Plasma hepcidin concentrations and factors associated with hemoglobin levels in infants and young children in Zimbabwe

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ABSTRACT

Iron metabolism is very dynamic over the first year of life. Anemia is common in sub-Saharan African infants, particularly in the context of HIV infection. Hepcidin, a peptide hormone whose synthesis is simultaneously regulated by iron status and the innate immune system, has evolved as the master regulator of iron metabolism, thereby linking iron homeostasis, inflammation, infection and anemia. However little is known about normal hepcidin values in infancy, its role in the pathogenesis of anemia during infancy or its role in the pathology of HIV infection or exposure. Plasma hepcidin concentrations were higher in 3-month-old (median 9.7 ng/mL [IQR 2.5, 19.3]), than in 6-month-old (4.5 ng/mL [IQR 0.5, 7.3]) and 12-month-old infants (1.9 ng/mL [IQR 0.7, 6.2)]) ($p<0.001$, Kruskal–Wallis) among healthy, non-anemic Zimbabwean infants with normal iron parameters at 3 (n=60), 6 (n=47) and 12 (n=40) months of age. The correspondingly lower levels of plasma hepcidin at 6 and 12 months in healthy non-anemic infants is likely a physiologic response to mobilize iron stores and increase iron absorption to prevent anemia. Plasma hepcidin concentrations were higher in HIV-infected compared to HIV exposed uninfected (HEU) and HIV-unexposed groups throughout infancy and correlated with levels of plasma ferritin and C- reactive protein (CRP). HEU infants also had higher levels of plasma hepcidin and inflammation (alpha-1-acid glycoprotein (AGP) and CRP) compared to HIV unexposed infants. Overall, anemia had no effect on plasma hepcidin concentrations during infancy except in HIV unexposed infants. Plasma hepcidin declined with age in all groups; girls had higher plasma hepcidin concentrations than boys. Plasma hepcidin concentrations appear to be driven by inflammation in infants, as has been shown in adults. We did not find a significant association between

infant and young child feeding (IYCF) indicators and hemoglobin levels in children 6-24 months using data from the 2010-11 Zimbabwe Demographic and Health Survey. However, Water, Sanitation and Hygiene (WASH) practice indicators were associated with hemoglobin levels in young Zimbabwean children adjusting for biological and social factors and warrant further investigation in randomized controlled trials.

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PREFACE

Hepcidin, a peptide hormone whose synthesis is simultaneously regulated by iron status and the innate immune system, has evolved as the master regulator of iron metabolism, linking iron homeostasis, inflammation, infection and anemia (Drakesmith& Prentice, 2008; Drakesmith& Prentice, 2012). Hepcidin blocks the activity of ferroportin, the only known mammalian iron exporter, (Ganz *et al*, 2008; Grebenchtchikov *et al*, 2009; Galesloot *et al*, 2011) effectively blocking iron efflux from duodenal enterocytes, hepatocytes, the placenta and macrophages (Nemeth *et al*, 2004; Nemeth *et al*, 2004; Drakesmith& Prentice, 2012). Since its discovery in 2000 there is burgeoning evidence that hepcidin measurements may reflect the dynamic iron metabolism typical of infancy. Rehu *et al* reported elevated plasma hepcidin in cord blood (Rehu *et al*, 2010) consistent with hemoglobin values that are highest at birth and decreased erythropoiesis during the first stage of iron nutrition. Neonatal serum hepcidin concentrations were comparatively lower in preterm infants (Muller *et al*, 2012) using the same assay, consistent with lower iron stores in preterm infants and a greater demand for iron compared to full term infants (Ganz *et al*, 2008; Rehu *et al*, 2010; Muller *et al*, 2012). A trial on the effects of iron supplementation reported increased serum hepcidin concentration over time in infants who were supplemented but not in the placebo group, consistent with higher iron stores and hemoglobin levels in the supplemented group (Berglund *et al*, 2011). We are only aware of one study in the literature that looks at the association between hepcidin and infection in infants and they reported a 4-fold increase in serum hepcidin concentrations in very low birth weight infants with compared to those without late onset neonatal sepsis

(Wu *et al*, 2013) inline with hepcidin's role in inflammation/infection. Although limited these studies suggest that hepcidin is associated with changes that have been described in infant iron nutrition and in the presence of infection (Rehu *et al*, 2010; Berglund *et al*, 2011; Muller *et al*, 2012; Young *et al*, 2012; Wu *et al*, 2013). These studies suggest that hepcidin could be useful as an indicator of iron status in infants however normative hepcidin values have not been described over the course of infancy and the role of hepcidin in other infectious conditions, in particular HIV, have not yet been fully characterized (Rehu *et al*, 2010; Berglund *et al*, 2011; Muller *et al*, 2012; Young *et al*, 2012; Wu *et al*, 2013).

The main objective of this study was to describe normative plasma hepcidin values in infancy and to determine the independent effects of HIV status, anemia and age on hepcidin concentrations during the first year of life. We also felt it necessary to determine if infant and young child feeding (IYCF) and water, sanitation and hygiene (WASH) indicators were associated with hemoglobin levels in infants and young children using nationally representative data.

In Chapter 2, we present normative values of plasma hepcidin generated from infants enrolled in the ZVITAMBO Vitamin A trial who had: a gestational age >37 weeks, birth weight > 2500g and never had abnormal iron indicators (i.e. hemoglobin < 105 g/L at 3 and 6 months and < 100g/L at 12months, serum ferritin < 12µg/l, sTfR > 8.3mg/L), inflammation (i.e. AGP > 1g/L or CRP> 5mg/L) or an acute illness (diarrhea, fever, in the prior week or measles in the prior 3 months) at 3, 6 and 12 months of age.

In Chapter 3, we characterize plasma hepcidin levels and inflammatory and iron status indicators in six groups of infants selected at 3, 6 and 12 months of age, based on HIV and anemia status: HIV-unexposed non-anemic infants (Group 1-Chapter 2), HIV-unexposed anemic infants (Group 2); HIV-exposed uninfected non-anemic infants (Group 3); HIV-exposed uninfected anemic infants (Group 4); HIV-infected non-anemic infants (Group 5) and HIV-infected anemic infants (Group 6). We describe the independent effects of HIV status, anemia and age on plasma hepcidin concentrations during the first year of life.

In Chapter 4, we examine the hypothesis that IYCF indicators will not be associated with hemoglobin because the iron-rich IYCF indicator primarily or solely counts intake of natural foods and so does not reflect iron consumption at levels that are relevant to infants iron needs. We also examine the hypothesize that WASH indicators that reflect fecal exposure among infants will be associated with hemoglobin levels during infancy based on our hypothesized causal pathway through Schitosomiasis, soil transmitted helminths (STH) and environmental enteropathy (EE), elevated hepcidin and anemia of inflammation in children aged 6-24 months using data from the 2010-11 Zimbabwe Demographic and Health Survey.

To my mother and father, the epitome of love, grace and kindness,

who gave me roots to know where home is and wings to fly away. And to my brother,

who reminds me not to take anything too seriously.

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Chapter 1

INTRODUCTION

1.1 Research objectives

Anemia is a serious public health problem affecting 2 billion people worldwide, particularly infants and young children in developing countries where its etiology is multifactorial (WHO.; Lokeshwar *et al*, 2011; Milman, 2011). Anemia in infancy is associated with impaired cognitive and behavioral development (Walter *et al*, 1989; Walter, 1994; Rao& Georgieff, 2002), impaired oxygen transport (Cullis, 2011) and a poorer prognosis in the context of many chronic diseases, including HIV (Xu *et al*, 2010).

Hepcidin, a peptide hormone whose synthesis is simultaneously regulated by iron status and the innate immune system, has evolved as the master regulator of iron metabolism, linking iron homeostasis, inflammation, infection and anemia (Drakesmith& Prentice, 2012). The molecular control of hepcidin is part of the innate immune response to pathogens and is stimulated by IL-6, IL-22, type I interferons, toll-like receptor (TLR) ligands, and the endoplasmic reticulum stress response (Drakesmith& Prentice, 2012). Hepcidin synthesis is stimulated by elevated plasma iron concentration, infection and/or inflammation, (Nemeth *et al*, 2004; Galesloot *et al*, 2011) and is suppressed in conditions that demand increased serum iron, such as increased or ineffective erythropoiesis, hypoxia, anemia and iron deficiency (Figure 1.1) (Kroot *et al*, 2010; Galesloot *et al*, 2011).

A limited number of studies have characterized hepcidin during infancy, (Rehu *et al*, 2010; Berglund *et al*, 2011; Muller *et al*, 2012; Wu *et al*, 2013) and none have studied infants in developing countries where the prevalence of anemia and infectious disease are both high. Specifically, only one study to date has reported hepcidin levels in HIV-infected adults, and none have described hepcidin in HIV-infected children.

We designed the hepcidin study to characterize normative plasma hepcidin concentrations in healthy non-anemic Zimbabwean infants and to determine the independent effects of HIV infection/ exposure and anemia on hepcidin concentrations in infancy. Understanding normative hepcidin levels in this age group will allow us to understand the hormonal regulatory mechanisms that allow healthy infants to avoid developing anemia. We set out to measure plasma hepcidin concentrations and to characterize normative values in healthy, non-anemic, Zimbabwean infants. We used an algorithm developed by Kroot *et al* to generate hepcidin consensus values, to allow for harmonization with different hepcidin assays (Kroot *et al*, 2012). Secondly, we determined plasma hepcidin levels in anemic and non-anemic infants in the context of HIV infection and exposure to better understand the pathophysiology underlying this highly prevalent condition of global health importance.

The multifactorial etiology of anemia in developing countries motivated us to conduct an epidemiological data analysis of the Zimbabwe Demographic and Health Survey (ZDHS) to better understand the primary processes implicated in the pathogenesis of anemia in developing countries. We hypothesized that indicators of Water Sanitation and Hygiene

(WASH) would be associated with anemia in young children because conditions of poor WASH are associated with *Schistosomiasis*, soil-transmitted helminths (STHs) and environmental enteropathy (EE). Furthermore, since most natural dietary sources of iron are not iron-concentrated enough to meet dietary iron requirements in 6 month – 3 year old children, and fortified infant foods are practically non-existent in Zimbabwe, we hypothesized that indicators of infant feeding practices would not be associated with anemia in this population (Lind *et al*, 2004; Baker& Greer, 2010; Domellof, 2011).

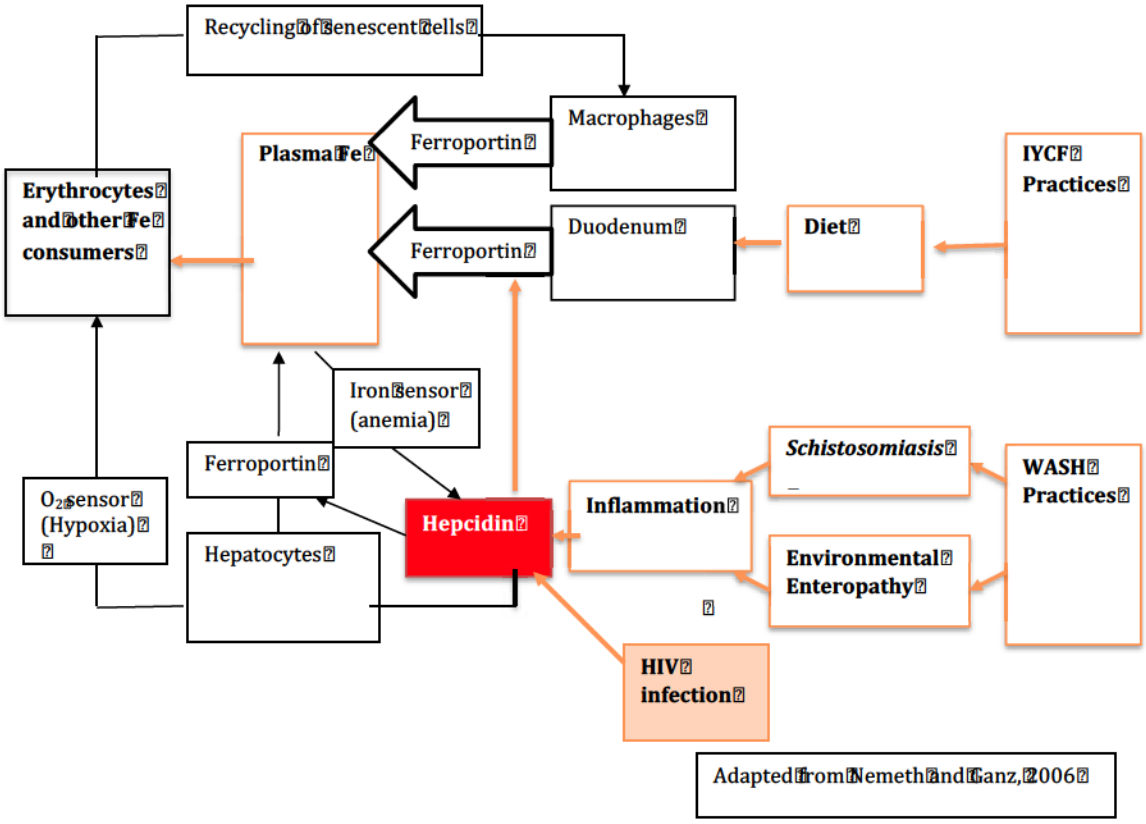

Figure 1.1. Hepcidin and Iron metabolism in infancy. Hepcidin binds to ferroportin, the only known mammalian iron exporter, (Ganz *et al*, 2008; Grebenchtchikov *et al*, 2009; Galesloot *et al*, 2011) inducing its degradation (Nemeth *et al*, 2004; De Domenico *et al*, 2007; Troutt *et al*, 2012) and inhibiting the release

of iron by macrophages and hepatocytes; and attenuates iron absorption by duodenal enterocytes (Grebenchtchikov *et al*, 2009; Pasricha *et al*, 2011). Hepcidin synthesis is stimulated by elevated plasma iron concentration, infection and/or inflammation, (Nemeth *et al*, 2004; Galesloot *et al*, 2011) resulting in decreased availability of circulating iron, hepcidin synthesis is decreased in conditions that demand increased serum iron concentrations, such as increased or ineffective erythropoiesis, hypoxia, anemia and iron deficiency (Kroot *et al*, 2010; Galesloot *et al*, 2011).

1.1.1. Specific aims

The specific aims of this study are presented in the corresponding chapters:

Chapter 2

- Estimate reference intervals for plasma hepcidin for non-anemic healthy infants at three ages (3, 6 and 12 months) where non-anemic healthy is defined as: gestational age at birth >37 weeks; birth weight > 2500 g; hemoglobin ≥ 105 g/L at 3 and 6 months and ≥ 100 g/L at 12 months; plasma ferritin ≥ 12 μg/L; soluble transferrin receptor (sTfR) ≤ 8.3 mg/L; AGP < 1 g/L and CRP < 5 mg/L at 3, 6, and 12 months of age; and no history of diarrhea or fever in the week prior or measles in 3 months prior to blood drawing at 3, 6, and 12 months of age from a well-characterized cryopreserved archive of plasma collected from HIV negative mother infants pairs participating in the ZVITAMBO study in Harare Zimbabwe (1998-2001).

Chapter 3

- Characterize the independent associations between HIV status, anemia and hepcidin concentration in infants, by measuring plasma hepcidin concentrations in cryopreserved samples from the ZVITAMBO trial collected from 6 groups of infants: HIV-unexposed non-anemic (chapter 2), HIV-unexposed anemic, HIV-exposed uninfected (HEU) non-anemic, HEU anemic, HIV-infected non-anemic; HIV-infected anemic at 3, 6, 12 months. Infants were classified as HIV unexposed if the mother and infant pair were HIV negative at delivery and did not seroconvert during the trial period; HEU infants were born to HIV positive

mothers but were HIV negative at delivery and did not seroconvert during the trial period. HIV infected infants were infected intrauterine (IU), intrapartum (IP) or postnatal (PN). Anemia was defined by hemoglobin ≤ 105 g/L at 3 and 6 months and ≤ 100g/L at 12 months.

- Determine the correlation between plasma hepcidin and other biomarkers: plasma ferritin and CRP.

Chapter 4

- Test the hypothesis that indicators of poor environmental sanitation and personal hygiene that reflect fecal exposure among infants will be associated with hemoglobin levels during infancy based on our hypothesized causal pathway through Schistosomiasis, STHs and EE. Together with the hypothesis that IYCF indicators will not be associated with hemoglobin levels because the iron-rich IYCF indicator lacks specificity with regard to iron consumption among infants and young children in Zimbabwe using data from the 2010-11 Zimbabwe Demographic and Health Survey.

1.2 Background and significance

1.2.1 Anemia

Anemia is a serious public health problem affecting 2 billion people worldwide particularly infants and children. (Lokeshwar *et al*, 2011; Milman, 2011). The presence of anemia is defined by a red blood cell count below the accepted lower level of the normal range that compromises oxygen carrying capacity (Tolentino& Friedman, 2007; Milman, 2011). Hence the underlying pathology of anemia is an insufficient supply of oxygen, which may result in fatigue, limited attention span, tachycardia and tachypnea (Wardrop *et al*, 1978; Cullis, 2011). Anemia is also associated with a poorer prognosis in many chronic diseases including HIV, although studies on whether anemia plays a causative role in determining prognosis are inconclusive (Humphrey *et al*, 2006; Xu *et al*, 2010). Iron deficiency anemia in particular is associated with impaired cognitive, motor and behavioral development in infancy (Walter *et al*, 1989; Walter, 1994; Rao& Georgieff, 2002). Infancy is a key period for brain development and hence iron uptake is at its peak during infancy (Taylor& Morgan, 1990). Iron is required for myelination of nerve fibers, energy metabolism, and is a cofactor for enzymes involved in neurotransmitter synthesis (Shaw& Friedman, 2011).

1.2.2 Iron metabolism in infancy

Iron metabolism is very dynamic during infancy, with iron requirements 10-fold higher than in adults, because of rapid infant growth (Tolentino& Friedman, 2007). The average term infant triples in body weight within the first year and body iron almost doubles

(Dallman, 1980). Iron metabolism in infancy can be divided into 3 stages (Figure 1.2). The term infant has on average 75 mg/kg total body iron at birth, 25% of which is in iron stores (Rao& Georgieff, 2002). Hemoglobin concentration is highest at birth, followed by a rapid decline in hemoglobin due to decreased erythropoiesis and shorter red blood cell lifespan, with a nadir between 6 and 8 weeks (Dallman, 1987) while iron stores increase due to recycling of senescent erythrocytes (Domellof, 2011). Erythropoiesis increases in the second stage reversing the decline in hemoglobin concentrations and storage iron gradually decreases (Dallman *et al*, 1980). Endogenous iron stores become depleted in the third stage, which occurs around 3 months of age in preterm and 4 months of age in term infants and dietary iron is needed to maintain hemoglobin concentrations. Endogenous iron stores become depleted earlier in low birth weight infants due to a more rapid rate of postnatal growth and the third stage of iron metabolism begins earlier than in term infants (Gorten& Cross, 1964; Lundstrom *et al*, 1977). Insufficient dietary iron during the third stage of iron nutrition is associated with a decline in hemoglobin concentration and a rising prevalence of iron deficiency anemia (Dallman *et al*, 1980).

