It was because of this wide range of values that we decided to compare several methods for determining iron concentrations in biological samples. Atomic absorption spectrophotometry is a widely used tool to determine mineral content in biological samples. We decided to examine 3 different methods for determining iron levels: graphite furnace atomic absorption spectrophotometry (direct measurement with use of prepared standards and method of standard additions) and flame atomic absorption spectrophotometry. Since the amount of iron these infants are consuming in their diet is of great importance, we analyzed a subset of 10 mothers' breast milk to determine the concentration of iron.

Differences were found between the breast milk iron concentrations of those mothers who provided milk for analysis both at different times and by different analytical method. There was a drop in mean iron concentration between 30-day and 3.5-month milk samples, which is in agreement with Siimes et al (1979) and those summarized by Casey (1996). Our findings demonstrated mean iron concentration ranging from 0.47 – 0.29 mg/L from 30-day to mature milk at 3.5-months (figure 3.14) using the GFAAS methods while the FAAS method produced a consistent mean concentration of iron in both samples (0.76 – 0.78 mg/L). The discrepancy between the results obtained by GFAAS and those by the FASS method illustrate the problems with reporting breast milk mineral concentrations by a variety of methods. FAAS and GFAAS are two common methods used to determine many minerals in biological fluids and have been used by

most when determining the iron concentrations (Siimes et al, 1979; Fransson et al, 1984; Fransson and Lonnerdal, 1980; Picciano et al, 1981; Arnaud et al, 1993; Zavaleta et al, 1995). The variety of ashing techniques and dilution protocols used by the different researchers are a possible reason for the wide range of results seen in the literature. A clear example of this effect is illustrated by Arnaud and associates (1993, 1995) who produced two different ranges for early breast milk iron by changing the procedure used. The use of a flame method on whole breast milk yielded mena iron concentration of 4.9 μmol/L while a GFAAS method using Triton X-100 as the diluent yielded between 6 - 14 μmol/L iron with a mean concentration of 9.4 μmol/L. The FAAS method with wet ashing of the sample used in our experiments, and used by other researchers for mineral determinations in milk products (Alkanani et al, 1994; Zavaleta et al, 1995), yielded consistently higher values for breast milk iron in our experiments compared to others (0.76 mg/L versus 0.40 mg/L by Zavalata et al, 1995).

We also examined the reliability of a high dilution direct method for the GFAAS, which also produced very reliable and reproducible values. The method of addition technique described in Chapter 2 (section 2.5.3) is reported to be the most effective method to remove organic matrix effects from biological samples (Perkin Elmer HGA 300 Method Manual; Lena Davidsson, personal communication). The values obtained by the method of addition and the direct diluted methods conform to the reported values of breast milk iron from Siimes et al (1979), Fransson et al (1984) and the mean of the various data summarized by Casey (1996).

4.2.3 Haematological and Iron Status

The supplementation of infants 1 – 6 months of age with 7.5 mg iron per day has clear benefit to the haematological status by 6-months. Haemoglobin and hematocrit levels were maintained by iron therapy compared to an 8% and 6% difference seen in the group receiving the placebo. While the MCV fell in both groups, those infants receiving iron had a higher cell volume at 6-months dropping only 16% of the 1-month level compared to those receiving placebo whose mean volume fell 19%. While no significant difference was detected in the mean plasma ferritin levels between the study group and the group receiving the placebo the latter did decrease between blood samples at a significantly higher rate (49% versus 68%).

The haematological data from this study compare closely to data on breast fed infants published by Saarinen (1978) when he examined iron status among breastfed, formula fed and cow milk fed infants in the first year of life. The same patterns observed by Saarinen are seen in the placebo group from 1 to 6-months. There is a striking difference between our supplemented group and the infants presented in Saarinen's study with respect to haemoglobin concentrations. The infants in Saarinen's study displayed a sharp decrease in haemoglobin concentration between 1 and 4 months followed by an increase and plateau by 6 months. The iron-supplemented infants were able to avoid this decline in haemoglobin while the other measures of iron status, MCV and plasma ferritin, progressed similar to that of Saarinen's infants.