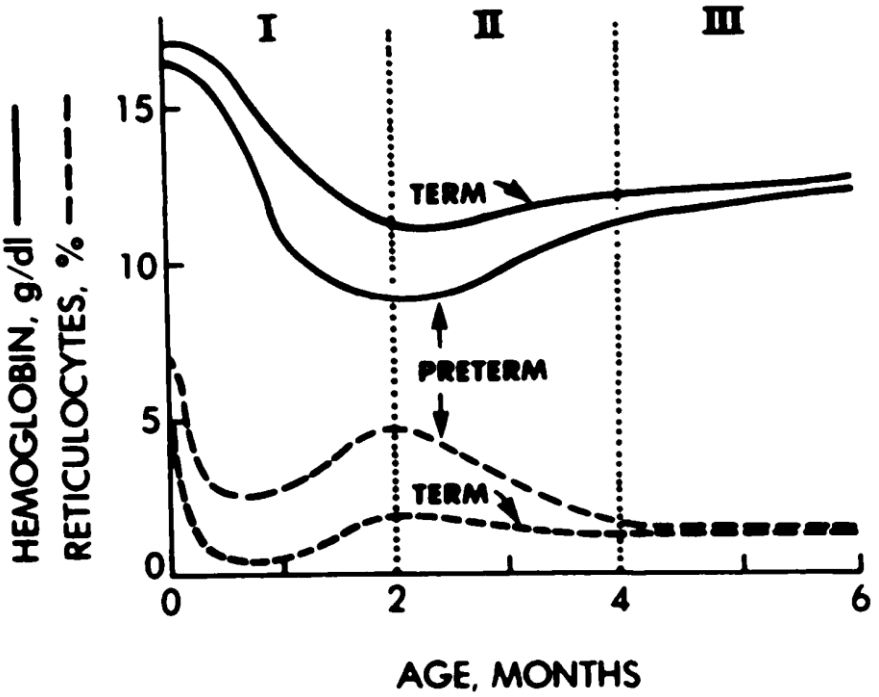

Figure 1.2. Iron metabolism in infancy. Stages I, II and III of iron nutriture. Mean values for hemoglobin (g/dL) and reticulocyte count (%) for term and preterm infants. (Reproduced from (Dallman *et al*, 1980)).

1.2.3 Development of iron deficiency

Reduced storage iron in the presence of normal levels of transport iron and hemoglobin is the first stage in the development of iron deficiency (Table 1.1) (Vendt *et al*, 2007). Iron deficient erythropoiesis, the second stage of iron deficiency, is marked by exhaustion of iron stores, decreased transferrin saturation and limited iron supply to the bone marrow. Limited iron for heme synthesis leads to accumulation of erythrocyte protoporphyrin, a heme precursor in the red blood cells(Gibson, 1990). However hemoglobin levels usually remain in the normal range until the third stage and characterize iron deficiency anemia (IDA). Exhaustion of iron stores, depressed levels of circulating iron, microcytic and

hypochromic anemia characterize IDA, the third stage of iron deficiency. Hence to identify IDA both hemoglobin an indicator of anemia and two indicators of iron deficiency such as low erythrocyte mean cell volume (MCV), low ferritin, high zinc protoporphyrin (ZPP) and/ high sTfR (Breymann et al, 2011; Domellof et al, 2007) are needed. The WHO for example defines IDA in infants as a combination of hemoglobin < 110 g/L, ferritin < 12 µg/L and sTfR > 8.3 mg/L (WHO., 2001; Carvalho *et al*, 2010) to reflect functional impairment, tissue avidity for iron, and iron storage, respectively in determining IDA (WHO., 2001; WHO/CDC., 2007), together with the use of acute phase protein in areas with high rates of infection (WHO/CDC., 2007).

	Iron depletion	Iron-deficient erythropoiesis	Iron deficiency anemia
Plasma ferritin (µg/L)	↓	↓↓	↓↓
Transferrin saturation (%)	Normal	↓	↓
sTfR	Normal	↑	↑↑
RBC protoporphyrin (µg/dL)	Normal	↑	↑↑
Hemoglobin g/dL	Normal	Normal	↓

Erythrocytes	Normal	Normal	Microcytic/ Hypochromic

Table 1.1: Stages in the development of iron deficiency. Adapted from (Herbert, 1987).

1.2.4 Iron Biomarkers

Soluble transferrin receptors (sTfR) are present on the surfaces of every iron incorporating cell and facilitate uptake of iron into the cell (Beguin, 2003). Increased sTfR indicates functional iron deficiency, increased erythropoiesis and increased iron needs (Breymann, 2002; Lewis *et al*, 2007; Jain *et al*, 2010). However sTfR is affected by the rate of erythropoiesis, which varies by age hence age cutoffs are still needed (Beguin, 2003; Kung'u *et al*, 2009). Ferritin is the most frequently used indicator of iron status. In healthy individuals a ferritin concentration of 1 μg/L (between 20-300 μg/L) is equivalent to ≈10 mg of storage iron (Gibson, 1990). Although low plasma ferritin is a reliable index of iron deficiency in the absence of inflammation, ferritin is a positive acute phase protein and thus normal or raised values maybe observed in deficient individuals in the presence of inflammation (Totin *et al*, 2002). Therefore to avoid misclassification of iron biomarkers in epidemiological or clinical studies, the WHO recommends measurement of an acute phase protein (APP) to aid in the interpretation of iron status indicators in areas with high rates of infection.

1.2.5 Acute Phase Proteins

The acute phase response (APR) is an innate immune response to inflammation triggered by cytokines including interleukin-1 (IL-1), tumour necrosis factor-alpha (TNF-a) and

IL-6 (Northrop-Clewes, 2008). Iron metabolism and iron status is influenced by infection and inflammation through the APR, obscuring the interpretation of iron biomarkers most notably ferritin a positive acute phase protein (APP), as the host modifies iron metabolism to make iron less available to pathogens. APPs frequently used in epidemiological and clinical studies include CRP, AGP and a-1-antichymotrypsin (ACT) (Rawat *et al*, 2009).

CRP increases rapidly within the first 5 hours of infection and peaks between 24 and 48 hours after infection followed by a rapid decline (Figure 1.3). AGP rises more slowly and peaks between 3–5 days after infection, and remains elevated in different infections (Rawat *et al*, 2009). ACT rises rapidly following infection, and remains elevated for some time after clinical symptoms disappear making it a good marker of both acute and chronic infection (Thurnham *et al*, 2005; Northrop-Clewes, 2008; Rawat *et al*, 2009).

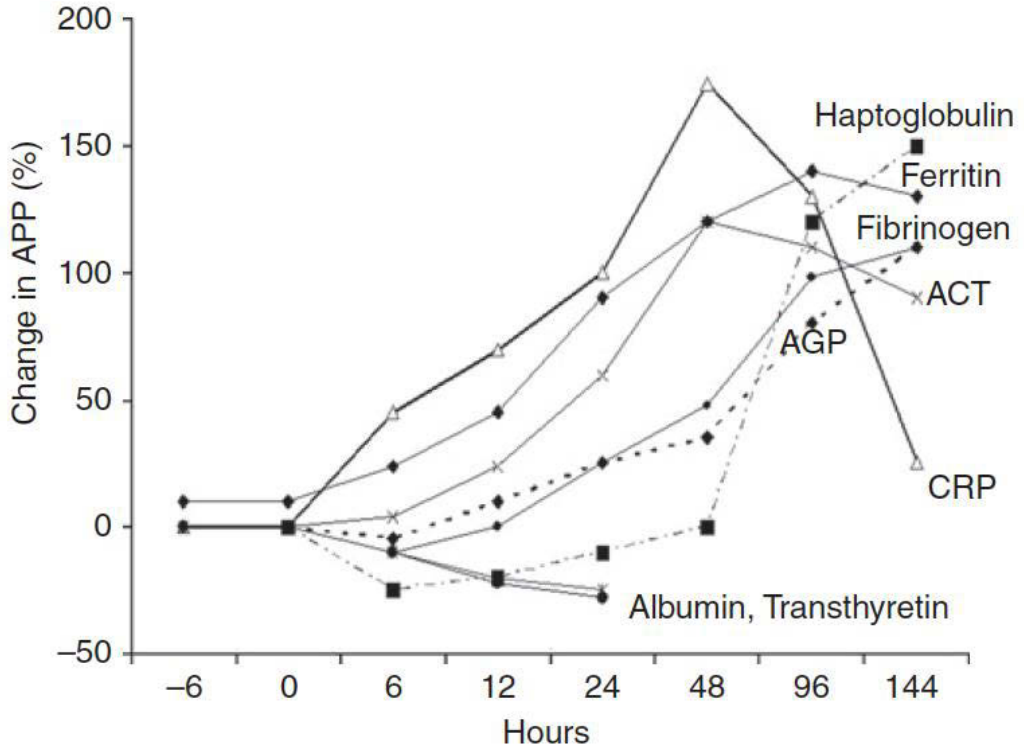

Figure 1.3. Percentage change in APP in response to inflammation/infection. (Reproduced from (Northrop-Clewes, 2008)).

Ferritin concentrations like CRP peak at day 2, but like AGP, remain elevated for several subsequent days (Feelders *et al*, 1998). Hence there is need to adjust for the APR in the presence of inflammation (Kung'u *et al*, 2009). Iron status indicators, ZPP, TfR, and EPO are less perturbed by inflammation than ferritin (Kung'u *et al*, 2009), although there is some evidence that sTfR is influenced by inflammation in HIV infected adults (Rawat *et al*, 2009). TfR concentrations are depressed in anemic HIV positive Zimbabwean women with evidence of inflammation (AGP >1 g/l). This may reflect suppression of erythropoiesis by pro-inflammatory cytokines including IL-1, IL-6 and TNF-a, which simultaneously result in increased AGP concentrations (Rawat *et al*, 2009).

1.2.6 Hepcidin

Originally named liver-expressed antimicrobial peptide (LEAP-1) (Krause *et al*, 2000; Park *et al*, 2001; Pigeon *et al*, 2001; Franchini *et al*, 2010) on its discovery by 3 independent research groups between 2000 and 2001(Krause *et al*, 2000; Park *et al*, 2001; Pigeon *et al*, 2001), hepcidin is considered the major regulator of iron metabolism (Ganz *et al*, 2008; Grebenchtchikov *et al*, 2009; Kroot *et al*, 2010; Galesloot *et al*, 2011; Pasricha *et al*, 2011; Troutt *et al*, 2012). Hepcidin, a 25-amino acid peptide hormone is secreted by hepatocytes as an 84-amino-acid pre-prohepcidin (Pigeon *et al*, 2001) encoded by the human hepcidin gene (HAMP; OMIM 606464), which contains three exons and is located on chromosome 19q13.1(Franchini *et al*, 2010). The prohormone is converted into a 64 amino acid prohepcidin during export from the cytoplasm and further posttranslationally converted into hepcidin-25 amino acid (Wessling-Resnick, 2008). Further processing generates two smaller N-terminal truncated isoforms (hepcidin-20 and -22) whose biological function has not yet been determined (Park *et al*, 2001; Ganz *et al*, 2008; Grebenchtchikov *et al*, 2009). The peptide hormone is a member of the defensins family, a group of small cationic, antibiotic peptides that form part of the innate immune system (Ganz& Lehrer, 1995).

Hepcidin regulates iron influx into the plasma by regulating tissues involved in iron absorption, storage, transport and recycling of iron from senescent erythrocytes: duodenal enterocytes, hepatocytes, the placenta and macrophages (Ganz *et al*, 2008; Grebenchtchikov *et al*, 2009; Franchini *et al*, 2010; Galesloot *et al*, 2011). Hepcidin

binds to ferroportin the only known mammalian iron exporter, inducing its internalization and lysosomal degradation, thereby effectively blocking iron efflux from macrophages, the placenta and hepatocytes; and attenuating iron absorption by duodenal enterocytes (Grebenchtchikov *et al*, 2009; Pasricha *et al*, 2011). Hepcidin synthesis is physiologically increased by elevated plasma iron concentration (Pigeon *et al*, 2001; Nemeth *et al*, 2004) and pathologically increased by infection and/or inflammation (Nemeth *et al*, 2004; Wessling-Resnick, 2010) resulting in decreased availability of circulating iron. Its synthesis is suppressed in conditions that demand increased serum iron, such as increased or ineffective erythropoiesis, hypoxia, anemia and iron deficiency (Kroot *et al*, 2010; Galesloot *et al*, 2011). Decreased hepcidin concentrations result in the release of macrophage iron and increase duodenal iron absorption (Nemeth& Ganz, 2006). Hepcidin deficiency is the cause of iron overload in most hereditary hemochromatosis and contributes to iron overload in β-thalassemia and other iron-loading anemia types (Ganz *et al*, 2008).

1.2.7 Hepcidin assay

Research on hepcidin has been hampered by difficulties in isolating and qualifying absolute values of hepcidin in blood or urine specimens and by the lack of congruence in hepcidin measurements between different assays (Kroot *et al*, 2009). Two types of hepcidin assays are currently available for research use: immunoassays based on anti-hepcidin antibodies, and mass spectrometric assays (MS) that detect the characteristic mass of the active 25 amino acid hepcidin species (Ganz& Nemeth, 2011). Immunoassays include competitive ELISA (Ganz *et al*, 2008; Galesloot *et al*, 2011),

enzyme immunoassay (EIA) (Berglund *et al*, 2011) and radioimmunoassay (RIA) (Grebenchtchikov *et al*, 2009). MS methods include surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS), matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and liquid chromatography tandem-MS techniques (LC-MS/MS). The lack of congruence between hepcidin measurements from different assays has been attributed to: the use of different calibration solutions; hepcidin binding to interfering substances such as albumin and presence of biologically inactive hepcidin isoforms hepcidin-20 and 22 (Kroot *et al*, 2009). These potential sources of measurement error are also thought to differentially affect immunochemical and mass spectrometry methods and sample type (urine, plasma and serum samples) (Kroot *et al*, 2009).

Hence there is currently no gold standard assay, each approach offering relative advantages and caveats (Table 1. 2). Immunoassays are limited by the hepcidin's highly conserved "compact hairpin," structure with four disulfide bonds among eight cysteine residues which restricts the number of antigenic epitopes (Castagna *et al*, 2010). They also lack specificity for hepcidin isoforms because of antibody cross-reactivity and over estimate hepcidin concentrations compared to mass spectrometry methods in diseases in which hepcidin isoforms are prevalent (Galesloot *et al*, 2011). It is, however, worth noting that such isoforms are rare in reference healthy populations (Galesloot *et al*, 2011). Advantages of immunoassays include: a lower limit of detection, lower cost, high-throughput and potential for wide application in clinical settings compared to MS. Mass spectrometry-based methods on the other hand are often semi-quantitative due to

competition during binding steps, and variations in ionization efficiency unless a proper internal standard is used (Ganz *et al*, 2008). Mass spectrometric techniques also rely on expensive equipment that is not widely available (Ganz *et al*, 2008) and require skilled personnel.

Assay	Advantages	Limitations
Immunoassays	Low limit of detection Low cost High-throughput	Lack specificity for hepcidin isoforms
Mass spectrometry-based methods	Can distinguish between hepcidin isoforms	Provide semi-quantitative measurements unless a proper internal standard is used Rely on expensive equipment that is not widely available Require skilled personnel.

Table 1.2 Advantages and limitations of hepcidin assays

An interim solution is an algorithm generated from the medians of duplicate native samples obtained by 2 highly correlated methods(Kroot *et al*, 2012). The algorithm can be used to generate hepcidin consensus values (HEPCON1) for 13 ‘‘well performing’’

assays (within sample variation (reliability) < 10% of the total variance and a spearman rank correlations > 0.90 with the other assays) from an initial list of 11 MS (Murao *et al*, 2007; Murphy *et al*, 2007; Kobold *et al*, 2008; Swinkels *et al*, 2008; Ward *et al*, 2008; Altamura *et al*, 2009; Bansal *et al*, 2009; Li *et al*, 2009; Crockett *et al*, 2010; Kroot *et al*, 2010; Anderson *et al*, 2011) and 10 immunoassays (De Domenico *et al*, 2008; Ganz *et al*, 2008; Busbridge *et al*, 2009; Grebenchtchikov *et al*, 2009; Koliaraki *et al*, 2009; Butterfield *et al*, 2010; Kroot *et al*, 2010; Schwarz *et al*, 2011; Kroot *et al*, 2012). A caveat however is that these algorithms have not yet been applied by the general research community and may be less reliable in the low and high hepcidin concentration range (Kroot *et al*, 2012).

1.2.8 Relationship between hepcidin and iron and inflammatory biomarkers

A limited number of studies have characterized the association between hepcidin and markers of infection, inflammation and iron status in humans. However studies have consistently reported a strong correlation between hepcidin and ferritin, the most important correlate of hepcidin concentration in healthy adults (Ganz *et al*, 2008; Schulze *et al*, 2008; Swinkels *et al*, 2008; Ashby *et al*, 2009; Galesloot *et al*, 2011) preterm infants (Muller *et al*, 2012) and among low birth weight infants (6 weeks, 12 weeks and 6 months-old) (Berglund *et al*, 2011). A 1% change in serum ferritin (μg/L) for example was associated with a 0.81% and 0.85% change in hepcidin concentration (nmol/L), in men and women respectively, in adjusted linear regression models in a large population based study in the Netherlands (Galesloot *et al*, 2011). Both hepcidin and ferritin are

positive APP and respond similarly to inflammation and changes in iron stores. However, hepcidin responses take place on the time scale of a few hours peaking 6 hours after infection, whereas changes in ferritin concentrations are much slower peaking 24 to 48 hours after initiation of inflammation (Ganz *et al*, 2008).

Although more modestly associated and inconsistent, hepcidin levels have also been associated with other indictors of iron status including MCV, sTfR, transferrin saturation, transferrin, TIBC and serum iron (Rehu *et al*, 2010; Berglund *et al*, 2011; Muller *et al*, 2012).

Although IL-6 triggers both CRP and hepcidin synthesis, studies on the correlations between hepcidin and CRP are inconsistent. A positive association between hepcidin and CRP has been reported in adults (Galesloot *et al*, 2011) and neonates with sepsis, making it a useful biomarker for diagnosing late onset neonatal sepsis (Wu *et al*, 2013). However hepcidin was not correlated with CRP in preterm infants (Muller *et al*, 2012). Hepcidin was modestly associated with AGP but not CRP in pregnant Bangladeshi women (Schulze *et al*, 2008). The inconsistent results may be due to the fact that hepcidin and CRP have different time course of synthesis: hepcidin concentrations change much faster and have been reported to peak 6 hours after initiation of inflammation in contrast to CRP concentrations that peaks between 24 to 48 hours after initiation of inflammation (Northrop-Clewes, 2008; Schulze *et al*, 2008; Muller *et al*, 2012; Wu *et al*, 2013).

1.2.9 Hepcidin studies in infancy

Given the pivotal role of hepcidin in understanding and characterizing iron metabolism in infancy, studies quantifying hepcidin levels across infancy are warranted. Establishing normative values of plasma hepcidin in healthy growing infants is particularly important because of the dynamic hematological changes associated with infancy. However a limited number of studies have characterized hepcidin levels in infancy and the use of different assays often precludes comparisons across studies.

Only one study has described reference values for hepcidin in cord blood. Rehu *et al* reported plasma hepcidin median (geometric mean concentration (GMC) range) 78.4 (20.5 and 231.9 ng/mL) using an ELISA from Intrinsic Life Sciences, CA, USA among 137 healthy full term infants (37- 44 weeks gestation) in Finland (Rehu *et al*, 2010) (Table 1.2). The study excluded newborns with anemia, iron deficiency, increased erythropoiesis, inflammation and iron overload as defined by hemoglobin ≤ 145 g/L, transferrin saturation ≤ 30%, erythropoietin (EPO) > 97.5th percentile, CRP ≥ 10 mg/L and serum ferritin > 1153.3 µg/L in cord blood, respectively (Rehu *et al*, 2010). A second study using the same assay reported similar hepcidin levels (median (SD) 61.7 (77.0 µg/L)) among 19 neonates at birth (Young *et al*, 2012). The high hepcidin levels reported by both these studies are consistent with the very high hemoglobin levels and slow rate of erythropoiesis during the first stage of iron nutrition.

Neonatal serum hepcidin concentrations were lower in a study among 31 preterm infants (23-32 weeks gestation) GMC (95% CI) 48.5 (33.0-71.3 ng/mL) at 35 days postnatal (IQR: 26-48 days) (Muller *et al*, 2012) using the same assay. This is consistent with lower iron stores in preterm infants and a greater demand for iron compared to full term

infants (Ganz *et al*, 2008; Rehu *et al*, 2010; Muller *et al*, 2012).

A trial on the effects of iron supplementation with 1 or 2 mg/kg of iron daily from 6 weeks to 6 months of age among 285 low birth weight infants (between 2000 and 2500 g) reported mean (SD) hepcidin 12.2 ng/mL (1.8 ng/mL) at 6 weeks using an ELISA from Bachem CA, USA (Berglund *et al*, 2011). Hepcidin concentration increased over time in infants who were supplemented but did not change over time in the placebo group reaching mean ± SD in the 2 mg/kg group of 19.2 ± 2.5 ng/mL compared to 13.0 ± 2.6 ng/mL in the placebo group (p=0.001) at 6 months (Berglund *et al*, 2011). Previous studies that have shown that infants have the ability to upregulate iron absorption when iron requirements increase (Domellof *et al*, 2002; Hicks *et al*, 2006). Hence these results offer a possible mechanism through which infants adapt iron absorption to iron status.