The observation that breast fed infants usually do not display signs of iron deficiency until after 6 months (Lönnerdal and Hernell, 1994; Walter et al, 1998; Makrides et al, 1998; Owen et al, 1981) may not be true as 55% of the infants in the present study were iron deficient. In addition, Lönnerdal and Hernell (1994) demonstrated that infants fed low iron formula became anaemic by 4

– 6 months and required early intervention to treat the problem. The iron content of breast milk may be of higher bioavailability then the iron found in humanized formulas but the absolute content is still relatively low. The infants in the Lönnerdal and Hernell (1994) study who received the higher iron formula (4 – 7 mg iron per litre; 2.6 – 5.6 mg iron per day) from 1.5 – 6 months of age improved iron stores until solid foods could be introduced. The breast fed infants receiving placebo in our study did not reach this level of iron intake until 6-months of age (Appendix A). Contrary to our findings, Ziegler and associates did not see any benefit supplementing breast fed infants with 7.0 mg of iron per day between 1 – 5.5 months. They did state that iron status in breastfed infants is not universally good (Ziegler, 2001). There has been agreement in the literature that less than 10 mg of iron per day is enough to maintain iron status in formula fed infants (Walter, 1998; Lönnerdal and Hernell, 1994). The poor iron status of the placebo infants of this study lend support to opinion that mild iron supplementation of breast fed infants until iron containing solids are introduced may be prudent to prevent ID (Ziegler, 2001; Fomon, 2001). Makrides and associates (1998) recommend high iron weaning foods as the solution to improving iron status, but points out there are infants who still receive no solids by 6 months.

Given the rates of ID and IDA seen in this study the case is stronger to either supplement breast-fed infants during the first 6 months of life or to develop a screening regiment appropriate to identify infants who may become anaemic by 6 months. Only 4 of 20 infants in the supplemented group developed ID by 6 months compared to 11 of 20 in the group receiving no iron. There was no difference in the prevalence of IDA, 2 of 20 in placebo group compared to 1 of 20 in the supplemented group. These values are similar to others in Canada for the breast-feeding population (Innis et al, 1997; Friel et al, 1997). While no difference was seen between the plasma

ferritin concentrations of either group, the rate of ferritin decrease between 1 and 3.5 was significantly higher (Δ 139 μg/L (placebo) versus Δ 82 μg/L at 3.5 months). The higher incidence of ID coupled with a high rate of iron store depletion are warning signs that this group are at a high risk for developing anaemia by 6-months. Since it has not been clarified what level of iron deficiency is detrimental to the cognitive and psychomotor development of infants it is of concern that such a high number of infants are suffering from this deficiency early but are left untreated until anaemia manifests itself.

4.2.4 Potential Adverse Effects of Iron Supplementation

4.2.4.1 Evidence of Oxidant Stress

Potential oxidative stress was measured by any differences in antioxidant response, as a function of plasma FRAP value, RBC catalase and SOD. Iron supplementation at 7.5 mg iron per day does not appear to cause any oxidative stress to the treated infants compared to those not receiving iron.

The plasma FRAP values, which measures the total antioxidant capacity of the infants' plasma showed no differences between groups. Theoretically, the infants that were under stress would demonstrate a lower value as there should be a decrease in non-enzymatic reducing factors in the plasma corresponding to the increased oxidation. The plasma FRAP values were within the range of healthy adults (Benzie and Strain, 1996) but more consistent with respect to elderly women (Cao et al, 1998) opposed to the 25-year old male standard, which was used throughout

this study. This is the first reported use of the assay on an infant population. Investigation of differences between sick infants under oxygen stress and healthy infants would be beneficial.

There were no differences observed between the RBC catalase and SOD activities of the two groups. During peak supplementation (3.5 months) the catalase and SOD activities were virtually identical. There are few reports of the activity of the antioxidant enzymes in the infant population but the data is similar to that previously reported (Friel et al, 1997; van Zoeren-Grobben et al, 1997; Silvers et al, 1998; Friel et al, 2000). It has been shown that human plasma is susceptible to oxidative damage via a ferrous iron – hydrogen peroxide mechanism (Agil et al, 1995). However no difference was detected in any of the measures of antioxidant response examined in the present study, which can be interpreted as iron supplementation at 7.5 mg per day does not put an infant at increase oxidative risk. It should be noted that the predictive value of this data is weak due to the low statistical power. A much larger sample size is needed for this type of enzyme analysis, far beyond the ability of this study. The FRAP assay appears to be a better suited test of oxidative stress for smaller groups.