Hepcidin levels were lower in low birth weight infants compared to normal birth weight infants (Rehu *et al*, 2010). This is in line with increased iron demand associated with a more rapid rate of postnatal growth in low birth weight infants. However, our ability to make quantitative inferences about the difference in the hepcidin values reported in this study compared to the studies reported above is limited by the lack of harmonization between different assays (Kroot *et al*, 2009).

We are only aware of one study in the literature in which the association between hepcidin and infection in infants was examined. Wu *et al* reported 4-fold higher levels of hepcidin concentrations in very low birth weight (VLBW) infant with late onset neonatal sepsis compared to VLBW infants without late onset neonatal sepsis and similar hepcidin concentrations between the two groups after therapy using capture enzyme-linked

immunosorbent assay (Wu *et al*, 2013).

Although limited, these studies suggest that hepcidin is influenced by both iron nutriture and infection in preterm and low birth weight infants (Rehu *et al*, 2010; Berglund *et al*, 2011; Muller *et al*, 2012; Young *et al*, 2012; Wu *et al*, 2013)..

1.2.10 Hepcidin studies in adults

A number of hepcidin studies have been conducted in adults (Ganz *et al*, 2008; Swinkels *et al*, 2008; Grebenchtchikov *et al*, 2009; Galesloot *et al*, 2011). Ganz *et al* created a reference for plasma hepcidin concentration in adult men and women and reported a 5% to 95% range of 29 to 254 ng/mL median 112 ng/mL in men (n=65) and 17 to 286 ng/mL median 65 ng/mL in women (n=49) using the first competitive ELISA (Ganz *et al*, 2008). Grebenchtchikov *et al* developed a new RIA (CV 4-6% range) and reported significantly higher median hepcidin concentration in men compared to women (Grebenchtchikov *et al*, 2009). However, a study using SELDI-TOF MS did not find a significant difference in hepcidin concentrations between men and women (Swinkels *et al*, 2008).

Galesloot *et al* extended these findings using a similar competitive ELISA to Ganz *et al* (Kroot *et al*, 2010) and established age- and sex-stratified reference ranges (median, 2.5th and 97.5th percentiles) for serum hepcidin concentration using a population-based sample from the Netherlands (n = 2998). Participants who were pregnant, had alanine aminotransferase (ALT) > 50 U/L, CRP >10 mg/L, estimated glomerular filtration rate < 60 mL/min/1.73 m^2, using iron supplements, anemic, or had a BMI > 30 kg/m^2 were not

eligible for the study, however no strict definition of 'healthy' was employed. Galesloot *et al* and Grebenchtchikov et *al* both reported diurnal variation in hepcidin levels, however their results are inconsistent (Grebenchtchikov *et al*, 2009; Galesloot *et al*, 2011). Galesloot *et al* reported lower hepcidin values from blood samples obtained in the morning (before 12pm) compared to blood samples obtained between 12 and 5 pm in both men and women. In contrast median hepcidin levels were 1.83 and 1.70 times higher at 9 am compared to 4 pm in men and women respectively in a study by Grebenchtchikov *et al* (Grebenchtchikov *et al*, 2009).

Hepcidin concentrations were constant over age in men median (2.5th and 97.5th percentiles): 7.8 nM (0.6 - 23.3 nM) but were higher in postmenopausal compared to premenopausal women (4.1 nM (0.4 - 19.7 nM) in women < 55 years and 8.5 nM (1.2-24.8 nM) in women > 55 years) (Galesloot *et al*, 2011)[1]. Galesoot *et al* reported lower median hepcidin concentrations compared to Ganz *et al* and found a less pronounced difference in hepcidin concentrations between men and women (Ganz *et al*, 2008; Galesloot *et al*, 2011). These differences might be due to the fact that the 2 studies used different assays and women in the study by Galesoot *et al* had a higher median age (55 years) compared to women in the study by Ganz *et al* (32.6 years) (Ganz *et al*, 2008; Galesloot *et al*, 2011). Although the two studies used similar immunochemical assays, they used different antibodies highlighting the need for harmonizing hepcidin assays

[1] To convert hepcidin-25 from nmol/L to nM or ng/mL, multiply by 2.789 Peters, H. P., Rumjon, A., Bansal, S. S., Laarakkers, C. M., van den Brand, J. A., Sarafidis, P., Musto, R., Malyszko, J., Swinkels, D. W., Wetzels, J. F. & Macdougall, I. C. (2012). Intra-individual variability of serum hepcidin-25 in haemodialysis patients using mass spectrometry and ELISA. Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association **27**(10): 3923-3929.

(Ganz *et al*, 2008; Galesloot *et al*, 2011).

Although the difference in absolute hepcidin values obtained by different assays precludes the comparison of hepcidin concentrations across different assays, among studies using the Ganz assay, hepcidin concentrations in infants are consistently lower compared to those in adults (Ganz *et al*, 2008; Rehu *et al*, 2010; Muller *et al*, 2012; Young *et al*, 2012). These infant studies included cord blood samples from healthy term infants (Rehu *et al*, 2010), neonates (Young *et al*, 2012) and preterm infants 35 days postnatal (Muller *et al*, 2012). This is consistent with higher iron needs in infants compared to adults particularly in preterm infants. We would expect hepcidin concentrations to be even lower in older infants as erythropoiesis increases and the infant becomes dependent on exogenous iron in the 2nd and 3rd stage of iron nutrition respectively.

Chapter 2 characterizes hepcidin levels in healthy non-anemic infants at 3 ages (3, 6 and 12 months), in the 2nd and 3rd stages of iron nutriture, when anemia is more prevalent, as erythropoiesis increases and the infant becomes dependent on exogenous supply of iron, respectively. Older infants are particularly vulnerable to anemia through mechanisms described above, however anemia has been described as early as 3 months. By selecting healthy non-anemic infants, we describe hepcidin levels that are apparently associated with effective mobilization of iron stores and adequate absorption of dietary iron.

1.2.11 The role of hepcidin in anemia of inflammation

Hepcidin's role as part of the innate immune system is associated with anemia of inflammation. Anemia of inflammation is defined as a mild-to-moderate, normochromic/ normocytic anemia associated with a chronic inflammatory, infectious or neoplastic illness and altered iron homeostasis (Lee, 1983; Weiss, 2002; Jain *et al*, 2010; Cullis, 2011). Anemia of inflammation is induced by a stimulated innate immune system through mechanisms mediated by inflammatory cytokines, acute phase proteins and hepcidin (Weiss, 2002; Means, 2004; Weiss& Goodnough, 2005; Weiss, 2009). The hypoferremia associated with anemia of inflammation is caused primarily by iron sequestration into reticuloendothelial macrophages (Lee, 1983; Tolentino& Friedman, 2007). Increased uptake and retention of iron within cells of the reticuloendothelial system leads to a diversion of iron from the circulatory system into storage sites of the reticuloendothelial system (Weiss& Goodnough, 2005). Macrophages recycle 30 mg of iron per day compared to 1-2 mg of iron absorbed from the diet, hence if iron is not released from the macrophages erythropoiesis is restricted (Weiss& Goodnough, 2005; Drakesmith& Prentice, 2008; Xu *et al*, 2010). Proinflammatory cytokines interleukin-1 (IL-1) and tumor necrosis factor α (TNF- α) induce ferritin synthesis, iron acquisition and iron storage by the reticuloendothelial system (Weiss, 2002). Hepcidin synthesis is stimulated by TNF-α and IL-6 (De Domenico *et al*, 2007) and is the primary mediator for altered iron homeostasis (Ganz, 2002; Ganz, 2003). Elevated hepcidin levels are associated with low serum iron and normal or elevated serum ferritin, the diagnostic factor in anemia of inflammation (Lee, 1983; Means& Krantz, 1992; Means, 2000). Hepcidin contributes to anemia of inflammation primarily through its effects on ferritin, (Means, 2004) which is typically elevated in anemia of inflammation (Coenen *et al*,

1991; Means, 2004). A second feature of anemia of inflammation is reduced erythrocyte survival. The decrease in erythrocyte survival has been linked to increased phagocytic activity by activated macrophages (Lee, 1983; Means, 2004). Cytokines and acute-phase proteins (IFN-γ, IFN-α, TNF-α and IL-1) also inhibit the growth and differentiation of erythroid progenitor cells (Weiss, 2009). Lastly a blunted erythropoietin (EPO) response to anemia has been described in anemia of inflammation (Weiss, 2002; Means, 2004; Weiss, 2009).

1.2.12 HIV infection

HIV disease progression is rapid in perinatally infected infants; without antiretroviral therapy (ART), over 50% of children die before 2 years of age (Newell *et al*, 2004). Anemia occurs in 42-90% of HIV-infected children in low-income countries and is a well-recognized risk factor for mortality (Clark *et al*, 2002; Totin *et al*, 2002; Belperio& Rhew, 2004; Adetifa *et al*, 2006; Bolton-Moore *et al*, 2007; Bong *et al*, 2007; 2008; Marazzi *et al*, 2008; Wamalwa *et al*, 2010). Anemia in HIV infection is caused by multiple mechanisms including: the direct action of HIV on bone marrow cells, adverse reactions to antiretroviral therapy, opportunistic infections, vitamin B12 and iron deficiency anemia (Semba& Gray, 2001; Totin *et al*, 2002; Tolentino& Friedman, 2007; Adetifa& Okomo, 2009). However anemia of inflammation is thought to be the predominant cause of anemia in HIV infected patients (Semba& Gray, 2001; Totin *et al*, 2002; Tolentino& Friedman, 2007; Adetifa& Okomo, 2009).

1.2.13 Hepcidin as a pathogenic factor in HIV infection

Apart from mediating anemia of inflammation, iron sequestration in reticuloendothelial macrophages through hepcidin (Miller *et al*, 2006) may encourage the growth and survival of the HIV virus(Xu *et al*, 2010). Elevated hepcidin concentration may protect the host against iron-dependent extracellular pathogens by decreasing iron supply to extracellular pathogens; however, elevated hepcidin concentration may also promote the growth and survival of iron dependent intracellular micro-organism such as HIV and *Mycobacterium tuberculosis* (Weiss, 2005; Weiss& Goodnough, 2005; Galesloot *et al*, 2011; Kroot *et al*, 2011). HIV infection of macrophages is an important component of HIV pathogenesis and survival of HIV patients correlates inversely with higher iron stores in bone marrow macrophages (Xu *et al*, 2010). *Mycobacterium tuberculosis* requires iron to grow and target macrophages to acquire iron (Boelaert *et al*, 2007; Drakesmith& Prentice, 2008). Hence macrophage iron loading is thought to increase the both the risk and virulence of *Mycobacterium tuberculosis* (Boelaert *et al*, 2007).

1.2.14 Hepcidin Studies in HIV Infection

However, only one study has reported hepcidin levels in HIV-infected individuals. They reported median (IQR) hepcidin concentration of 25.1 ng/mL (6.4–39.7) in 188 Rwandan HIV-positive women without ferroportin mutations using a monoclonal antibody immunoassay (Schwarz *et al*, 2011; Masaisa *et al*, 2012). While a smaller cohort of 12 women with a ferroportin mutation (Q248H mutation in the gene SLC40A1) associated with resistance to hepcidin (Masaisa *et al*, 2012), had a median hepcidin concentration of 3.3 ng/mL (IQR 1.3-7.5).

1.2.15 HIV exposure

The large success of Prevention of Mother-to-Child Transmission (PMTCT) of HIV responses in the most affected African countries means that an increasing number of HIV exposed uninfected (HEU) infants are being born (Filteau, 2009). More than 18% of infants for example will be HEU if MTCT is reduced to 10% in countries with an antenatal HIV prevalence of more than 20% (Marinda *et al*, 2007; Kumwenda *et al*, 2008; Filteau, 2009). HEU infants have reduced growth, increased rates of infections and higher mortality than HIV-unexposed infants (Thea *et al*, 1993; Brahmbhatt *et al*, 2006; Otieno *et al*, 2006; Makasa *et al*, 2007; Marinda *et al*, 2007; McNally *et al*, 2007; Shapiro *et al*, 2007). Several studies have also highlighted immune abnormalities in HEU infants; however, the relationship between specific cytokine abnormalities and increased morbidity and mortality is still unclear (Clerici *et al*, 2000; Kuhn *et al*, 2001). This increased risk has been hypothesized to result from immune abnormalities, greater exposure to infections and social and economic causes (poorer care due to ill or deceased parents) (Marinda *et al*, 2007). Suboptimal infant feeding practices in particular have been implicated as causes for poor health and nutrition (Coovadia& Bland, 2007; Marinda *et al*, 2007). HEU infants are also exposed to infections carried by immune-compromised parents (Marinda *et al*, 2007). Furthermore, increased vulnerability to anemia has been associated with infant exposure to maternal ART (Le Chenadec *et al*, 2003; Feiterna-Sperling *et al*, 2007; Dryden-Peterson *et al*, 2011; Ziske *et al*, 2013) in HEU infants.

Chapter 3 characterizes the independent effects of HIV status, anemia and age on hepcidin concentration in infants from a well-defined cohort in Zimbabwe. Normative hepcidin values established in chapter 2 serve as the reference group in chapter 3. The study reported in chapter 3 of this thesis is, to our knowledge, the first to characterize hepcidin concentrations in HIV-infected sub-Saharan African infants, who are at particularly high risk of anemia.

Table 1.3: Review of Hepcidin Studies.

Assay	Study population	Inclusion criteria	Hepcidin concentration	Coefficient of variation (CV)	References
c-ELISA (serum) Intrinsic Life Sciences	19 pregnant women (16-32 years) 19 neonates (cord blood) median gestational age 39.9 weeks range 36.0-41.6 weeks	Healthy, nonsmoking females with uncomplicated pregnancies were included. Participants were excluded if they had diabetes, underlying malabsorption diseases or medical conditions known to affect Fe homeostasis	Median (SD) at delivery: Mothers 9.30 (50.1µg/L) Neonates 61.7 (77.0 µg/L)	Intra-assay CV 5-19% Inter-assay CV 12%	(Young *et al*, 2012)
c-ELISA (Bachem)	11 post malarial children mean age 28.1 months 16 non malarial anemia children mean age 28.7 months (n=48)	Post malarial anemia was defined by a fever and peripheral parasitemia. Non malarial anemia was defined by the absence of fever in the previous 7 days and no record of clinical malaria episodes or antimalarial treatment	Mean ± SE Day 1 of Fe supplementation Post malarial anemia 25.5 ± 7.4 ng/mL Non malarial anemia 12.8 ± 5.8 ng/mL Day 15 of Fe supplementation Post malarial anemia 16.3 ± 5.3 ng/mL Non malarial anemia 17.5 ± 4.7 ng/mL	Not provided	(Prentice *et al*, 2012)
c-ELISA (Bachem)	100 Kenyan children aged 6 months to 10 years with acute malaria	Children with fever (> 38.5°C), anemia, and *P. falciparum parasitemia were included*. Children treated with antimalarials during the week before admissions were	Median (IQR) baseline: 8.57 (1.00-39.05) ng/mL, one week after treatment: 1.69 (0-6.31) ng/mL one month after treatment: 0.43 (0.37-	Not provided	(Casals-Pascual *et al*, 2012)

		excluded.	0.75) ng/mL		
c-ELISA (Intrinsic Life Sciences, CA, USA)	44 very low birth weight (VLBW) infants Mean (SD) birth weight 885 (245) g and mean gestational age was 26.2 (1.7) weeks. 21 healthy term infants mean (SD) birth weight 3297 (348) g and mean gestational age was 38.8 (1.2) weeks.	Infants born with congenital anomalies, twin–twin transfusion syndrome, placenta abruption, or immediate postnatal hemoglobin level <10 g/dL on admission were excluded. Sepsis was defined post hoc as a positive blood culture and/or antibiotic therapy for 5 or more days in infants with clinical signs of infection.	Range of hepcidin 26.8-67.7 ng/mL in healthy term infants. Infants without sepsis (means 43.9 ng/mL, CV 13.7%) infants with sepsis with negative blood cultures mean 99.2 ng/mL, CV 22.4%) infants with sepsis with positive blood cultures (mean 244.8 ng/mL, CV 10.7%)	Intra-assay CV 5-19% Inter-assay CV 12%	(Wu *et al*, 2013)
c-ELISA (Intrinsic Life Sciences, CA, USA)	191 pregnant women and their full term babies (gestational age 37-42 weeks) Reference ranges were calculated in 116 mothers and 137 cord blood samples	Pregnant women with Hb ≤ 110 g/L, % hypochromic red blood cells ≥ 3.4%, ferritin > 64 µg/L, Erythropoietin (EPO) > 97th percentile, CRP >10 mg/L New born infants with cord blood Hb ≤ 145 g/L, ferritin > 115.3 µg/L transferrin saturation ≤ 30%, EPO > 97th percentile, CRP>10 mg/L	Reference ranges Geometric mean (5th and 95th percentile) Pregnant women at term 10.6 (< 5.0 - 58.8 ng/mL) Cord blood 72.3 (< 5.0-231 ng/mL)	Intra-assay CV 5-19% Inter-assay CV 12%	(Rehu *et al*, 2010)
c-ELISA	2998 participants from the general population, 48% male Median age in men and women 63 and 54 years respectively	References were created by excluding subjects who were pregnant, had an Alanine transaminase (ALT) > 50 U/L, CRP > 10 mg/L, epidermal	Reference ranges median (2.5th and 97.5th percentiles) per 5 year age group were constant in men 7.8 nM (0.6-23.3 nM)	Intra-assay CV 6.3% Inter-assay CV 11.9%	(Galesloot *et al*, 2011)

		growth factor receptor (eGFR) < 60 Ml/min/1.73 m^2, used iron supplements, were anemic or had a BMI > 30 kg/m^2	Women < 55: 4.1 nM (0.4-19.7 nM) and Women > 55: 8.5 nM (1.2-24.8 nM)		
EIA kit S-1337; Bachem	285 healthy Low Birth weight babies between 6 weeks and 6 months. 49% were boys, 56% were preterm mean gestational age 36.5 ± 1.9 weeks	Birth weight 2000-2500 g, no disease symptoms at inclusion, no chronic disease, no previous blood transfusion, no history of iron supplementation	12.2 ± 1.8 ng/mL at 6 weeks 19.9 ± 2.1 ng/mL at 6 months in iron supplemented infants and 13.0 ± 2.6ng/mL in placebo group	Detection range of 0 to 25 ng/mL CV not provided	(Berglund *et al*, 2011)
EIA Bachem kit	Patients with vasculitic disease (ANCA-AAV) and healthy controls	Not provided	92.93 ± 15.37 ng/mL in patients 15.43 ± 2.11ng/mL in healthy controls	Not provided	(Konopásek *et al*, 2011)
EIA Bachem kit UK	134 recipients of heart allografts	Prevalent heart allograft recipients	24.98 ± 9.79 ng/mL in anemic patients 20.59 ± 9.59 ng/mL in non anemic patients	Not provided	(Przybylowski *et al*, 2010)
ELISA using recombinant functional hepcidin 25-His peptide	32 healthy controls 7 patients with HJV associated juvenile hemochromatosis 10 patients with IDA 7 patients with Hodgkin's Lymphoma	Not provided	Mean hepcidin Healthy controls – 42.7 µg/L Patients with juvenile hemochromatosis - 12.8 µg/L Patients with IDA- 15.7 µg/L Patients with Hodgkin's Lymphoma- 116.7 µg/L	Intra- and inter-assay CV ranged from 8-15% and 5-16% respectively	(Koliaraki *et al*, 2009)
C-ELISA	Healthy volunteers 18-81 years, 65 men	Normal health history	5-95%range (median)	Intra-assay CV	(Ganz *et al*, 2008)

(Ganz lab)	and 49 women from the USA and Italy	based on local standards, hemoglobin, serum iron, total iron binding capacity, ferritin, serum transferrin receptor and CRP	29-254 ng/mL (112 ng/mL) in men 17-286 ng/mL (65 ng/mL) in women	5-19% Inter-assay CV mean and median 12 and 7% respectively	
Radioimmunoassay (RIA)					
RIA (Bachem)	47 Normal patients samples 40 patients with ulcerative colitis 15 patients with IDA 45 patients with chronic kidney disease (CKD) not requiring dialysis 95 patients with CKD requiring dialysis	Not provided	Reference range of 1.1-55 ng/mL in normal patients	Intra-assay CV 5.8-7.2% Inter-assay CV 6.7-7.6%	(Busbridge *et al*, 2009)
c-RIA (Bachem)	61 patients with Inflammatory Bowel Disease (IBD) (mean age 44.03 years) 25 healthy controls (mean age 36.02 years)	Not provided	Mean hepcidin in IBD was 6.13 ng/ml compared to 15.3 ng/ml in healthy controls p=0.009	Intra-assay CV 2.7- 7.2% Inter-assay CV 2.4 - 4.7%	(Arnold *et al*, 2009)
RIA Grebenchtchikov *et al*, 2009 Lab (Developed RIA instead of a sandwich ELISA because of the size of hepcidin)	64 healthy individuals 29 men 35 women	Not provided	Median (range) Men at 9 am: 5.77 (0.37 - 29.15 µg/l) Women at 9 am: 3.60 (0.25-12.56µg/l) Men at 4pm: 15.59 (0.26 - 40.63 µg/l) Women at 4 pm- 5.89 (0.18-19.11 µg/l)	Intra- and inter assay CVs of 4.4% and 6.2% respectively (Currently has the lowest limit of detection - 0.02 µg/l)	(Grebenchtchikov *et al*, 2009)
Sandwich ELISA (Butterfield et al Lab)	34 Cancer patients 76 rheumatoid arthritis patients 100 Healthy volunteers (18-64, mean 37 years, 50 women)	Not provided	Median (25-75% range) Cancer patients 54.8 (23.2-93.5 µg/L) Rheumatoid arthritis patients 10.6 (5.9-18.4 µg/L) Healthy volunteers 1.20 (0.42-3.07 µg/L)	Intra-assay CVs were 3.4%, 4.5%, 3.5% for human serum samples containing 0.16, 4.5, and 15.1 µg/L of	(Butterfield *et al*, 2010)

				hepcidin	

Mass Spectrometry (MS)

Liquid Chromatography (LC-MS/MS)					
LC-MS/MS	12 healthy controls (24-50 years, 6 males, 6 females) 12 patients with hereditary hemochromatosis (32-65 year old males) 6 patients with systemic infection and inflammation (45-61 years, 4 males, 2 females) 15 patients with sickle cell anemia (32-65 years, 8 males, 7 females)	Not provided	Mean Hepcidin 5.2 ± 1.92 nmol/mmol creatinine $^{-1}$ in controls 0.55 ± 0.52 nmol/mmol creatinine $^{-1}$ in patients with hemochromatosis 26.5 ± 14.5 nmol/mmol creatinine $^{-1}$ in patients with inflammation	< 10%	(Bansal *et al*, 2009)
LC-MS/MS	231 apparently healthy adults partitioned into age groups 18-30, 31-50, 51-70 and > 70 years with 21-37 people per group	"Apparently healthy adults"	Reference values Females aged 18-50 years: 0.4 - 9.2 nmol/L Females > 50 years: 0.7-16.8 nmol/L Males ≥ 18 years 1.1-15.6 nmol/L Median (range) 3.2 (0.4-15.3 nmol/L)	Inter-assay CV ≤ 5%, Total variation ≤ 7.6%	(Itkonen *et al*, 2012)
	95 apparently healthy adults		Median (range) 5.7 (1.4-35.9 nmol/L)	Not calculated	
LC-MS/MS	10 healthy individual subjects	Not provided	Median plasma and serum hepcidin levels of 4.9 and 3.6 ng/mL respectively in healthy males and median plasma and serum levels of 2.8 and 3.6 ng/mL in women	Inter-assay CV range 11.0-15.3%	(Murphy *et al*, 2007)

Weak Cation-Exchange chromatography and (WCX- TOF MS) internal standard synthetic hepcidin24 Peptide					
WCX- TOF MS	23 Beninese women mean (SD) age was 20.2 (4.8) years			Inter-assay CV 6.5% Intra-assay CV 2.7%	(Cercamondi *et al*, 2010)
Matrix-assisted laser desorption/ionization time of flight MS (MALDI)-TOF MS)					
MALDI-TOF MS (Bruker Daltonics)	Indonesian school children, aged 5-15 years 73 with Asymptomatic P. falciparum, 18 with Asymptomatic P.vivax and 17 controls	Asymptomatic parasitemia was defined as asexual *P. falciparum* or *P. vivax* parasitemia in the absence of fever (temperature ≤ 37.9°C) and of clinical signs or symptoms suggestive of malaria or another infectious disease.	Serum hepcidin *P. falciparum* 5.2 nM *P.vivax* 5.6 nM Controls 3.1 nM	Inter-assay CV- 2.7% Intra-assay CV-6.5%	(de Mast *et al*, 2010)
MALDI-TOF MS	62 female and 64 healthy male pediatric patients and 86 HIV-negative adults males	Not provided	11.7 ng/mL in pediatric samples 16.1 ng/mL in adult males	Inter-day CV-14.83% Intraday CV – 18.48%	(Anderson *et al*, 2011)
Surface-enhanced laser desorption/ionization time of flight (SELDI)-TOF MS					
SELDI-TOF MS	181 African children less than 16 years, 93 male, mean age was 8 years	Children were excluded if they had received antibiotics or specific treatment for *H.pylori* in the preceding month, had a diagnosis of immunodeficiency or active tuberculosis, or is H.pylori fecal antigen was not performed	Urinary hepcidin levels Median (IQR) 25 children with IDA - 0.1 (0.05 - 0.6 nmol/mmol Cr 156 children without IDA- 3.0 (0.9 - 6.7 nmol/mmol Cr)	Intra-assay CV 6.1-7.3% Inter-assay CV 7.9-10.9%	(Cherian *et al*, 2008)
SELDI-TOF MS	199 patients in Ghana (86 children, 82 adults, and 31 pregnant women) with malaria 79.1%, 82.9%, and 90.3%	Subjects were eligible for the study if they were diagnosed with *P. falciparum* malaria, were	Hepcidin (intensity/mmol creatinine) median (25^{th} and 75^{th}	Not provided	(Howard *et al*, 2007)

	anemic, respectively	not admitted for transfusion, had a hemoglobin level > 50 g/L, and had no evidence of cerebral malaria.	percentile) Children 4.2 (1.4, 9.5) Adults 0.7 (0.3, 3.6) Pregnant women 0.7 (0.2, 5.9)		
Weak cation-exchange chromatography and SELDI-TOF MS synthetic analog hepcidin-24 of hepcidin-25 was used as an internal standard	33 men over 40 years	Not provided	Range 0 to 7.8 nmol/L	Intra-assay CV 3.5% Inter-assay CV 7.5%	(Roe *et al*, 2009)
SELDI-TOF MS hepcidin-24 peptide (Peptide International) was used as an internal reference standard.	Children 2 months to 13 years in Tanzania	Febrile at admission (axillary temperature, > 37.5°C), *P. falciparum* (i.e., with detection of at least 125 parasites/200 white blood cells, a negative HIV test result, a negative blood culture result, anemia (Hb level, < 10 g/dL), and no history of iron therapy in the preceding 3 weeks	Median (IQR) Urinary hepcidin level, nmol/mmol creatinine 177.5 (91.9–376.4) on admission	Not provided	(de Mast *et al*, 2009)
SELDI-TOF MS	23 healthy volunteers (12 men, 11 women)	Normal values for complete blood count (CBC), serum iron, transferrin saturation, ferritin, CRP, sTfR, liver function tests,	All volunteers 5.3 nmol/l (3.5-8.3 nmol/l) Men 7.2 nmol/l (3.3-16.0 nmol/l) Women 3.9 nmol/l (2.7-5.4 nmol/l) p=0.139	Intra-assay CV 5.7-11.7%	(Swinkels *et al*, 2008)

1.2.16 Etiology of Anemia in developing countries

Primary processes implicated in the pathogenesis of anemia in developing countries include: 1) inadequate iron intake; 2) iron deficiency caused by extra-corporeal blood loss due to *Schistosomiasis* and soil-transmitted helminths (STHs): hookworm, and *Trichuris trichiur*a infection and 3) anemia of inflammation resulting from high rates of infectious diseases, as well as subclinical infections and inflammation including malaria and HIV.(Weatherall, 1990; Stoltzfus *et al*, 1997; Friedman *et al*, 2005; Tolentino& Friedman, 2007; Midzi *et al*, 2010). Recently, the prevalent subclinical gut disorder termed environmental enteropathy (EE) has been hypothesized to contribute to anemia of inflammation, even in the absence of clinical disease (Prendergast& Kelly, 2012).

1.2.17 Environmental Enteropathy (EE)

EE is a subclinical intestinal pathology that is ubiquitous among people living in unhygienic conditions. EE results from chronic fecal-oral transmission of microorganisms (Figure 1.4) (Humphrey, 2009). The intestinal barrier is compromised in EE: increasing intestinal permeability, enabling microbial translocation and resulting in chronic systemic inflammation(Lunn *et al*, 1991; Campbell *et al*, 2003).

Intestinal permeability studies in infants with EE demonstrated that both absorptive and barrier functions of the small bowel mucosa were compromised. EE may result in anemia through impaired digestive/absorptive and barrier functions of the small intestine (Campbell *et al*, 2003). The most common histological features described in EE is some

degree of villus atrophy (Beaton& Ghassemi, 1982; Martorell, 1995; Stephensen, 1999) including a reduction in villous height, broadening of the villi, and increased crypt depth (Cook *et al*, 1969; Carneiro Chaves *et al*, 1981). The degree of intestinal permeability correlated with the presence of endotoxin (LPS) and anti-endotoxin core antibodies (EndoCAb) in the bloodstream in Gambian infants among whom EE was reported as early as 3–6 months.(Campbell *et al*, 2003). EE in Gambian infants was also associated with chronic, low level immunostimulation, including raised lymphocyte and platelet counts, CRP, and plasma immunoglobulins (Campbell *et al*, 2003). Reduced absorptive capacity and subclinical malabsorption of carbohydrates (Keusch, 1972; Northrop-Clewes *et al*, 1997), fat and vitamin B12 (Lindenbaum *et al*, 1966) has also been observed in EE.

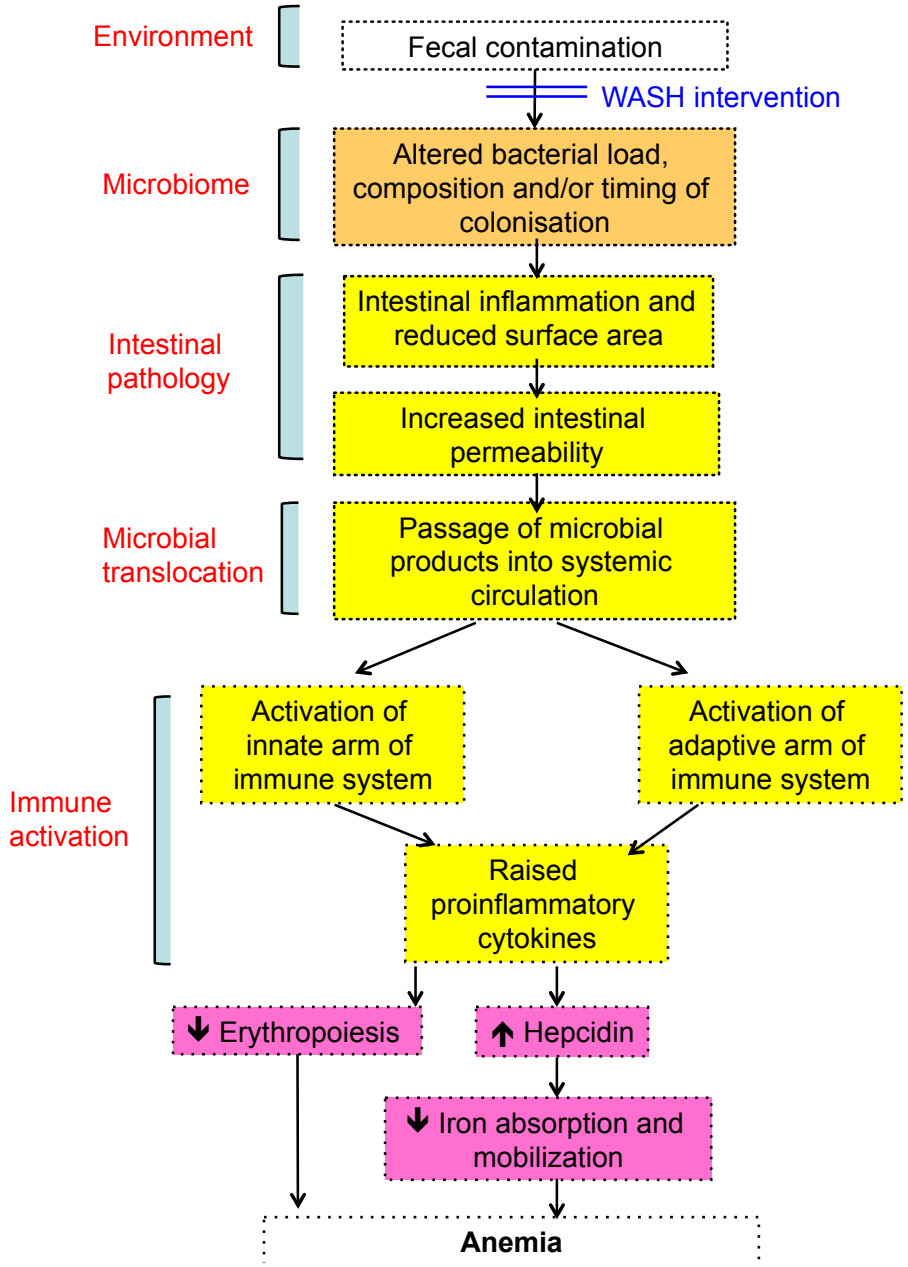

Figure 1.4. Hypothesized causal pathway linking fecal contamination and Environmental Enteropathy (EE) through poor WASH practices with anemia. Adapted from (Prendergast& Kelly, 2012).

1.2.18 Schistosomiasis and soil transmitted helminths

Poor WASH practices are associated with fecal exposure which can contain over 50 known pathogens including *Schistosomiasis* and STHs (Brown *et al*, 2013). Hence Schistosomiasis (*S*. *haematobium* and *S*. *mansoni*) and STHs: hookworm, *Trichuris trichiur*a and *Ascaris lumbricoides* infection are endemic in developing countries with poor WASH practices (Hotez *et al*, 2005). Children 12-24 months who are at highest risk for anemia often acquire their first STH infection during the same time period (Stoltzfus *et al*, 2004; Kung'u *et al*, 2009). *Schistosomiasis* is among the top 14 out patient treated diseases in Zimbabwe (Midzi *et al*, 2011) with overall mean prevalence of S. *haematobium*, S. *mansion* and STHs of 20.8%, 9.0% and 6.0% respectively and prevalence rates of S. *haematobium*, up to 70% in some regions of the country (Taylor P, 1985; Chandiwana *et al*, 1988; Midzi *et al*, 2011) (available from, the Ministry of Health and Child Welfare (MoHCW) of Zimbabwe). 29.6% of Zimbabwean households have access to improved toilet facilities (Zimbabwe National Statistics Agency & ICF International Inc., 2012). 20.3%, 9.3% and 6.0% of individuals infected with *S*. *haematobium, S*. *mansoni* and STHs respectively had a toilet at home (available from MoHCW of Zimbabwe).

1.2.19 Infant and young child feeding practices

Iron requirements are high during the second half of infancy as endogenous iron stores become depleted and the infant is no longer self-reliant for iron to meet the needs for

erythropoiesis and growth (Lind *et al*, 2004; Baker& Greer, 2010; Domellof, 2011). Because of the large iron stores at birth, the Adequate intake (AI) and Recommended Dietary Allowance (RDA) for iron in term infants during the first 6 months of life is only 0.27 mg/day (equivalent to the amount provided by breastmilk for an exclusively breast fed infant). Between 7-12 months of age, when stores at birth are mostly depleted yet growth remains rapid, iron requirements increase to 11 mg/day (a 40-fold increase over the first half of infancy); this requirement is almost 10 times higher per kg bodyweight, that of an adult male (0.9 - 1.3 mg/kg body weight in 6-12 mo infant vs. 0.14 mg/kg body weight requirement of adult male (Domellof, 2011; World Health Organization, Food and Agriculture Organization of the United Nations 2004). During the 1-3 year age period, requirements decrease to 7 mg/d, which is still much greater than the adult requirement in mg/Kg body weight terms (0.35 mg/kg body weight) (Baker& Greer, 2010; World Health Organization, Food and Agriculture Organization of the United Nations 2004). Hence, the period between 6 months and 3 years of age when infants have depleted birth stores yet still grow rapidly, and which coincides with transition from breast milk to the family diet represents a particularly vulnerable period for infants to develop anemia (Zongrone *et al*, 2012). This is especially true for infants in developing countries where complementary foods are largely plant based and high in substances that inhibit iron absorption and rarely include meat, poultry or iron fortified cereal or formula (Dewey, 2003). The need for simple indicators of diet quality in young children that can be implemented in population-based surveys such as the Demographic and Health Survey (DHS) led to the development of the WHO core and optional IYCF practice indicators(Arimond *et al*, 2008; WHO., 2008).

Iron sources are divided into heme and non-heme. Heme iron is found in animal foods that originally contained hemoglobin and is more bioavailable while plant sources make up non-heme iron. Very good sources of heme iron (≥ 3.5 mg per serving) include chicken liver (9.9 mg/3oz) and beef liver (5.6 mg/3oz) and very good sources of non-heme iron include fortified formula (10-12.8 mg/L) and fortified baby cereal (4.5-18 mg) (Table 1.3).

While good sources of heme iron include beef (≥ 2.1 mg per serving). Thus, with the possible exception of chicken liver, only foods that are specially fortified for infants will meet infant iron requirements: natural dietary sources of iron are not iron-concentrated enough to provide 11 mg elemental iron in the small volumes consumed by infants. For example, to meet this requirement, an infant would need to consume 400 g red meat, 700 g chicken, 11 eggs, or 6 cups of cooked legumes daily.

Hence the provision of iron-fortified weaning foods or low dose iron supplements is advocated by the WHO and UNICEF (Stoltzfus, 1998; Stoltzfus *et al*, 2004). However there are no national iron supplementation programs for infants and young children and national consumption foods fortified in the home with a micronutrient powder containing iron or a lipid based nutrient supplement (LNS) containing iron is practically non-existent (Zimbabwe National Statistics Agency & ICF International Inc., 2012).

Hence we hypothesize that the key IYCF indicator “consumption of iron-rich/iron-fortified food for children 6-23 months” is not associated with hemoglobin levels in

Zimbabwean children because the iron-rich IYCF indicator lacks specificity with regard to iron consumption

		Amount needed/day to meet Fe needs	
Food source	Elemental Iron (mg/100 g)	7-12 mo RDA: 11 mg/day	12-36 mo RDA: 7 mg/day
Animal liver/ organ meat	8-25	100 g (3 oz)	100 g (3 oz)
Red Meat	2.5	400 g (13 oz)	300 g (8 oz)
Chicken	1.5	730 g (24 oz)	500 g (14 oz)
Fish	0.7	1 600 g (47 oz)	1 000 g (30 oz)
Beans, peas, lentils, nuts, peanuts	2.1	6 cups cooked	3.5 cups cooked
Egg	1.0	11 eggs	7 eggs

Table 1.4. Iron content of unfortified food sources (Baker& Greer, 2010; US Department of Agriculture, Agricultural Research Service., 2013)

Chapter 4 tests the hypothesis that WASH indicators that reflect fecal exposure among infants will be associated with hemoglobin levels during infancy based on our hypothesized causal pathway through *Schistosomiasis*, STHs and EE. We also test the hypothesis that IYCF indicators will not be associated with hemoglobin because the iron-rich IYCF indicator lacks specificity with regard to iron consumption

1.3 Research Design

Chapters 2 and 3 utilized data and stored samples from ZVITAMBO, a randomized controlled trial of maternal and neonatal vitamin A supplementation (Humphrey *et al*, 2006). The ZVITAMBO trail recruited a total of 14,110 mother-infant pairs from 14 maternity clinics and hospitals in greater Harare between November 1997 and January 2000 (Figure 1.5). Pairs were recruited within 96 h of delivery at maternity clinics and hospitals in Harare and were eligible if neither the mother nor the baby had an acutely life threatening condition and the infant was a singleton with birth weight >1500 g and the mother planned to stay in Harare after delivery. Written, informed consent was obtained from mothers. A 2×2 factorial design was used in the study, and all pairs were randomized ≤ 96 h after delivery to one of 4 treatment groups. The treatment groups were Aa, Ap, Pa, and Pp, where "A" was maternal vitamin A supplementation (400,000 IU), "P" was maternal placebo, "a" was infant vitamin A supplementation (50,000 IU), and "p" was infant placebo.