Analysis of problems associated with taking the supplement also showed no difference between the groups (Appendix B). These results provide answers to the common public perceptions that iron will cause fussiness, constipation and stomach cramps. There has been some indication that iron supplementation will have adverse effects on weight gain in young children (Idjradinata, 1994). Our data indicate that infants 0 – 6 months receiving 7.5 mg iron/day do not have growth rates compromised. Idjradinata's study examined 12 - 18 month infants using iron at 3.0-mg/kg-body weight, somewhat higher than is commonly accepted as the upper limit of

normal dietary supplementation, 2.5 – 3.12 mg/100 kcal (Idjradinata, 1994) versus 1.8 – 2.0 mg/100 kcal (Fomon, 1993).

4.2.4.2 Plasma Mineral Concentrations

Plasma zinc and copper concentrations did not differ as a function of treatment or placebo. The plasma zinc and copper concentration were consistent with values previously reported by other researchers (Walravens et al, 1976; Hambidge et al, 1979; Gibson et al, 1981; Friel et al, 1997). There was however an increase in the mean plasma zinc concentrations of the supplemented group between the 3.5 and 6 month blood sample. Given that not all infants completed the entire protocol of supplement between 3.5 and 6 months there are several possibilities which could cause this rise in plasma Zn in the supplemented group: a) inherent increase in zinc absorption caused the increase in plasma zinc due to the cessation of iron supplements, b) dietary intake between groups differed resulting in a higher mean zinc intake and consequently higher plasma zinc concentrations, c) another factor or combination of factors beyond the scope of this investigation played a part in the increased zinc absorption between 3.5 and 6 months.

It has been shown that iron supplements without zinc added could reduce zinc absorption by close to 50% as well as reduces the plasma zinc concentration in pregnant women (O'Brien, 2000). However, the plasma zinc concentrations compared well with infants from the sample geographic area (Walravens et al, 1976; Hambidge et al, 1979). No difference was seen between the mean dietary zinc intakes of the supplemented group compared to the placebo group. We were unable to elucidate the cause of this rise in plasma zinc concentrations. There would appear

to be an interaction occurring with plasma zinc that requires further investigation and this study was not equipped to answer that particular question.

4.3 Clinical Significance

Our findings appear to support the recommendations of Fomon (2001), which call for the supplementation of breast-fed infants. While wide spread supplementation is an issue which should be approached with caution we have demonstrated that there is a need to further develop the screen procedures and predictors of infant iron status. Further investigation into the use of early haematological data and rate of iron store depletion should be undertaken to help clinicians accurately identify infants at risk of ID and IDA. Our data also show that infants in the first six months of life whom are exclusively breastfed are at increased risk of developing ID or IDA by six months in contrast to others have reported. While the choice to breast-feed is the healthiest choice for mothers to make for their infants, precautions with respect to iron nutrition should be taken and promoted. Whether these infants are screened more closely than the infants whom are formula fed or the breastfed infants are supplemented with iron during the first 4 - 6 months, it does appear that intervention is warranted. Mild iron supplementation does not appear to have any deleterious effects for the infants and is safe to give at the level used in this study. The question of zinc absorption could be addressed by adding zinc to the iron supplement (O'Brien et al, 2000; Fomon, 2001). Given the possible cognitive and psychomotor effects iron deficiency can have on infants, improved screen or supplementation appears to be a reasonable course of action.

Chapter 5: Conclusion

There are a number of risks associated with an infant depleting its iron stores to the point of nutritional deficiency. The literature points to the possibility of irreversible damage to the CNS, cognitive and psychomotor developmental delays and anaemia as the most severe and common effects of this deficiency. Given that each of these conditions would occur within the first year of life there is a small window of prevention time that must be seized if these effects are to be avoided. There are a number of conclusions, which can be drawn from the data presented within this thesis, which are listed below:

1. The concentration of iron in human breast milk appears to be consistent with the lower ranges reported by other researchers. The iron concentrations of human breast milk decline significantly between 1 and 3.5 months of lactation.

2. The direct method for GFAAS determination of human breast milk iron concentration described in the present study appears to be validated as a simple accurate and reliable procedure.