Hemoglobin was measured in a 34% subsample of infants (Anemia sub-study), over-selecting for those born to HIV-positive women, for a total of 2314 and 535 infants born to HIV-positive and HIV-negative women, respectively (Miller *et al*, 2003). Mothers and their babies were followed-up at 6 wk, 3 mo and every 3 mo until 12 mo of age by study midwives in a study clinic or at home. Blood was collected at recruitment and follow-up, and Hb measured in baseline samples and repeated in infant samples from 3 mo of age onward.

A sample of 907 infants was recruited from the Anemia sub-study into the Hepcidin sub-study. We conducted a cross-sectional study of 3, 6 and 12mo infants. For each respective age, 6 groups of infants were selected, based on HIV and anemia status: HIV-unexposed non-anemic infants (Group 1), HIV-unexposed anemic infants (Group 2); HIV-exposed uninfected non-anemic infants (Group 3); HIV-exposed uninfected anemic infants (Group 4); HIV-infected non-anemic infants (Group 5) and HIV-infected anemic infants (Group 6). Infants were classified as HIV unexposed if mother-infant pair was HIV negative at delivery and did not seroconvert during the trial period; HEU infants were born to HIV positive mothers but were HIV negative at delivery and did not seroconvert during the trial period. HIV infected infants were infected intrauterine, intrapartum or postnatal. Anemia was defined by a hemoglobin measurement <105 g/L at 3 and 6 months, or <100 g/L at 12 months; these criteria were modified from those of the World Health Organization because of the growing literature supporting lower hemoglobin cut-offs in infancy (Domellof *et al*, 2002; Miller *et al*, 2003; Eneroth *et al*, 2011). Further recruitment criteria were applied to group 1 to generate normative hepcidin values in chapter 2: gestational age > 37 weeks; birth weight > 2500 g; absence of iron-deficiency anemia (defined as hemoglobin >105 g/L at 3 mo and 6 mo, or > 100 g/L at 12 mo; plasma ferritin >12 μg/L; and soluble transferrin receptor (sTfR) < 8.3 mg/L); absence of inflammation (defined as AGP <1g/L and CRP < 5 mg/L); absence of acute illness (defined as diarrhea or fever in preceding week, or measles in preceding 3 months). Indicators of iron (sTfR and ferritin) and inflammatory (CRP and AGP) status and hepcidin were measured in cryopreserved samples at 3, 6 and 12 months.

Chapter 4 presents a secondary analysis of data collected in the 2010-2011 Zimbabwe Demographic Health Survey, available on the Demographic Health Survey website (Zimbabwe National Statistics Agency & ICF International Inc., 2012). The Zimbabwe Demographic Health Survey collected information from a nationally representative probability sample of all 10 Zimbabwean provinces using a stratified, two-stage cluster design yielding 9,756 households and a household response rate of 96%. Hemoglobin testing was carried out among children aged 6-59 months and was successfully measured in 80% of eligible children. We analyzed data from children 6-24 months of age for whom age-appropriate IYCF indicators could be constructed from available data. The initial population comprised 1741 children. Our analysis was restricted to the youngest child in a household in this age range with an available hemoglobin measurement, whose mother was interviewed. Hence 51 and 314 children were excluded because they were the older child in a household with more than one child between 6 and 24 months and because they did not have a valid hemoglobin measurement respectively. 105 children were excluded from the multivariate analysis because they were not "*de jure*" household members or lacked information on included variables. Therefore, 1376 children were selected and studied in the univariate analysis and 1271 children were included in the multivariate analysis.

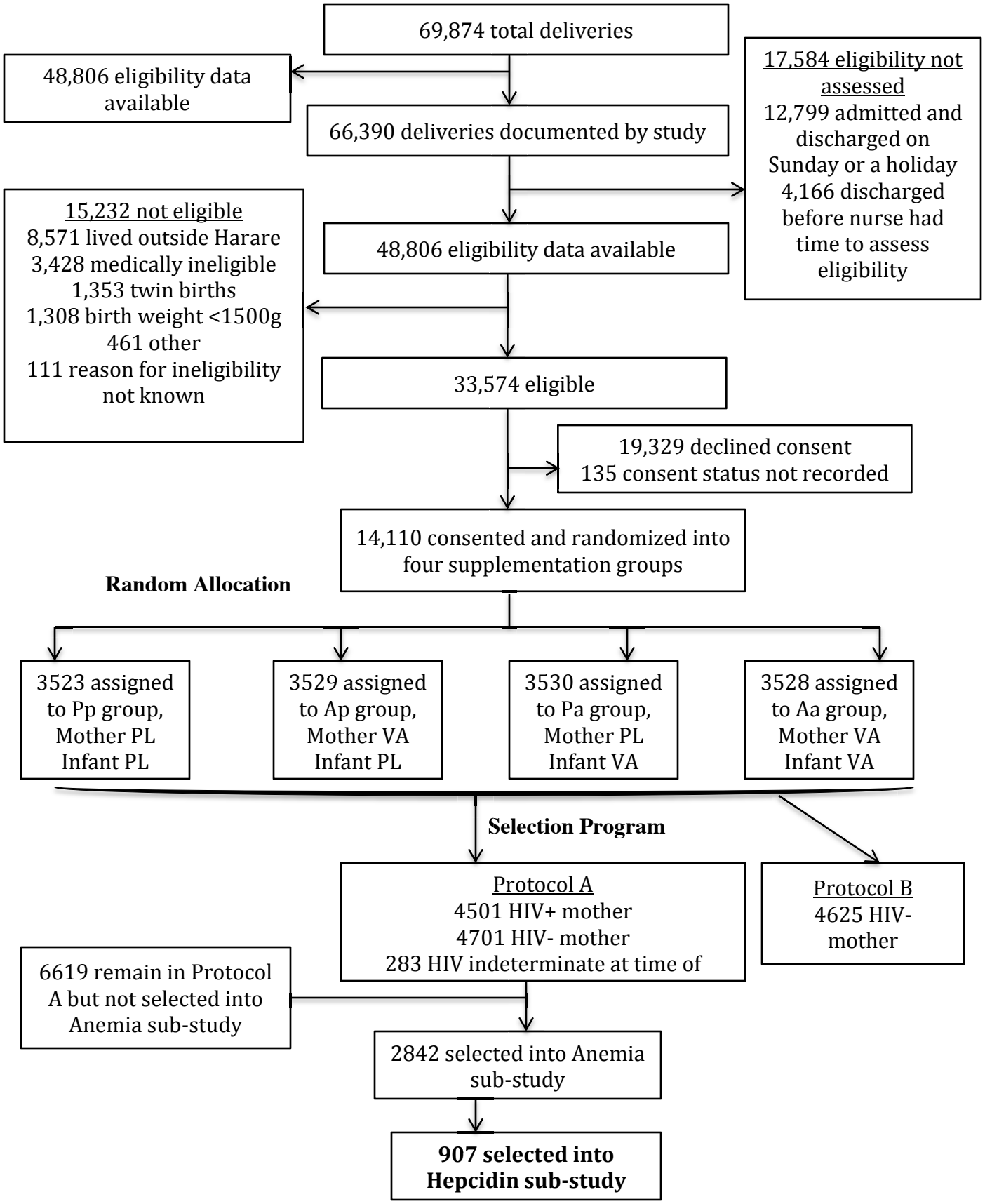
69,874 total deliveries
48,806 eligibility data available
17,584 eligibility not assessed
12,799 admitted and discharged on Sunday or a holiday
4,166 discharged before nurse had time to assess eligibility
66,390 deliveries documented by study
15,232 not eligible
8,571 lived outside Harare
3,428 medically ineligible
1,353 twin births
1,308 birth weight <1500g
461 other
111 reason for ineligibility not known
48,806 eligibility data available
33,574 eligible
19,329 declined consent
135 consent status not recorded
14,110 consented and randomized into four supplementation groups
Random Allocation
3523 assigned to Pp group, Mother PL Infant PL
3529 assigned to Ap group, Mother VA Infant PL
3530 assigned to Pa group, Mother PL Infant VA
3528 assigned to Aa group, Mother VA Infant VA
Selection Program
Protocol A
4501 HIV+ mother
4701 HIV- mother
283 HIV indeterminate at time of
Protocol B
4625 HIV- mother
6619 remain in Protocol A but not selected into Anemia sub-study
2842 selected into Anemia sub-study
907 selected into Hepcidin sub-study

Figure 1.5. The ZVITAMBO trial profile including the Hepcidin sub-study

Hepcidin decreases over the first year of life in healthy African infants

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Short title: Hepcidin In Infancy

ABSTRACT

Iron metabolism is very dynamic over the first year of life. Hepcidin is the master regulator of iron metabolism, but little is known about normal hepcidin values in infancy. We selected healthy, non-anemic Zimbabwean infants with normal iron parameters at 3 (n=60), 6 (n=47) and 12 (n=40) months of age and measured hepcidin, soluble transferrin receptor, ferritin, α1-acid glycoprotein and C-reactive protein on stored plasma samples. Hepcidin concentrations were higher in 3-month-old (median 9.7 ng/mL [IQR 2.5, 19.3]), than in 6-month-old (4.5 ng/mL [IQR 0.5, 7.3]) and 12-month-old infants (1.9 ng/mL [IQR 0.7, 6.2)]) ($p<0.001$, Kruskal–Wallis). Hepcidin concentrations were non-significantly higher in girls than boys in each age group, and were lower than those reported in adults, supporting the higher metabolic need for iron in infancy. Hepcidin decline during infancy is likely a physiologic response to mobilize iron stores and increase iron absorption to prevent anemia.

INTRODUCTION

Iron metabolism is very dynamic over the first year of life, with iron requirements almost 10 times higher per kilogram body weight than in adults because of the rapid growth that characterizes infancy (Tolentino& Friedman, 2007; Shaw& Friedman, 2011). Hepcidin, a 25-amino acid peptide hormone secreted by hepatocytes, is considered the major regulator of iron metabolism (Ganz *et al*, 2008; Grebenchtchikov *et al*, 2009; Kroot *et al*, 2010; Galesloot *et al*, 2011; Pasricha *et al*, 2011; Troutt *et al*, 2012). Hepcidin binds to

ferroportin, the only known mammalian iron exporter, (Ganz *et al*, 2008; Grebenchtchikov *et al*, 2009; Galesloot *et al*, 2011) inducing its degradation (Nemeth *et al*, 2004; De Domenico *et al*, 2007; Troutt *et al*, 2012) and inhibiting the release of iron by macrophages and hepatocytes; and attenuates iron absorption by duodenal enterocytes (Grebenchtchikov *et al*, 2009; Pasricha *et al*, 2011). Hepcidin synthesis is stimulated by elevated plasma iron concentration, infection and/or inflammation, (Nemeth *et al*, 2004; Galesloot *et al*, 2011) resulting in decreased availability of circulating iron. Hepcidin concentrations are decreased in conditions that demand increased serum iron concentrations, such as increased or ineffective erythropoiesis, hypoxia, anemia and iron deficiency (Kroot *et al*, 2010; Galesloot *et al*, 2011).

Infants are at high risk of developing anemia, particularly in developing countries; however, little is known about circulating hepcidin levels over the first year of life, even in healthy infants (Rehu *et al*, 2010; Berglund *et al*, 2011; Muller *et al*, 2012; Wu *et al*, 2013). We set out to measure plasma hepcidin concentrations and to characterize normative values in healthy, non-anemic, Zimbabwean infants.

METHODS

The current study utilized data and stored samples from the ZVITAMBO trial, a randomized controlled trial of maternal and neonatal vitamin A supplementation (Humphrey *et al*, 2006). 14,110 mother-infant pairs were recruited within 96 hours of delivery at maternity clinics and hospitals in Harare, Zimbabwe, between November

1997 and January 2000. Maternal-infant pairs were eligible if neither the mother nor infant had an acute life-threatening condition and the infant was a singleton with birth weight >1500 g. Follow-up was conducted at 6 weeks, 3 months and every 3 months thereafter, until the child was 12-24 months old. Hemoglobin was measured using the HemoCue hemoglobinometer (HemoCue, Mission Viejo, CA) in a 34% subsample of infants, over selecting for babies born of HIV positive women, for a total of 2314 and 535 infants born to HIV positive and negative women respectively (Miller *et al*, 2003). Written informed consent was obtained from the mother at recruitment. The original ZVITAMBO trial and this sub-study were approved by the Medical Research Council of Zimbabwe and the Committee on Human Research of The Johns Hopkins Bloomberg School of Public Health.

Study subjects for hepcidin study

HIV unexposed infants with a hemoglobin measurement were selected at 3, 6 and 12 months of age based on the following criteria: gestational age >37 weeks; birth weight >2500 g; absence of iron-deficiency anemia (defined as hemoglobin >105 g/L at 3 and 6 months, or >100 g/L at 12 months; plasma ferritin >12 μg/L; and soluble transferrin receptor (sTfR) < 8.3 mg/L); absence of inflammation (defined as alpha-1 acid glycoprotein (AGP) <1g/L and C-reactive protein (CRP) < 5 mg/L); absence of acute illness (defined as diarrhea or fever in the preceding week, or measles in the preceding 3 months). This yielded a sample size of 139 infants with three cross-sectional groups at 3mo (n=60), 6mo (n=47) and 12mo (n=40), with 132 infants contributing data at one time

point, 6 infants contributing data to 2 time-points and one infant contributing data to all three time-points. The criteria used to diagnose anemia were modified from those of the World Health Organization (WHO., 2011) because of the growing literature supporting lower hemoglobin cut-offs in infancy (Domellof *et al*, 2002; Miller *et al*, 2003; Eneroth *et al*, 2011).

Laboratory assays

Plasma samples were stored at -70°C until analysis, with freeze-thaw cycles kept to a minimum. Soluble transferrin receptor concentration was measured by enzyme immunoassay (Ramco Laboratories Inc, Houston, TX), with mean intra-assay coefficient of variation (CV) 5.4% and inter-assay CV 4.4%. Plasma ferritin was measured by enzyme immunoassay (Ramco Laboratories Inc, Houston, TX), with mean intra-assay CV 8.4% and inter-assay CV 8.3%. Plasma AGP and CRP were measured by ELISA (R&D Systems Inc, Minneapolis, MN), with manufacturer-reported intra-assay mean CV of 6.1% and 5.9%, respectively, and inter-assay mean CV of 6.5% and 7.4%, respectively.

Hepcidin was measured in plasma by competition ELISA, using the hepcidin-25 (human) enzyme immunoassay kit (S-1337; Bachem, San Carlos, CA) with detection range between 0.02 and 25 ng/mL, according to the manufacturer's protocol. Plasma samples were diluted 1 in 4 in supplied standard diluent (peptide-cleared human serum). Standards were run in duplicate and samples in triplicate. Samples giving readings outside the linear region of the curve were re-run at alternative dilutions. The intra-assay

CV was mean 6.3% (range 5.7 - 6.9%), and inter-assay CV was 6.3%.

Statistical analysis

Baseline and iron variables are reported as median with interquartile range (IQR), or mean with standard deviation (SD). Comparisons between groups were made using Kruskal–Wallis, ANOVA and regression analysis. Hepcidin values below the detection limit of 0.02 ng/mL were imputed using the limit of detection (LOD)/√2, thereby assigning a value of 0.014 ng/mL to avoid zero values dropping out when data were log transformed in regression analysis (Barr *et al*, 2006). Hepcidin consensus values (hepcon1) were calculated using the algorithm developed by Kroot *et al*, to enable interpretation across studies where different assays are used (Kroot *et al*, 2012). All statistical analyses were performed with STATA version 12 (StataCorp, College Station, TX).

RESULTS AND DISCUSSION

At 3, 6 and 12 months of age, 60, 47 and 40 infants, respectively, met the eligibility criteria and had sufficient plasma volume. Infant baseline characteristics are shown in Table 2.1. Mean (SD) hemoglobin in 3, 6 and 12-month infants was 117.9 (13.4), 118.0 (9.4) and 117.1 (11.8) g/L, respectively. Ferritin was lower at older age groups ($p< 0.001$; Kruskal–Wallis test).

Hepcidin concentrations were higher in 3-month-old infants (median 9.7 ng/mL [IQR 2.5, 19.3]), than in infants at 6 months (4.5 ng/mL [IQR 0.5, 7.3]) and 12 months (1.9 ng/mL [IQR 0.7, 6.2)]) ($p<0.001$; Kruskal–Wallis test; Table 1). Hepcidin was undetectable in one infant at 6 months of age. Hepcon1 values were median (IQR) 6.2 (0.6, 13.7), 2.2 (-1.0, 4.4) and 0.1 (-0.8, 3.5) in 3, 6 and 12 month infants, respectively. Hepcidin concentrations were higher in girls than boys in each age group, but sex differences were not significant in univariate regression analysis and after adjustment for age (Figure 2.1).

Iron metabolism is very dynamic in infancy, with hemoglobin and plasma ferritin concentrations falling dramatically after birth as the breakdown of fetal red blood cells exceeds the formation of new red blood cells (Dallman, 1987). Anemia prevalence therefore tends to increase over infancy as endogenous supplies of iron become depleted and dietary intake is insufficient to meet demands. We show for the first time in healthy, non-anemic infants that hepcidin concentrations decrease over infancy, with most of the drop in hepcidin occurring between 3 and 6 months. This is presumably a physiologic response to mobilize iron stores and absorb more dietary iron, thus preventing iron deficiency anemia.

Comparison of these infant values with adult values is complicated by the variation between hepcidin assays currently in use; hence we also provided hepcidin consensus values for future comparisons (Ganz *et al*, 2008; Grebenchtchikov *et al*, 2009; Galesloot *et al*, 2011). Konopasek *et al*. reported mean (SD) hepcidin concentration of 15.43 (2.11) ng/mL in healthy adults, using the same assay (Konopásek *et al*, 2011). Infant values

therefore appear lower than those reported in adults, supporting the higher metabolic need for iron in infancy compared to adulthood. Sex and age differences in hepcidin levels have been observed in adults (Galesloot *et al*, 2011); however, the higher values we observed in girls compared to boys were not significant in our study population, perhaps because of the small sample size. Boys have been observed to be more vulnerable to anemia (Emond *et al*, 1996; Thorisdottir *et al*, 2011) and the lower hepcidin concentrations among boys might be a physiologic response to inherently lower iron stores in infancy (Emond *et al*, 1996; Thorisdottir *et al*, 2011). Further studies are needed to determine hepcidin levels in anemic infants to better understand the pathophysiology underlying this highly prevalent condition of global health importance.

Table 2.1: Baseline characteristics of healthy, non-iron-deficient, non-anemic Zimbabwean infants at 3, 6 and 12-months of age[1,2,3]

Baseline Characteristics	3-month-old infants (N=60)	6-month-old infants (N=47)	12-month-old infants (N=40)
Infant characteristics			
Male sex[1],	46.7 (28)	46.8 (22)	52.5 (21)
Gestational age, weeks[2]	39.6 (1.2)	39.7 (1.2)	39.5 (1.2)
Birth weight, g[2]	3105 (376)	3186 (359)	3230 (459)
Apgar score at 5 minutes[3]	10 (10, 10)	10 (9, 10)	10 (10, 10)
Hemoglobin at blood sampling, g/L[2]	117.9 (13.4)	118.0 (9.4)	117.1 (11.8)
Ferritin at blood sampling, µg/l[3]	44.1(27.9, 103.6)	25.2 (17.6, 42.7)	22.2 (15.9, 32.9)
Soluble transferrin receptor at blood sampling, mg/L[3]	5.71 (4.61, 6.67)	5.91 (4.55, 6.44)	6.00 (3.94, 6.74)
AGP at blood sampling, g/L[3]	0.35 (0.27, 0.48)	0.51 (0.30, 0.61)	0.49 (0.39, 0.63)
CRP at blood sampling, mg/L[3]	0.21 (0.11, 0.91)	0.32 (0.13, 1.17)	0.54 (0.18, 1.20)

Hepcidin at blood sampling, ng/mL[3]	9.7 (2.5, 19.3)	4.5 (0.5, 7.3)	1.9 (0.7, 6.2)
Hepcidin consensus values[a] at blood sampling[3]	6.2 (0.6, 13.7)	2.2 (-1.0, 4.4)	0.1 (-0.8, 3.5)
WAZ at birth[2]	-0.42 (0.79)	-0.24 (0.75)	-0.18 (0 .95)
WAZ at blood sampling[2]	-0.07 (0.91)	-0.07 (1.09)	-0.39 (1.23)
LAZ at birth[2]	0.13 (0.90)	0.11 (0.71)	0.32 (1.44)
LAZ at blood sampling[2]	-0.32 (0.83)	-0.36 (1.23)	-0.84 (1.36)
Neonatal vitamin A treatment[1, b]	51.7 (31)	55.3 (26)	52.5 (21)
Maternal vitamin A treatment[1, b]	55.0 (33)	48.9 (23)	50.0 (20)
Maternal and household Characteristics			
Maternal Education, years[3]	11 (9, 11)	11 (9, 11)	11 (9, 11)
Mothers Employed[1]	16.7 (10)	10.9 (5)	15.0 (6)
Maternal MUAC, cm[2]	26.4 (2.8)	25.9 (2.4)	26.6 (2.7)
Household income per month, US dollars[3]	1220 (801, 1693.5)	1220 (855, 1830)	1221 (879, 2103)

[1]Values are % (n)

[2]Values are mean (SD)

[3]Values are median (IQR)

[a]Hepcidin consensus values were calculated using the algorithm Y= -1.36 + 0.78*hepcidin(Kroot *et al*, 2012)

[b]In the ZVITAMBO trial, mother-infant pairs were randomized within 96 h of birth to one of 4 treatment groups (Aa, Ap, Pa, Pp), where 'A' was maternal vitamin A supplementation (400,000 IU), 'P' was maternal placebo, 'a' was infant vitamin A supplementation (50,000 IU) and 'p' was infant placebo. Full details of the trial have been published elsewhere(Humphrey *et al*, 2006).

WAZ: weight-for-age Z-score; LAZ: length-for-age Z-score; MUAC: Mid-upper arm circumference, SD: standard deviation, IQR: interquartile range

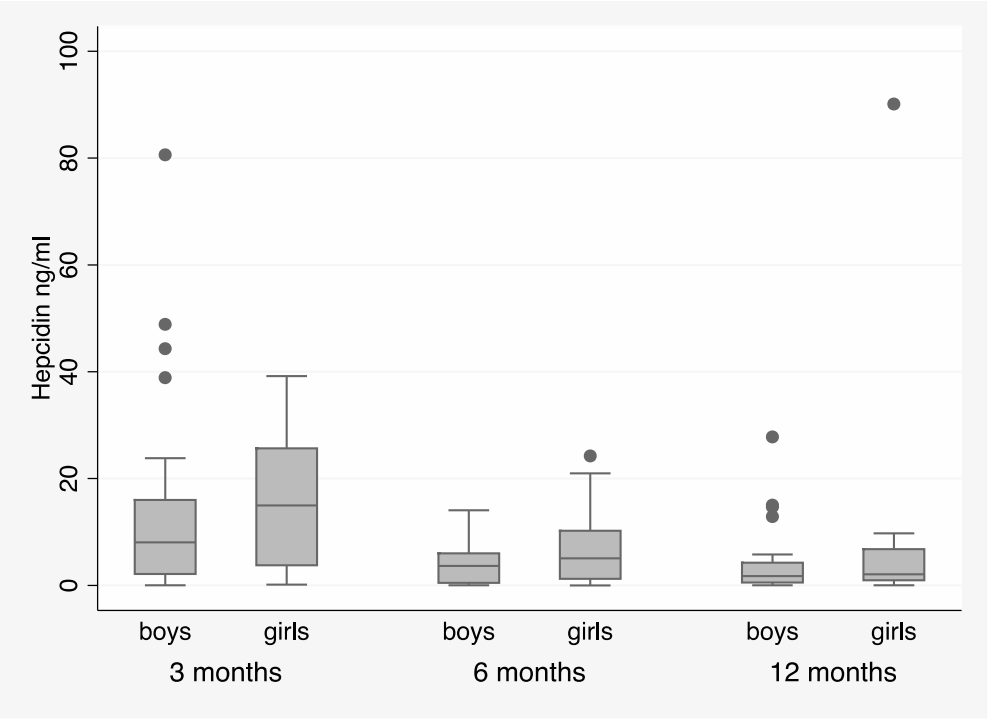

Figure 2.1. Plasma hepcidin concentrations in healthy non-anemic infants by age and gender. The boxes indicate the 25th and 75th percentiles and the horizontal line indicates the median. Differences by sex are non-significant in both univariate regression analysis and after adjusting for age (p=0.224).

Elevated hepcidin levels in HIV infected and HIV exposed Zimbabwean infants and the role of hepcidin in the pathogenesis of anemia in infants

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Short title: Hepcidin in the pathogenesis of infant anemia

Key words: Anemia, infants, hepcidin, inflammation, HIV

ABSTRACT

Anemia is common in sub-Saharan African infants, particularly in the context of HIV infection. Hepcidin is the major hormone regulating iron metabolism but its role in the pathogenesis of anemia during infancy is unclear. The aim of this study was to characterize hepcidin concentrations in anemic and non-anemic infants and in HIV-infected and HIV-exposed uninfected (HEU) infants. Archived plasma samples from HIV-unexposed (n=289), HEU (n=354) and HIV-infected (n=264) anemic and non-anemic Zimbabwean infants at 3, 6 and 12mo of age were used to measure hepcidin, ferritin, transferrin receptor, α-glycoprotein (AGP) and C-reactive protein (CRP) by ELISA. We undertook multivariate analysis of hepcidin, HIV and anemia status, adjusting for sex and age. Hepcidin concentrations were higher in HIV-infected compared to HEU and HIV-unexposed groups throughout infancy and higher in HEU compared to HIV-unexposed infants. Hepcidin concentrations were correlated with levels of ferritin and CRP. Overall, anemia was not associated with hepcidin concentrations during infancy, except in HIV-unexposed infants. Hepcidin declined with age in all groups; girls had higher hepcidin concentrations than boys. Plasma hepcidin concentrations are positively associated with inflammation in infants, as has been shown in adults. Future studies should explore whether elevated hepcidin contributes to the pathogenesis of HIV infection in infancy.

INTRODUCTION

Anemia is a serious public health problem affecting 2 billion people worldwide, particularly infants in developing countries (Lokeshwar *et al*, 2011; Milman, 2011). In many regions of Africa anemia occurs in the context of high HIV prevalence. An estimated 3.4 million children are infected with HIV globally, 90% of whom live in sub-Saharan Africa (WHO.; McDermid *et al*, 2007). HIV disease progression is rapid in perinatally infected infants without antiretroviral therapy (ART), with over 50% of children dying before 2 years of age (Newell *et al*, 2004; Marinda *et al*, 2007). Anemia occurs in 42-90% of HIV-infected children in low-income countries and is a well-recognized risk factor for mortality (Clark *et al*, 2002; Totin *et al*, 2002; Belperio& Rhew, 2004; Adetifa *et al*, 2006; Bolton-Moore *et al*, 2007; Bong *et al*, 2007; Gibb, 2008; Marazzi *et al*, 2008; Wamalwa *et al*, 2010). The cause of anemia is likely multifactorial, but the pathophysiology has not been well described in HIV-infected children (Calis *et al*, 2008). Furthermore, exposure to maternal HIV infection, even in the absence of infection, is associated with increased morbidity and mortality in early life: HIV-exposed uninfected (HEU) infants have higher mortality, increased rates of infections, impaired growth (Thea *et al*, 1993; Brahmbhatt *et al*, 2006; Makasa *et al*, 2007; Marinda *et al*, 2007; McNally *et al*, 2007; Shapiro *et al*, 2007) and increased vulnerability to anemia has been associated with infant exposure to maternal ART (Le Chenadec *et al*, 2003; Feiterna-Sperling *et al*, 2007; Dryden-Peterson *et al*, 2011; Ziske *et al*, 2013) in HEU infants.

Hepcidin in the pathogenesis of infant anemia

Hepcidin, a peptide hormone whose synthesis is simultaneously regulated by iron status and the innate immune system, has evolved as the master regulator of iron metabolism, linking iron homeostasis, inflammation, infection and anemia (Drakesmith& Prentice, 2008; Drakesmith& Prentice, 2012). The molecular control of hepcidin is part of the innate immune response to pathogens through IL-6, IL-22, type I interferons, toll-like receptor (TLR) ligands, and the endoplasmic reticulum stress response (Drakesmith& Prentice, 2012). Hepcidin synthesis is stimulated by elevated plasma iron concentration, infection and/or inflammation, (Nemeth *et al*, 2004; Galesloot *et al*, 2011) and is suppressed in conditions that demand increased serum iron, such as increased or ineffective erythropoiesis, hypoxia, anemia and iron deficiency (Kroot *et al*, 2010; Galesloot *et al*, 2011). The increased production of hepcidin in inflammation blocks the activity of ferroportin, the only known mammalian iron exporter, (Ganz *et al*, 2008; Grebenchtchikov *et al*, 2009; Galesloot *et al*, 2011) effectively blocking iron efflux from duodenal enterocytes, hepatocytes, the placenta and macrophages (Nemeth *et al*, 2004; Nemeth *et al*, 2004; Drakesmith& Prentice, 2012). Lowering circulating iron levels is an adaptive innate defense mechanism against iron-dependent extracellular organisms; however, diverting iron towards macrophages may enhance proliferation of intracellular organisms such as HIV and *Mycobacterium tuberculosis* (de Monye *et al*, 1999; Gordeuk *et al*, 2001; Montaner *et al*, 2006; Boelaert *et al*, 2007; Drakesmith& Prentice, 2012).

Only one study to date has reported hepcidin levels in HIV-infected adults, and none have described hepcidin in HIV-infected children. A limited number of studies have characterized hepcidin during infancy, when prevalence of anemia is high even in the

absence of HIV exposure, particularly in developing countries. We recently described hepcidin concentrations in healthy Zimbabwean infants (Mupfudze *et al*, 2013). In the current study, we aimed to characterize the independent associations between HIV status, anemia and age with hepcidin concentration in infants using archived samples from a well-defined cohort in Zimbabwe. We hypothesized that hepcidin levels are progressively elevated in HEU and HIV infected infants compared to HIV unexposed infants and are not able to distinguish between anemic and non-anemic infants in the presence of inflammation characteristic of HEU and HIV infected infants.

METHODS

The current study used data and stored samples from ZVITAMBO, a randomized controlled trial of maternal and neonatal vitamin A supplementation (Humphrey *et al*, 2006). Briefly, 14110 mother-infant pairs were recruited within 96 hours of delivery at maternity clinics and hospitals in Harare, Zimbabwe, between November 1997 and January 2000. Mother-infant pairs were eligible if neither had an acute life-threatening condition and the infant was a singleton with birth weight >1500 g. Follow-up was conducted at 6 weeks, 3 months and every 3 months thereafter, until the child was 12-24 months old. Demographic and obstetric data were collected through questionnaires at recruitment. Gestational age was assessed using the method of Capurro (Capurro *et al*, 1978). Maternal mid-upper arm circumference (MUAC) and height were measured within 96 hours of delivery; maternal weight was measured at 6 weeks postpartum. Infant weight and height were measured using an electronic scale (Seca Model 727, Hanover, MD, USA), and length board (ShorrBoard, Olney, MD, USA), respectively, using methods described by Gibson (Gibson, 1990). Weight-for-age (WAZ), height-for-age (HAZ), and weight-for-height (WHZ) Z-scores were calculated using WHO Anthro version 3.0.1.

Mothers were tested for HIV at enrolment by two parallel ELISAs (HIV 1.0.2 ICE [Murex Diagnostics]; GeneScreen HIV 1/2 [Sanofi Diagnostics Pasteur]); discordant results were resolved by Western blot (HIV Blot 2.2; Genelabs Diagnostics). The last available sample from each infant was tested for HIV (by serology for samples collected at ≥18 months of age; by PCR for samples collected <18 months, using Amplicor HIV-1

DNA test version 1.5 [Roche Diagnostics]). If the last available sample was negative, the child was classified as HIV-uninfected; if the last available sample was positive, the child was classified as HIV-infected and prior samples were tested to determine the timing of infection (Humphrey *et al*, 2006).

Hemoglobin was measured using the HemoCue hemoglobinometer (HemoCue, Mission Viejo, CA) in a 34% subsample of infants, over-selecting for those born to HIV-positive women, for a total of 2314 and 535 infants born to HIV-positive and HIV-negative women, respectively (Miller *et al*, 2003). Caregivers of infants with hemoglobin < 70 g/L were encouraged to take the child to a health facility for assessment. The trial was conducted prior to availability of cotrimoxazole prophylaxis or ART in Zimbabwe.

Written informed consent was obtained from mothers at recruitment. The original ZVITAMBO trial and this sub-study were approved by the Medical Research Council of Zimbabwe and the Committee on Human Research of The Johns Hopkins Bloomberg School of Public Health.

Study subjects for hepcidin study

We conducted a cross-sectional study of 3, 6 and 12mo infants. For each respective age, 6 groups of infants were selected, based on HIV and anemia status: HIV-unexposed non-anemic infants (Group 1), HIV-unexposed anemic infants (Group 2); HIV-exposed uninfected non-anemic infants (Group 3); HIV-exposed uninfected anemic infants (Group 4); HIV-infected non-anemic infants (Group 5) and HIV-infected anemic infants

(Group 6). Anemia was defined by a hemoglobin measurement < 105 g/L at 3 and 6 months, or < 100 g/L at 12 months; these criteria were modified from those of the World Health Organization because of the growing literature supporting lower hemoglobin cut-offs in infancy (Domellof *et al*, 2002; Miller *et al*, 2003; Eneroth *et al*, 2011). A total of 907 infants met the above criteria and had sufficient plasma volume available (≥ 120 μL). We therefore had three cross-sectional cohorts at ages 3, 6 and 12mo; 736 infants contributed data to one time-point, 153 infants to 2 time-points and 18 infants contributed data to all three time-points.

Laboratory assays

Plasma samples were stored at -70°C until analysis. Soluble transferrin receptor (sTFR) concentration was measured by enzyme immunoassay (Ramco Laboratories Inc, Houston, TX), with mean intra-assay coefficient of variation (CV) 5.4% and inter-assay CV 4.4%. Plasma ferritin was measured by enzyme immunoassay (Ramco Laboratories Inc, Houston, TX), with mean intra-assay CV 8.4% and inter-assay CV 8.3%. Plasma α-glycoprotein (AGP) and C-reactive protein (CRP) were measured by ELISA (R&D Systems Inc, Minneapolis, MN), with intra-assay mean CV of 6.1% and 5.9%, respectively, and inter-assay mean CV of 6.5% and 7.4%, respectively.

Hepcidin was measured in plasma by competition ELISA, using the hepcidin-25 (human) enzyme immunoassay kit (S-1337; Bachem, San Carlos, CA) with detection range between 0.02 and 25 ng/mL, according to the manufacturer's protocol. Plasma samples were diluted 1 in 4 in supplied standard diluent (peptide-cleared human serum).

Standards were run in duplicate and samples in singlicate. Samples giving readings outside the linear region of the curve were re-run at alternative dilutions. The intra-assay CV was mean 6.3% (range 5.7 - 6.9%), and inter-assay CV was 6.3%. Hepcidin levels in healthy non-anemic HIV-unexposed infants (Group 1) were previously published by our group as normative values (Mupfudze *et al*, 2013).

Statistical analysis

Baseline and iron variables are reported as median with interquartile range (IQR), or mean with standard deviation (SD). Comparisons between groups were made using Kruskal–Wallis and Mann-Whitney tests for skewed data and chi-square test for categorical data. Ferritin and hepcidin values below the detection limits of 0.59 μg/L and 0.02 ng/mL, respectively, were imputed using the limit of detection (LOD)/√2, thereby assigning a value of 0.42 μg/L for ferritin and 0.014 ng/mL for hepcidin (Barr *et al*, 2006). Hepcidin consensus values (hepcon1) were calculated using the algorithm developed by Kroot *et al*, to enable interpretation across studies where different assays are used (Kroot *et al*, 2012). Iron (ferritin and transferrin receptor) and inflammatory biomarkers (AGP and CRP) and hepcidin were log transformed prior to inclusion in multivariate analysis and correlation analysis. The associations of HIV and anemia status with plasma hepcidin concentration were assessed in a multivariate model adjusting for age and sex. All statistical analyses were performed using STATA version 12 (StataCorp, College Station, TX).

RESULTS

3-month-old infants

HIV-unexposed: Among 3-month old HIV-unexposed infants, those who were anemic were more stunted, had significantly higher plasma concentrations of CRP and hepcidin compared to those who were not anemic (Table 3.1).

HEU: Among 3-month-old HEU infants, those with anemia had lower birth weight, lower median concentrations of plasma hepcidin and ferritin, and a higher proportion had ferritin <12 μg/L compared to those without anemia.

HIV-infected: Amongst 3-month-old HIV-infected infants, those who were anemic had mothers with lower weight and BMI and reduced household income, compared to non-anemic infants. Plasma concentrations of AGP, CRP, ferritin and hepcidin were elevated in HIV-infected compared to other groups of 3-month-old infants, but no biomarkers were significantly different between anemic and non-anemic HIV-infected infants.

6-month-old infants

HIV-unexposed: HIV-unexposed infants who became anemic by 6 mo of age had a lower birthweight and were more stunted at birth than non-anemic infants (Table 3.2). Hepcidin was significantly higher in anemic compared to non-anemic infants. There was evidence of both iron deficiency and inflammation in anemic infants at 6 months: almost half of anemic infants had ferritin <12 μg/L and CRP levels were higher in anemic compared to non-anemic infants.

HEU: Amongst HEU infants, those who were anemic at 6 mo of age had lower gestational age and birthweight and had mothers with lower weight and BMI compared to non-anemic infants. Moreover, among HEU infants who were anemic at 6 months of age compared to those who were not, mean concentration of sTfR was higher, but no significant differences were detected between the groups in median concentrations of plasma ferritin, CRP, AGP or hepcidin.

HIV-infected: Mean plasma concentrations of AGP, CRP, sTfR, ferritin and hepcidin were higher among HIV-infected infants compared to all other infant groups. Within HIV-infected infants, those with anemia at 6 mo of age had higher mean sTfR concentrations compared to those without anemia at 6 months; no other significant differences were detected between the anemic and non-anemic groups in the other iron indices measured or in any baseline or 6 month maternal or infant characteristics.

12-month-old infants

HIV-unexposed: Among 12-month old HIV-unexposed infants, those who were anemic were more stunted at birth and had lower birth weight compared to non-anemic infants (Table 3.3). There was a greater degree of iron deficiency among HIV-unexposed infants at 12 mo compared to earlier ages. Hepcidin was lower in anemic compared to non-anemic infants, consistent with predominantly iron-deficiency anemia in this anemic group.

HEU: Among HEU infants, those who were anemic at 12 mo had lower birth weight and were more likely to be male compared to non-anemic HEU infants. Anemic HEU infants had higher levels of sTfR and lower levels of ferritin. Of note, however, more than half of infants who were not anemic also had ferritin concentrations in the iron-deficient range (<12 μg/L). Levels of hepcidin and inflammatory biomarkers (AGP and CRP) were similar between anemic and non-anemic HEU infants.