3. Maternal haemoglobin at delivery is neither a predictor of human breast milk iron concentrations nor of infant 1-month iron status as measured by haemoglobin concentration.

4. Mild iron supplementation at 7.5 mg per day for exclusively breast fed infants will promote a more healthy iron status compared to those exclusively breast fed infants not receiving supplemental iron. Mean haemoglobin, hematocrit and MCV

are improved, ferritin stores decline at a slower rate and incidence of iron deficiency is lower at six months in the supplemented infants compared to the infants receiving a placebo.

5. Infant 1-month haemoglobin and the rate of decline in plasma ferritin concentration between a 1 and 3.5-month blood sample may provide a useful tool to predict breast-fed infant ferritin concentration at 6 months.

6. Antioxidant status in blood, as defined by the infant erythrocyte catalase and superoxide dismutase activities and the plasma FRAP value, does not indicate that the iron is causing in vivo oxidative damage. Activities of antioxidant enzymes and the plasma FRAP value were in the expected ranges.

7. There is an increased free radical generating potential, for those infants who have received supplemental iron in fecal samples collected at 6-months, as measured by the production of methanesulfinic acid production. Nothing is known as to the effect of the free radical production in the infant gastrointestinal tract.

8. No difference was detected between the plasma copper concentrations of infants receiving supplements and those receiving the placebo. All values were within the expected range for the subjects. There was however a significant rise between the 3.5 month and 6 month plasma zinc concentrations of the supplemented infants, which could not be explained by dietary analysis.

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Appendix A

Table 6.1 Dietary Composition Reported by Three Day Diet Record – Supplemented Infants

3-Day Diet Record Summary
Specific Nutritional Composition and Nutrients Recorded as Mean (+/- Standard Deviation) Daily Intake
Iron Supplemented Group (N = 16)

Age	Energy (Kcals)	Protein (g)	Fat (g)	Carbohydrate (g)	Fe (mg)	Cu (mg)	Zn (mg)	Se (µg)	Ascorbic Acid (mg)
1-Month	525 (0)	9 (0)	33 (0)	57 (0)	0.3 (0)	0.3 (0)	2.4 (0)	10.5 (0)	30 (0)
3.5-Month	526 (29)	9 (1)	32 (3)	58 (3)	1.5 (2.9)	0.3 (0.03)	2.6 (0.3)	9.3 (3.2)	31 (3)
6-Month	632 (121)	14 (4)	32 (6)	78 (16)	7.8 (5.6)	0.5 (0.15)	3.8 (1.5)	12.9 (5.7)	48 (15)

Table 6.2 Dietary Composition Reported by Three Day Diet Record – Placebo Infants

3-Day Diet Record Summary
Specific Nutritional Composition and Nutrients Recorded as Mean (+/- Standard Deviation) Daily Intake
Placebo Group (N = 17)

Age	Energy (Kcals)	Protein (g)	Fat (g)	Carbohydrate (g)	Fe (mg)	Cu (mg)	Zn (mg)	Se (µg)	Ascorbic Acid (mg)
1-Month	525 (0)	9 (0)	33 (0)	57 (0)	0.3 (0)	0.3 (0)	2.4 (0)	10.5 (0)	30 (0)
3.5-Month	519 (35)	9 (1)	32 (2)	56 (4)	0.4 (0.2)	0.3 (0)	2.4 (0.1)	10.4 (0.3)	30 (2)
6-Month	667 (113)	14 (3)	35 (6)	81 (15)	8.5 (6.3)	0.5 (0.1)	3.7 (1.0)	13.3 (4.8)	48 (15)

Appendix B

Table 6.3. Reported Problems/Discomfort With Taking Medication

Symptom	Supplemented Group		Placebo Group	
	Reported	% of Infants	Reported	% of Infants
No Problem	16/38	42	20/32	63
Gas	11/38	29	9/32	28
Constipation	13/38	34	6/32	19
Cramps	2/38	5	0/32	0
Spit-up	4/38	11	3/32	9
Blood in Stool	1/38	3	0/32	0
Diarrhea	1/38	3	0/32	0

No statistical difference in problems encountered between groups by Fisher exact test ($p>0.05$).