HIV-infected: Among HIV-infected infants, those who were anemic at 12 mo were born to mothers with lower weight, BMI and MUAC, were more stunted at birth and had lower birth weight compared to non-anemic infants. The majority of anemic infants acquired HIV infection intrapartum or *in utero*. As at younger ages, hepcidin was elevated in HIV-infected infants compared to other groups, but levels were not significantly different between anemic and non-anemic HIV-infected infants. Levels of CRP were also significantly higher in anemic compared to non-anemic infants.

Characteristics associated with HIV exposure status across all age groups

Across HIV-unexposed, HIV-exposed and HIV-infected groups, infants were progressively more underweight and stunted, both at birth and throughout infancy. There was a general trend towards higher AGP, CRP and hepcidin across HIV-unexposed, HEU and HIV-infected infant groups, and maternal hemoglobin tended to decline progressively across groups.

Table 3.1. Characteristics of 3-month-old infants

Characteristic	HIV-Negative Mother		HIV-positive Mother			
			Non-infected infants		Infected infants	
	Non-anemic	Anemic	Non-anemic	Anemic	Non-anemic	Anemic
	N= 60	N= 61	N=69	N=67	N=50	N=66
Maternal factors						
Age, years[2]	27 (6)	24 (6) [a]	27 (5) [b]	26 (5) [b]	28 (5) [b, d]	26 (5) [b]
Hemoglobin, g/L[3]	135	133	123	125	117	111
	(128, 137)	(126, 140)	(115, 129) [a, b]	(113, 130) [a, b]	(113, 128) [a, b]	(91, 127) [a, b, c, d]
Height, m[3]	1.62	1.60	1.59	1.59	1.60	1.60
	(1.57, 1.66)	(1.56, 1.64)	(1.55, 1.63) [a]	(1.56, 1.64)	(1.57, 1.63)	(1.56, 1.65)
Weight at 6 weeks						56.5
postpartum, kg[3]	61.0	62.8	58.0	58.8	63.0	(52.8, 63.3) [a, b,
	(55.6, 70.5)	(56.1, 75.0)	(53.5, 64.4) [b]	(49.5, 67) [b]	(55.4, 83.2) [c, d]	d, e]
BMI[3]	23.6	25.1	22.8	22.5	23.7	21.8

	(20.8, 25.8)	(21.5, 27.2)	(21.6, 25.5)	(20.6, 24.5) [b]	(21.8, 28.8) [d]	(20.3, 23.7) [b, e]
Maternal MUAC,	26.4	25.8	25.89	25.6	26.4	25.6
cm^2	(2.8)	(2.7)	(2.6)	(3.4)	(2.8)	(2.4)
CD4 count, per	N/A	N/A	384	380	378	319
microliter[3]			(223, 558)	(209, 522)	(271, 535)	(179, 580)
Viral load, copies	N/A	N/A				26481
per ml[3]			7367	20641	13910	(10200,
			(3061, 38032)	(1335, 88481)	(4534, 35073)	100052) [c]
Vitamin A	55 (33)	53 (32)	45 (31)	43 (29)	56 (28)	59 (39)
treatment[¥ 1]						
Education, years[3]	11 (9, 11)	11 (9,11) [a]	11 (9,11)	10 (9,11)	11 (9,11)	10 (8,11)
Mothers	16.7 (10)	11.5 (7)	18.8 (13)	20.9 (14)	18.0 (9)	24.2 (16)
Employed[1]						
Household income	1220	913	914	913	1994	918
per month, US	(801, 1694)	(730, 1455)	(704,1546)	(629, 2033)	(913, 2992)	(412, 1369)

dollars[3]						
Infant characteristics						
Male sex[1]	47 (28)	48 (29)	42 (29)	58 (39)	40 (20)	47(31)
Gestational age, weeks[2]	39.6 (1.2)	39.7 (1.1)	39.5 (1.3)	39.2 (1.6)	39.1 (1.4)	39.0 (1.3) [a, b]
Birth weight, g[2]	3105 (376)	3065 (304)	3023 (429)	2838 (457) [a, b, c]	2906 (495) [a, b]	2879 (513) [a, b]
WAZ at birth[2]	-0.42 (0.79)	-0.50 (0.66)	-0.60 (0.94)	-1.07 (0.94) [a, b, c]	-1.07 (1.09) [a, b]	-0.88 (1.12) [a, b]
WAZ at 3 mo[2]	-0.07 (0.91)	-0.26 (0.88)	-0.50 (1.07) [a]	-0.81 (1.39) [a, b]	-1.44 (1.42) [a, b, c, d]	-1.72 (1.36) [a, b, c, d]
LAZ at birth[2]	0.13 (0.90)	-0.14 (1.03)	-0.37 (1.04) [a]	-0.69 (1.04) [a, b]	-0.69 (1.36) [a]	-0.39 (1.21) [a]
LAZ at 3 mo[2]	-0.32 (0.83)	-0.95 (1.04) [a]	-0.79 (1.39) [a]	-1.21 (1.36) [a]	-1.57 (1.17) [a, b, c]	-1.93 (1.50) [a, b, c, d]
Breastfeeding pattern at 3 months[1]						
Exclusive	26.7 (16)	11.5 (7)	14.5 (10)	14.9 (10)	12.0 (6)	6.1 (4) [a, b]

Predominant	40.0 (24)	41.0 (25)	53.6 (37)	47.8 (32)	50.0 (25)	69.7 (46)
Mixed	20.0 (12)	29.5 (18)	24.6 (17)	20.9 (14)	18.0 (9)	15.2 (10)
Timing of infection[1]						
IP					16.0 (8)	31.8 (21)
IU					56.0 (28)	51.5 (34)
PN					28.0 (14)	16.7 (11)
Infant mortality[1]	0	0	0	0	12 (6) [a, b, c, d]	25.76 (17) [a, b, c, d]
Neonatal vitamin A treatment[¥ 1]	52 (31)	44 (27)	39 (27)	61 (41) [c]	50 (25)	58 (38) [c]
Infant Biomarkers at 3months						
Hemoglobin, g/L[2]	117.9 (13.4)	92.6 (13.7) [a]	118.6 (10.5) [b]	90.1 (16.6) [a, c]	116.6 (8.0) [b, d]	89.4 (13.7) [a, b, e]
Hepcidin, ng/mL[3]	9.7 (2.5, 19.3)	14.7 (6.8, 29.5) [a]	14.3 (8.1, 28.4) [a]	10.3 (3.7, 24.0) [c]	20.[3] (8.5, 40.0) [a, d]	23.9 (7.9, 42.9) [a, b, d]

Hepcidin consensus values [ψ]	6.2 (0.6, 13.7)	10.1 (3.9, 21.7) [a]	9.8 (4.9, 20.8) [a]	6.7 (1.5, 17.4) [c]	14.4 (5.3, 29.8) [a, d]	17.3 (4.8, 32.1) [a, b, d]
Ferritin, µg/l [3]	44.10 (27.94, 103.55)	49.25 (28.68, 91.76)	66.57 (42.79, 100.44)	51.09 (25.51, 74.87) [c]	104.98 (39.80, 194.28) [a, b, c, d]	116.74 (55.84, 351.15) [a, b, c, d]
Ferritin < 12µg/l [1]	0	8.20 (5) [a]	4.35 (3)	10.45 (7) [a]	0 [b, d]	0 [b, d]
Soluble transferrin receptor, mg/L [3]	5.71 (4.61, 6.69)	5.96 (5.25, 7.32)	7.25 (5.48, 8.71) [a, b]	6.96 (5.65, 8.2) [a, b]	8.50 (6.64, 12.14) [a, b, c, d]	9.11 (6.77, 12.03) [a, b, c, d]
Soluble transferrin receptor > 8.3mg/L [1]	0	14.75 (9) [a]	31.88 (22) [a, b]	23.88 (16) [a]	54.00 (27) [a, b, c, d]	63.64 (42) [a, b, c, d]

	0.21	0.45	0.46	0.95	1.4	3.06
CRP, mg/L[3]	(0.11, 0.91)	(0.18, 3.05) [a]	(0.21, 1.33) [a]	(0.22, 3.04) [a]	(0.70, 5.39) [a, b, c]	(0.84, 10.40) [a, b, c, d]
	0.35	0.32	0.55	0.59	0.67	0.74
AGP, g/L[3]	(0.27,0.48)	(0.23, 0.53)	(0.37, 0.74)	(0.40, 0.87) [a, b]	(0.53, 1.04) [a, b, c]	(0.51, 1.15) [a, b, c, d]

WAZ: weight-for-age Z-score, LAZ: length-for-age Z-score, MUAC: Mid-upper arm circumference, SD: standard deviation, IQR: interquartile range

[1]Values are % (n), [2]Values are mean (SD), [3]Values are median (IQR)

*All characteristics measured <96 hours of delivery except where noted

[ψ] Hepcidin consensus values were calculated using the algorithm Y= -1.36 + 0.78*hepcidin (Kroot *et al*, 2012)

[¥]In the ZVITAMBO trial, mother-infant pairs were randomized within 96 h of birth to one of 4 treatment groups (Aa, Ap, Pa, Pp), where 'A' was maternal vitamin A supplementation (400,000 IU), 'P' was maternal placebo, 'a' was infant vitamin A supplementation (50,000 IU) and 'p' was infant placebo. Full details of the trial have been published elsewhere(Humphrey *et al*, 2006).

For all variables, pair-wise comparisons were made by Mann-Whitney test and superscript letter denotes a raw $p<0.05$ for the difference between that group and:

[a] HIV unexposed non anemic infants

[b] HIV unexposed anemic infants

[c] HIV exposed uninfected non anemic infants

[d] HIV unexposed uninfected anemic infants

[e] HIV infected non anemic infants

Table 3.2. Characteristics of 6-month-old infants

Characteristic	HIV-Negative Mother		HIV-positive Mother			
			Non-infected infants		Infected infants	
	Non-anemic	Anemic	Non-anemic	Anemic	Non-anemic	Anemic
	N= 47	N= 66	N=66	N=65	N=33	N=69
Maternal factors						
Age, years[2]	24 (5)	25 (6)	26 (5)	26 (4)	27 (5) [a, b]	27 (5) [a, b]
Hemoglobin, g/L[3]	136	137	125	122	128	123
	(132, 142)	(128, 144.5)	(118, 133) [a, b]	(109, 134) [a, b]	(117, 132) [a, b]	(113, 128) [a, b]
Height, m[3]	1.59	1.58	1.59	1.59	1.62	1.60
	(1.56, 1.65)	(1.55, 1.63)	(1.55, 1.64)	(1.54, 1.65)	(1.57, 1.68)	(1.57, 1.66)
Weight at 6 weeks	61.5	58.3	61.0	57.3	62.5	60.0
postpartum, kg[3]	(53.0, 70.0)	(53.0, 68.0)	(54.7, 65.4)	(49.0, 66.2) [c]	(52.0, 66.5)	(55.0, 69.3)
BMI[3]	23.4	23.2	24.1	21.3	21.8	22.7
	(20.5, 25.6)	(20.2, 26.2)	(21.8, 26.3)	(20.0, 23.8) [c]	(19.5, 25.2)	(20.7, 27.3)

Maternal MUAC, cm[2]	25.9 (2.4)	26.0 (3.3)	27.1 (3.5) [a, b]	26.1 (2.5)	25.5 (2.5) [c]	26.1 (3.1)
CD4 count, per microliter[3]	N/A	N/A	403 (295, 550)	437 (250, 619)	386 (190, 452)	333 (268, 453)
Viral load, copies per ml[3]	N/A	N/A	9380 (2552, 34063)	5970 (1565, 65289)	27783 (6493, 82959)	23517 (8850, 79113) [d]
Vitamin A treatment[¥ 1]	49(23)	47 (31)	52 (36)	40 (27)	42 (14)	54 (37)
Education, years[3]	11 (9, 11)	10 (9,11)	11 (9,11)	10 (9,11)	11 (9,11)	10 (9,11)
Mothers Employed[1]	10.9 (5)	19.7 (13)	21.7 (15)	33.8 (23)	27.3 (9)	18.8 (13)
Household income per month, US dollars[3]	1220 (855, 1830)	913 (610, 1322) [a]	881 (503, 1555)	949 (711, 1815)	1763.00 (855, 2253) [b, d]	970 (671, 1902)
Infant characteristics						

Male sex[1]	47 (22)	59 (39)	52 (36)	44 (30)	55 (18)	55 (38)
Gestational age, weeks[2]	39.7 (1.2)	39.5 (1.1)	39.5 (1.4)	38.7 (1.6) [a, b, c]	39.3 (1.4) [d]	39.2 (1.2) [d]
Birth weight, g[2]	3186 (359)	3068 (386) [a]	3008 (451.04) [a]	2818 (497) [a, b]	3053 (452) [d]	2960 (457) [a, d]
WAZ at birth[2]	-0.24 (0.75)	-0.53 (0.80)	-0.74 (0.97) [a]	-1.12 (0.97) [a, b, c]	-1.12 (1.20) [d]	-0.57 (1.02) [a]
WAZ at 6 mo[2]	-0.07 (1.09)	-0.20 (1.17)	-0.45 (1.15) [a, b, d]	-0.27 (1.27)	-1.25 (1.70) [a, b, c, d]	-1.30 (1.38) [a, b, c, d]
LAZ at birth[2]	0.11 (0.71)	-0.29 (1.09)	-0.13 (0.96)	-0.42 (0.96) [a]	-0.42 (1.31) [a]	-0.53 (1.13)
LAZ at 6 mo[2]	-0.36 (1.23)	-0.79 (1.08)	-0.85 (1.19) [a]	-1.00 (1.27) [a]	-1.13 (1.61) [a]	-1.63 (1.25) [a, b, c, d]
Breastfeeding pattern at 3 months[1]						
Exclusive	17.0 (8)	10.6 (7)	9.1 (6)	9.2 (6)	6.1 (2)	5.8 (4)
Predominant	36.2 (17)	51.5 (34)	51.5 (34)	47.7 (31)	39.4 (13)	56.5 (39)
Mixed	14.9 (7)	19.7 (13)	16.7 (11)	20.0 (13)	24.2 (8)	11.6 (8)

Timing of infection[1]						
IP					24.2 (8)	36.2 (25)
IU					39.4 (13)	37.7 (26)
PN					36.4 (12)	26.1 (18)
Infant mortality[1]	0	1.52 (1)	0	0	6.06 (2) [c, d]	10.14 (7) [a, b, c, d]
Neonatal vitamin A treatment[¥ 1]	55 (26)	47 (31)	54 (37)	40 (27)	52(17)	57 (39)
Infant Biomarkers at 6 months						
Hemoglobin, g/L[2]	118.0 (9.4)	971.6 (13.2) [a]	118.0 (12.7) [b]	87.7 (17.5) [a, c]	116.3 (13.7) [b, d]	91.0 (11.7) [a, c]
Hepcidin, ng/mL[3]	4.5 (0.5, 7.3)	7.9 (1.6, 22.7) [a]	7.6 (3.2, 13.3) [a]	6.1 (1.2, 14.7) [a]	7.5 (4.3, 39.2) [a, d]	10.0 (1.6, 25.6) [a]
Hepcidin consensus values [ψ3]	2.2 (-1.0, 4.4)	4.8 (-0.1, 16.3) [a]	4.6 (1.11, 9.0) [a]	3.4 (-0.4, 10.1) [a]	4.5 (2.0, 29.2) [a, d]	6.4 (-0.1, 18.6) [a]

Ferritin, µg/l[3]	25.15 (17.64, 42.67)	13.16 (6.69, 25.08) [a]	17.48 (9.32, 30.68) [a]	12.36 (7.06, 22.76) [a]	37.20 (17.17, 115.28) [b, c, d]	30.69 (14.93, 52.84) [b, c, d]
Ferritin < 12µg/l[1]	0	46.97 (31) [a]	34.85 (23) [a]	46.15 (30) [a]	12.12 (4) [a, b, c, d]	17.39 (12) [a, b, c, d]
Soluble transferrin receptor, mg/L[3]	5.91 (4.55, 6.44)	6.81 (5.68, 8.68) [a]	7.51 (6.06, 8.28) [a]	8.69 (6.96, 10.96) [a, b, c]	7.45 (6.46, 9.81) [a]	8.66 (7.20, 11.34) [a, b, c, e]
Soluble transferrin receptor > 8.3mg/L[1]	0	32.31 (21) [a]	24.24 (16) [a]	55.38 (36) [a, b, c]	36.36 (12) [a]	56.52 (39) [a, b, c]
CRP, mg/L[3]	0.32 (0.13, 1.17)	2.33 (0.49, 7.52) [a]	2.08 (0.44, 7.03) [a]	1.97 (0.51, 6.06) [a]	3.10 (0.62, 7.69) [a]	4.64 (1.79, 14.00) [a, b, c, d]

	0.50	0.40	0.67	0.68	0.77	0.81
AGP, g/L[3]	(0.30, 0.61)	(0.27, 0.57)	(0.47, 0.90) [a, b]	(0.51, 0.84) [a, b]	(0.58, 1.02) [a, b]	(0.60, 1.20) [a, b, c, d]

WAZ: weight-for-age Z-score, LAZ: length-for-age Z-score, MUAC: Mid-upper arm circumference, SD: standard deviation, IQR: interquartile range

[1]Values are % (n), [2]Values are mean (SD), [3]Values are median (IQR)

*All characteristics measured <96 hours of delivery except where noted

[Ψ] Hepcidin consensus values were calculated using the algorithm Y= -1.36 + 0.78*hepcidin (Kroot *et al*, 2012)

[¥]In the ZVITAMBO trial, mother-infant pairs were randomized within 96 h of birth to one of 4 treatment groups (Aa, Ap, Pa, Pp), where 'A' was maternal vitamin A supplementation (400,000 IU), 'P' was maternal placebo, 'a' was infant vitamin A supplementation (50,000 IU) and 'p' was infant placebo. Full details of the trial have been published elsewhere(Humphrey *et al*, 2006).