Table 6.4. Problems/Discomfort Reporting Options

Option #	Description	Option #	Description
0	No Problem	8	Constipation, Spit-up
1	Gas	9	Spit-up, Constipation
2	Constipation	10	Constipation, Gas
3	Gas, Constipation	11	Blood in Stool
4	Cramps	12	Gas, Cramps, Constipation
5	Spit-up	13	Gas, Cramps, Spit-up
6	Gas, Spit-up	14	Diarrhea
7	Gas, Cramps		

Appendix C

DEPARTMENT OF BIOCHEMISTRY
MEMORIAL UNIVERSITY OF NEWFOUNDLAND
ST. JOHN'S, NEWFOUNDLAND, A1B 3X9

CONSENT TO PARTICIPATE IN RESEARCH

TITLE: IRON SUPPLEMENTATION OF HUMAN MILK FED FULL-TERM INFANTS
INVESTIGATORS: Drs. James Friel, Wayne Andrews, Khalid Aziz,
Drs. Mary Courage, Russell Adams, Poh Gin Kwa

You and your child have been asked to participate in a research project. Participation in the study is entirely voluntary. You may decide not to participate or you may decide to withdraw from the study at any time without affecting your normal treatment. Confidentiality of information concerning participants will be maintained by the investigators. The investigator(s) will be available during the study should you have any problems or questions about the study.

Below is a list of points which provide information about the study and what will be expected of you and your child should you decide to participate.

1. PURPOSE: We plan to compare the iron levels of breast-fed infants who have been provided with either an iron supplement or a placebo. Iron is an essential element for all of us and we want to ascertain that breast-fed infants are receiving a sufficient amount of it. We are also interested in cognition (learning, thinking, remembering), and language. Certain of these abilities may develop at a different rate depending on whether or not infants receive enough iron in early life. How much iron is needed in unknown. For this study, a breast-fed infant is someone who has received most of his/her food intake as breast-milk for a period of at least four months, preferably for six months.

2. DESCRIPTION OF PROCEDURES AND TESTS: All infants will be divided into two equal sized groups. One group will receive the iron supplement and the other the placebo. None of the Doctors involved will know into which group your infant is placed. In this manner all infants will be treated equally and the results will not be influenced by prior knowledge of group placement. For each infant in the study, we wish to take a small blood sample (about half a teaspoon) from your infant four times, once each at 1,3 ½, 6 and 12 months of age. This blood sample will be collected by Dr. Andrews, Dr. Aziz or their delegate who is experienced in this procedure. At 12 months, we wish to administer two tests of infant development. These will be conducted by psychologists who are well trained in these procedures. You will be asked to attend our Infant Nutrition Clinic at these times and to fill out a 3-day, dietary record of what your child eats and drinks, before you come to the clinic. You will also be asked to bring a stool sample in a plastic bag which we will supply to the 1 and 3 ½ month clinics and also to bring a 5 ml. (1 tsp.) breast milk sample to these clinics as well. We will ask you to supply a dietary record for the infant at 9 months of age although there will be no clinic visit at this time.

3. DURATION OF THE PROJECT: The project will last for 12 months.

4. FORESEEABLE RISKS, DISCOMFORTS, OR INCONVENIENCES: None of the developmental tests that are described above involve any risks or discomforts to your child. The only inconvenience involved is that if you live in

the St. John's area you will have to bring your child to Memorial University for testing at 12 months after he or she leaves the hospital. However, we will provide convenient, free parking. Also, we will ask you to fill out three day dietary records at 1, 3 ½, 6, 9 and 12 months of age. The only discomfort to your baby will be blood collection on four occasions.

5. BENEFITS TO YOU. The main benefit of participation is that you will be given a regular and immediate progress report on your child's development and also their iron status. If your child's development is below the norm for his or her age group in any way, you will know about it right away and can consult your family doctor for further referral. Early discovery of many problems can result in a better outcome.

6. ALTERNATIVE TREATMENT IF YOU DECIDE NOT TO PARTICIPATE: If you decide not to participate in this project, you can still have your child's developmental progress assessed by your family doctor or referral to other health care professionals. You may also wish to consult your family doctor about over the counter iron supplementation.

Finally, your signature on this form indicates that you have understood to your satisfaction the information regarding your participation in the research project and agree to participate as a subject. In no way does this waive your legal rights nor release the Investigators, sponsors, or involved institutions from their legal and professional responsibilities.

I, ----------, the undersigned, agree to my participation and to the participation

of ---------- my child or ward in the research project described.

Any questions have been answered and I understand what is involved in the study. I realize that participation is voluntary and that I can withdraw my participation at any time, and that there is no guarantee that I will benefit from my involvement. I acknowledge that a copy of this form has been given to me.

---------- ----------

Signatures of Parent/Guardian of Participant Date

---------- ----------

Signatures of witness Date

To be signed by investigator:

To the best of my ability I have fully explained to the subject the nature of this research project. I have invited questions and provided answers. I believe that the subject fully understands the implications and voluntary nature of the study.

---------- ----------

Signature of the Investigator Date

Appendix D

Breastfeeding Iron Study – Parent Introduction

How long do you plan to Breastfed?

This is a voluntary study in which we (Memorial University with the cooperation of the neonatologists at the Janeway, and Dr. Kwa at the Grace), are looking at whether Breastfed infants are receiving enough iron from Mother's milk. Iron is very important for growth, including brain functioning in the infant and all of us. We do not know at this time how much is needed.

Infants are placed in two groups:

1. One group will receive cherry syrup containing iron,
2. One group will receive cherry syrup with no iron.

Only the pharmacist preparing the study medication will know which babies are in the individual groups. We would like for you to attend the clinic at the Janeway where we will:

1. Weigh the baby
2. Take measurements of HT. and HC.
3. Take a small blood sample to check the iron content of the blood.
   1 ml of blood is taken only 4 times at:
   1 month
   31/2 months
   6 months
   12 months

At 12 months we would like to perform a developmental assessment of your child and that will tell us how they are progressing through normal development of learning, language development, behavior, & motor skills.

Your part in the study:

1. Three days before you bring your baby to the JCHC clinic, keep a record of what the baby feeds. So, for the first 4 months record the time and duration of Breastfeeding. After that time if you decide to introduce rice cereal, you would also record the amount and time.
2. We require 1 tsp of expressed breastmilk and a small stool sample of the baby. We will send the containers for these samples to you.

The clinics are at the JCHC and generally there is no waiting. We allow an appointment time of 45 minutes for each visit. Subsequent visits are usually a lot faster.

We are happy to provide compensation for:

1. Parking meters
2. Mileage allowance for gas
3. Taxi - should you require transportation.

You may also bring your other children if you need to.
You will also receive final results of the study.
I would like to call you again tomorrow to answer any questions and receive your answer.

Memorial University of Newfoundland
Department of Biochemistry
St. John's, NF, Canada
A1B 3X9

June 20, 2001

Dear:

I hope that all is well with you and your family.

We are requesting a three day dietary record from the mothers of the babies who participated in the Iron Supplementation Study. We would really appreciate it if you would take the time to complete this record and mail it in the self addressed envelope enclosed.

Thank you for you continued interest in our research. It is much appreciated.

All the Best,

Allison McDonald R.N.

Family Doctor Information Letter

July 28,1999

Dear Dr. _____:

_____ son/daughter of_____

is a participant in the Research Project entitled "The Iron Supplementation of Human Milk Fed Full Term Infants." The subjects in this study are randomized into one of two groups of which one group receives an iron supplement and the other a placebo. Please do not prescribe any iron preparation for this infant without first contacting Dr. Wayne Andrews or Dr. Khalid Aziz at the Janeway Child Health Centre. The telephone number is 778-4622.

For your information we are enclosing a copy of a study which shows that Iron does not cause constipation. Thank you very much for your cooperation.

Very truly yours,

_____

Dr. James K. Friel

_____

Dr. Wayne Andrews

_____

Dr. Khalid Aziz

Appendix E

Fecal Iron and Free Radical Production – Pilot Study

Fecal iron and free radical generating potential was measured to establish whether the supplemented infants have an increased free radical generating system in the gastrointestinal tract due to the higher concentration of iron passing through in the fecal materials. Iron concentrations were determined in 1 and 3.5-month stool samples to establish a baseline and peak supplementation iron concentration in the fecal material. The free radical generating capacity was determined using the methanesulfinic acid assay for hydroxyl radical generation. Due to insufficient amounts of fecal samples at 3.5-months it was not possible to obtain a value of methanesulfinic acid at this point. Therefore, methanesulfinic acid was only determined at 1 and 6 months. This makes the direct comparison between the fecal sample iron and methanesulfinic acid production impossible.

Iron was determined in a total of 20-paired fecal samples using FAAS at both 1 and 3.5-months. The methanesulfinic acid assay was carried out on 7 samples from the above noted 20 samples. The data are summarized in Table E1 and the relationships between methanesulfinic acid are shown in figure E.1 and fecal iron concentrations in figure E.2.

There was a higher level of methanesulfinic acid produced in the stool collected from infants at 6 months who had been receiving the iron supplement. This rise indicates the supplemental iron leads to increased free radical production in the feces as it passes through the colon. These findings are in keeping with data collected from iron-supplemented adults in the UK (Lund et al, 1999). Since no elevation was seen in the antioxidant defences and no decreases were observed in the FRAP values there does not appear to be any harm caused by this increase in free radical generation. Animal studies focusing on fecal MSA would be beneficial to elucidate

the actual effects on the colon and the rest of gastrointestinal tract compared to the responses of the antioxidant defence systems.

Table 6.5. Iron Content and Methanesulfinic Acid Production of Fecal Samples

Age	Supplemented Group		Placebo Group	
	Iron (mg/g dry feces)	Methanesulfinic Acid (mM/g wet feces)	Iron (mg/g dry feces)	Methanesulfinic Acid (mM/g wet feces)
N	*11*	*3*	*9*	*4*
1 Month	0.14 (0.09)	0.13 (0.06)	0.15 (0.13)	0.25 (0.12)
3.5 Month	1.32 (1.04)	-	0.36 (0.72)	-
6 Month	-	0.46 (0.03)	-	0.10 (0.06)

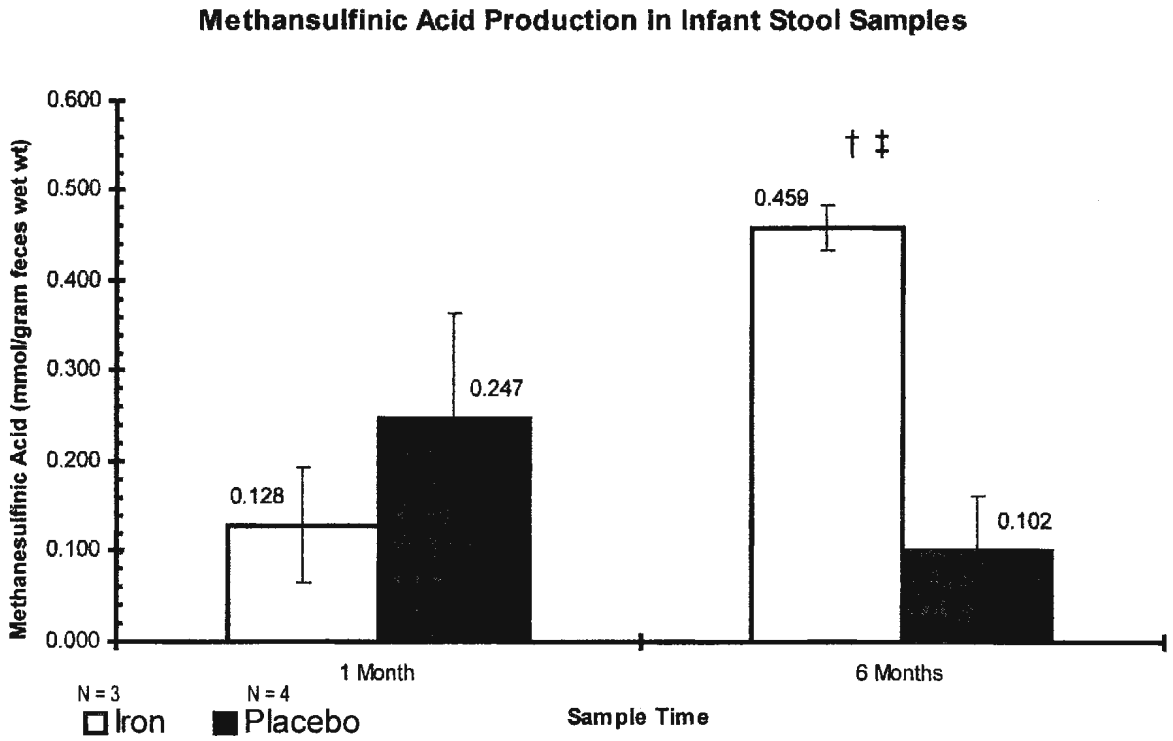

Figure 6.1 Methanesulfinic acid production from infant stool samples collected at 1 and 6 month clinic visits. Methanesulfinic acid is a measure of free radical generation. Mean +/- SD (error bars). † indicates difference between times within group; $p<0.05$. ‡ indicates difference between groups at same time; $p<0.05$.

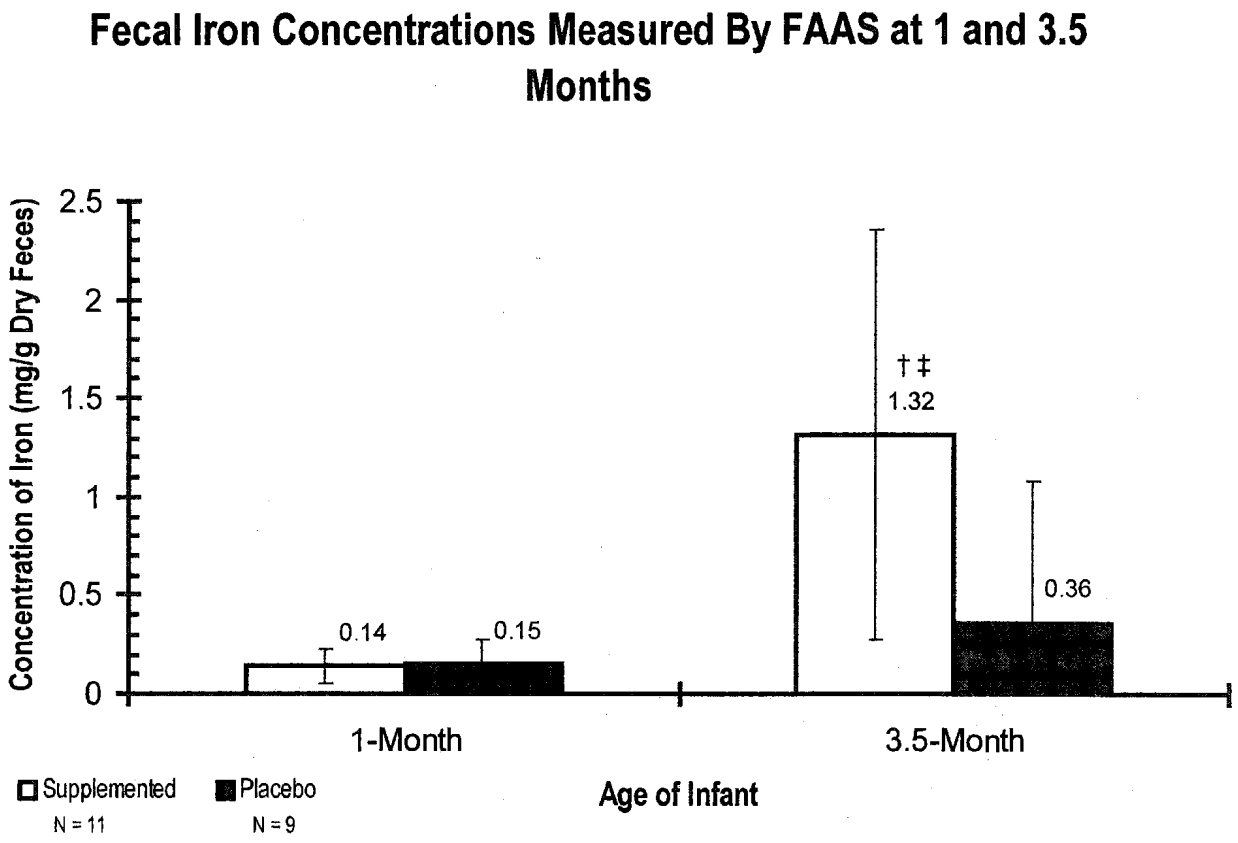

Figure 6.2 Fecal iron concentrations measured at 1 and 3.5-months. † denotes significant difference between time points within groups. ‡ denotes significant difference between group within particular time point.