For all variables, pair-wise comparisons were made by Mann-Whitney test and superscript letter denotes a raw $p<0.05$ for the difference between that group and:

[a] HIV unexposed non anemic infants

[b] HIV unexposed anemic infants

[c] HIV exposed uninfected non anemic infants

[d] HIV unexposed uninfected anemic infants

[e] HIV infected non anemic infants

Table 3.3. Characteristics of 12-month-old infants

Characteristic	HIV-Negative Mother		HIV-positive Mother			
			Non-infected infants		Infected infants	
	Non-anemic	Anemic	Non-anemic	Anemic	Non-anemic	Anemic
	N= 40	N= 66	N=69	N=68	N=68	N=66
Maternal factors						
Age, years[2]	26 (6)	24 (6)	25 (5)	25 (4) [b]	27 (5) [b]	26 (5) [b]
Hemoglobin, g/L[3]	141	133	129	125	127	125
	(130, 142)	(125, 142)	(113, 136) [a, b]	(115, 135) [a, b]	(116, 138) [a, b]	(114, 130) [a, b]
Height, m[3]	1.61	1.59	1.61	1.60	1.61	1.59
	(1.54, 1.65)	(1.55, 1.62)	(1.57, 1.66)	(1.55, 1.66)	(1.58, 1.65) [b]	(1.55, 1.63)
Weight at 6 weeks	61.0	58.0	60.3	58.2	61.5	56.4
postpartum, kg[3]	(53.3, 68.5)	(53.0, 63.5)	(56.0, 65.8)	(51.0, 63.5)	(55.0, 74.4) [d]	(52.2, 63.0) [e]
BMI[3]	23.4	22.6	23.1	22.1	23.5	22.2
	(21.0, 26.1)	(20.5, 26.6)	(21.3, 25.2)	(20.7, 25.0)	(21.7, 28.4) [d]	(20.1, 24.3) [e]

Maternal MUAC, cm^2	26.6 (2.7)	25.7 (2.7)	26.2 (3.3)	26.1 (2.4)	27.1 (3.4) [a, b]	25.1 (2.4) [d, e]
CD4 count, per microliter[3]	N/A	N/A	385 (187, 556)	493 (261, 576)	341 (289, 417)	375 (212, 563)
Viral load, copies per ml[3]	N/A	N/A	6399 (1734, 40323)	4635[g] (1282, 29643)	14269 (6015, 69921)	24697 (8484, 119308) [c, d]
Vitamin A treatment[¥ 1]	50.0 (20)	54.6 (36)	52.2 (36)	50.0 (34)	39.7 (27)	50.0 (33)
Education, years[3]	11 (9, 11)	11 (9, 11)	11 (9, 11)	11 (9, 11)	11 (9, 11)	10 (9, 11)
Mothers Employed[1]	15.0 (6)	18.2 (12)	18.8 (13)	17.7 (12)	19.1 (13)	18.2 (12)
Household income per month, US dollars[3]	1221 (879, 2103)	913 (676, 1322)	933 (529, 1831)	917 (575.5, 1576) [b]	1017 (680, 1526)	1164 (711, 2426)

Infant characteristics

Male sex[1]	53(21)	62 (41)	42 (29) [b]	66 (45) [c]	47 (32) [d]	52 (34)
Gestational age, weeks[2]	39.5 (1.2)	39.4 (1.1)	39.2 (1.6)	39.1 (1.6)	39.1 (1.3)	39.1 (1.4)
Birth weight, g[2]	3230 (459)	2995 (359) [a]	2973 (427)	2853 (420) [a, c]	3020 (498) [d]	2883 (466) [a]
WAZ at birth[2]	-0.18 (0.95)	-0.69 (0.78) [a]	-0.72 (1.01) [a]	-1.04 (1.01) [a]	-1.04 (0.96) [a, d]	-0.64 (1.13) [a]
WAZ at 12 mo[2]	-0.39 (1.23)	-0.58 (0.92)	-0.45 (1.19)	-0.71 (1.37)	-1.45 (1.32) [a, b, c, d]	-2.00 (1.49) [a, b, c, d, e]
LAZ at birth[2]	0.32 (1.44)	-0.19 (0.96) [a]	-0.30 (1.16) [a]	-0.63 (1.16) [a, b]	-0.63 (1.17) [d]	-0.17 (1.36) [a]
LAZ at 12 mo[2]	-0.84 (1.36)	-1.22 (0.96)	-0.88 (1.10)	-1.16 (1.70)	-1.71 (1.14) [a, b, c, d]	-2.32 (1.34) [a, b, c, d, e]
Timing of infection[1]						
IP					13.2 (9)	19.7 (13) [e]
IU					36.8 (25)	54.6 (36)

PN					50.0 (34)	25.8 (17)
Infant mortality[1]	0	0	1.45 (1)	0	5.88 (4) [b]	12.12 (8) [a, b, c, d]
Neonatal vitamin A treatment[¥ 1]	52.5 (21)	51.5 (34)	55.1 (38)	58.8 (40)	42.7 (29)	53.0 (35)
Infant Biomarkers at 12 months						
Hemoglobin, g/L[2]	117.1 (11.8)	89.4 (11.2) [a]	116.4 (10.1) [b]	83.2 (15.4) [a, b, c]	112.2 (14.7) [a, b, c, d]	84.0 (13.7) [a, b, e]
Hepcidin, ng/mL[3]	1.90 (0.73, 6.17)	0.89 (0.01, 3.88) [a]	0.82 (0.12, 5.21)	0.98 (0.01, 2.98) [a]	3.87 (0.72, 12.52) [b, c, d]	2.56 (0.25, 13.98) [b, c, d]
Hepcidin consensus values [ψ 3]	0.12 (-0.79, 3.45)	-0.66 (-1.35, 1.67) [a]	-0.72 (-1.27, 2.71)	-0.60 (-1.35, 0.97) [a]	1.66 (-0.80, 8.40) [b, c, d]	0.64 (-1.16, 9.54) [b, c, d]
Ferritin, µg/l[3]	22.21 (15.86, 32.85)	3.19 (0.61, 15.56) [a]	9.00 (4.44, 18.37) [a, b]	4.68 (1.74, 16.61) [a,	19.35 (9.37, 39.99) [b, c,	18.83 (8.71, 55.77) [b, c,

				[c, d]	[d]	[d]
Ferritin < 12µg/l[1]	0	71.21 (47) [a]	59.42 (41) [a]	73.53 (50) [a]	32.35 (22) [a, b, c, d]	40.91 (27) [a, b, c, d]
Soluble transferrin receptor, mg/L[3]	6.00 (3.94, 6.74)	6.71 (4.93, 9.16) [a]	6.21 (4.41, 8.00)	8.42 (6.61,10.82) [a, b, c]	6.99 (5.15,8.76) [a, d]	9.68 (6.18,15.30) [a, b, c, e]
Soluble transferrin receptor > 8.3mg/L[1]	0	36.92 (24) [a]	21.74 (15) [a, b]	50.00 (33) [a, c]	29.41 (20) [a, d]	56.92 (37) [a, b, c, e]
CRP, mg/L[3]	0.54 (0.18, 1.20)	1.43 (0.44, 5.52) [a]	0.84 (0.16, 3.98)	1.40 (0.28,4.64) [a]	2.64 (0.79,7.08) [a, c, d]	4.75 (1.81,14.80) [a, b, c, d, e]

	0.49	0.57	0.65	0.62	0.85	1.03 [a, b, c, d, g]
AGP, g/L[3]	(0.38, 0.63)	(0.42, 0.79)	(0.45, 0.89) [a]	(0.45,0.96) [a]	(0.57,1.14) [a, b, c, d]	(0.57,1.57)

WAZ: weight-for-age Z-score, LAZ: length-for-age Z-score, MUAC: Mid-upper arm circumference, SD: standard deviation, IQR: interquartile range

[1]Values are % (n), [2]Values are mean (SD), [3]Values are median (IQR)

*All characteristics measured <96 hours of delivery except where noted

[ψ] Hepcidin consensus values were calculated using the algorithm Y= -1.36 + 0.78*hepcidin (Kroot *et al*, 2012)

[¥]In the ZVITAMBO trial, mother-infant pairs were randomized within 96 h of birth to one of 4 treatment groups (Aa, Ap, Pa, Pp), where 'A' was maternal vitamin A supplementation (400,000 IU), 'P' was maternal placebo, 'a' was infant vitamin A supplementation (50,000 IU) and 'p' was infant placebo. Full details of the trial have been published elsewhere(Humphrey *et al*, 2006).

For all variables, pair-wise comparisons were made by Mann-Whitney test and superscript letter denotes a raw $p<0.05$ for the difference between that group and:

[a] HIV unexposed non anemic infants

[b] HIV unexposed anemic infants

[c] HIV exposed uninfected non anemic infants

[d] HIV unexposed uninfected anemic infants

[e] HIV infected non anemic infants

Determinants of hepcidin levels during infancy

To characterize the main determinants of hepcidin concentrations during infancy, we undertook multivariate analysis of hepcidin, HIV and anemia status, adjusting for age and sex (Table 3.4). Log hepcidin concentrations were 42% higher in HEU infants compared to HIV-unexposed infants, after adjusting for anemia status, age and sex ($p=0.017$); among HIV-infected infants log hepcidin concentrations were 176% higher than in HIV-unexposed infants ($p<0.001$). Overall, anemia status was not associated with hepcidin concentrations during infancy; however, infant age modified the association between anemia and hepcidin. Anemic 3 mo infants had 62% higher hepcidin levels compared to non-anemic 3 mo infants ($p=0.02$), with the same trend seen in 6 mo infants. However, at 12 mo of age, differences in hepcidin were observed in the opposite direction ($p<0.001$), with lower hepcidin levels in anemic compared to non-anemic infants.

Hepcidin levels were progressively lower with age in each group, with 44% and 84% decreases in 6 mo and 12 mo non-anemic infants, respectively, compared to 3 mo non-anemic infants ($p=0.008$ and $p<0.0001$, respectively) and 62% and 94% lower hepcidin levels in 6 mo and 12 mo anemic infants respectively, compared to 3 mo anemic infants ($p<0.001$ and $p<0.001$, respectively, 2.d.f.). Overall, girls had 83% higher hepcidin concentrations than boys ($p<0.001$) during infancy. Taken together, age, sex and HIV status were the main determinants of hepcidin levels during infancy.

Table 3.4. Results of linear regression analyses for log plasma hepcidin concentrations (ng/mL)

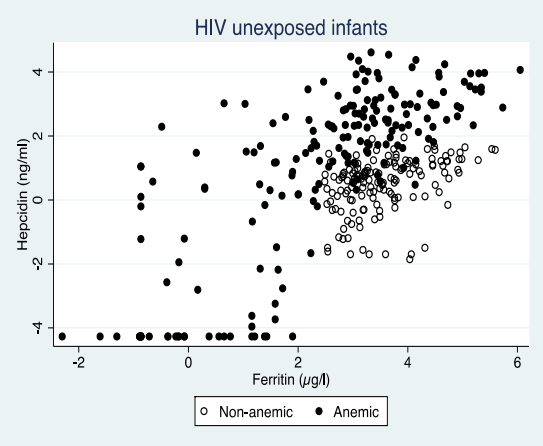
	Univariate model			Multivariate model		
	β	95% CI	P-value	β	95% CI	P-value
Sex						
Boys[R]						
Girls	2.08	1.59, 2.72	< 0.001	1.82	1.44, 2.31	<0.001
HIV status						
HIV unexposed[R]						
HIV exposed	0.14	1.00, 1.92	0.051	1.42	1.07, 1.90	0.02
HIV infected	2.57	1.84, 3.60	<0.001	2.76	2.05, 3.72	<0.001
Anemia						
Non-anemic[R]						
Anemic	-0.97	0.74, 1.27	0.818	1.62	1.08, 2.42	0.02
Age						
3 months[R]						
6 months	0.44	0.32, 0.59	<0.001	0.56	0.36, 0.86	0.008
12 months	0.09	0.07, 0.13	<0.001	0.16	0.11, 0.24	<0.001
Interaction:				0.68	0.38, 1.22	0.19

6 months × Anemic			
Interaction:	0.36	0.20, 0.64	<0.001
12 months × Anemic			

*The dependent variable hepcidin was log-transformed before inclusion in the models. Thus, the β values express the percentage change in hepcidin (ng/mL) that is associated with each group compared to the reference group.

Associations between haematological biomarkers

We next undertook analyses to explore the relationships between hepcidin and other hematological biomarkers (Fig 3.1). We assessed the relationship between hepcidin, ferritin and CRP within HIV-unexposed, HEU and HIV-infected infant groups at each age. Anemic and non-anemic infants were combined within each group in order to extend the ranges of hepcidin, ferritin and CRP concentrations. Hepcidin was positively correlated with both ferritin and CRP in all groups at each age ($p<0.001$ for all correlations). In all infant groups, hepcidin was more strongly correlated with ferritin than with CRP (Fig 3.1).



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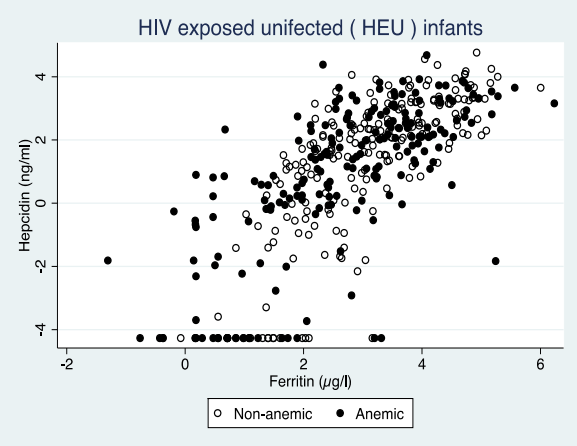

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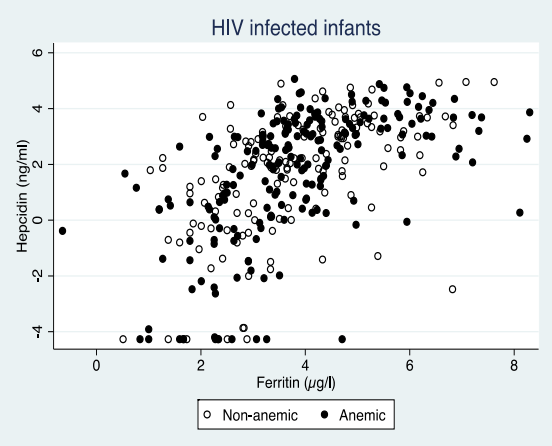

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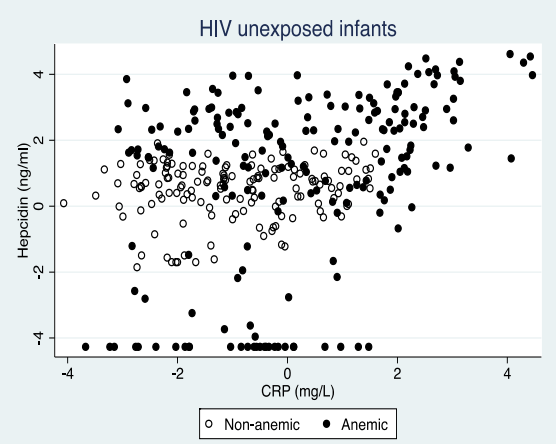

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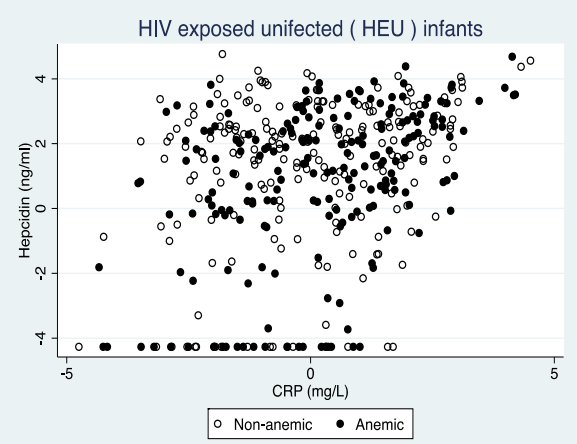

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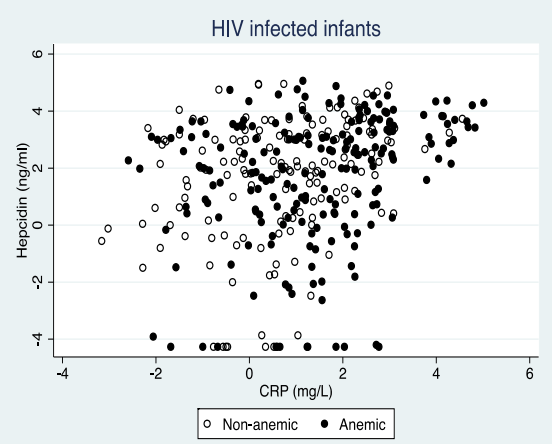

r=0.27

Figure 3.1. Relationships between hepcidin and other biomarkers

Correlation of hepcidin with ferritin and CRP in HIV-unexposed, HIV-exposed uninfected (HEU) and HIV-infected infants. Values for non-anemic (empty) and anemic (solid) infants ($p<0.001$ for all Pearson correlations). Hepcidin, ferritin and CRP are expressed on a log scale.

DISCUSSION

This study is, to our knowledge, the first to characterize hepcidin concentrations in HIV-infected and HIV-exposed infants, and focused specifically on sub-Saharan African infants, who are at particularly high risk of anemia. We show, first, that hepcidin levels are strikingly elevated in HIV-infected infants throughout the first year of life; second, that HIV-exposed uninfected infants have higher hepcidin levels than HIV-unexposed infants; third, that hepcidin levels in infancy are related to inflammatory biomarkers, which are elevated in HEU and HIV-infected infants; fourth, that hepcidin overall is not strongly associated with anemia during infancy in the context of HIV; fifth, that hepcidin concentrations are progressively lower at 6 and 12 months compared to 3 months; and, sixth, that hepcidin levels are higher in girls than boys.

Hepcidin concentrations were markedly elevated in HIV-infected infants from as early as 3 months of age, and remained high throughout infancy. HIV infection is characterized by immune activation, and we found a direct relationship between hepcidin and levels of inflammatory biomarkers in infants, as others have found in adults (Ganz *et al*, 2008; Galesloot *et al*, 2011). Hepcidin blocks the activity of the iron exporter ferroportin, leading to iron sequestration in macrophages. Consistent with this, we found that ferritin was higher in HIV-infected infants compared to other infant groups, and did not differ between anemic and non-anemic HIV-infected infants, highlighting both the iron sequestration that occurs during HIV infection and the role of ferritin as an acute phase protein.

Only one other study has reported hepcidin levels in HIV-infected individuals, and used a monoclonal antibody immunoassay (Schwarz *et al*, 2011). They reported median (IQR) hepcidin concentration of 25.1 ng/mL (6.4–39.7) in 188 Rwandan HIV-positive women without ferroportin mutations, whilst a smaller cohort of 12 women with a ferroportin mutation (Q248H mutation in the gene SLC40A1), associated with resistance to hepcidin (Masaisa *et al*, 2012), had a median hepcidin concentration of 3.3 ng/mL (IQR 1.3-7.5). Despite the depressed hepcidin concentration, they observed elevated ferritin in HIV-positive women with the ferroportin mutation and concluded that the increased availability of iron may explain the higher prevalence of opportunistic infections, including pulmonary tuberculosis and *Pneumocystis jiroveci* pneumonia, in this group compared to HIV-positive women without the ferroportin mutation (Masaisa *et al*, 2012).

Survival of HIV-infected individuals has been shown to correlate inversely with higher iron stores in bone marrow macrophages (Xu *et al*, 2010). Macrophages recycle 25-30 mg iron daily (compared to 1mg/day absorbed from a healthy diet), hence erythropoiesis is extremely sensitive to hepcidin levels. Elevated hepcidin levels may simultaneously halt erythropoiesis while promoting the growth and survival of HIV and intracellular co-pathogens such as *Mycobacterium tuberculosis* (Montaner *et al*, 2006; Boelaert *et al*, 2007; Drakesmith& Prentice, 2012). Anemia is a risk factor for mortality in pediatric HIV infection and is likely to reflect advanced disease; consistent with this, HIV-infected infants were more likely to be underweight and stunted in this cohort.

The expansion of prevention of mother-to-child transmission (PMTCT) interventions in

countries with high HIV prevalence means that an increasing number of HEU infants are being born in sub-Saharan Africa (Filteau, 2009). HEU infants have poor growth and increased morbidity and mortality compared to HIV-unexposed infants (Thea *et al*, 1993; Brahmbhatt *et al*, 2006; Otieno *et al*, 2006; Makasa *et al*, 2007; Marinda *et al*, 2007; McNally *et al*, 2007; Shapiro *et al*, 2007). In addition, several studies have cited hematologic toxicity and increased risk of anemia in HEU infants associated with exposure to maternal ART (Paul *et al*, 2005; Feiterna-Sperling *et al*, 2007; Dryden-Peterson *et al*, 2011; Ziske *et al*, 2013). Poor maternal health and suboptimal infant feeding practices may contribute to increased rates of illness and impaired nutritional status (Coovadia& Bland, 2007; Marinda *et al*, 2007). Several studies have also highlighted immune abnormalities in HEU infants; however, the relationship between specific cytokine abnormalities and increased morbidity and mortality is still unclear (Clerici *et al*, 2000; Kuhn *et al*, 2001). We observed elevated hepcidin and markers of inflammation (AGP and CRP) in HEU compared to HIV-unexposed infants. This suggests that exposure to maternal immune activation *in utero* may lead to low-grade inflammation in HEU infants and may partially contribute to the poor growth, increased morbidity and mortality consistently reported in HEU infants (Thea *et al*, 1993; Brahmbhatt *et al*, 2006; Otieno *et al*, 2006; Makasa *et al*, 2007; Marinda *et al*, 2007; McNally *et al*, 2007; Shapiro *et al*, 2007). However it should be noted that maternal ART was not available in Zimbabwe at the time of the study. If inflammation contributes to anemia in HEU infants, it does not appear to be driven by elevated hepcidin levels in this group.

Interpretation of anemia in HEU infants at 3 mo of age in this cohort was complicated by the finding that a higher proportion of anemic infants were randomized to vitamin A supplementation at birth in the ZVITAMBO trial. Although vitamin A supplementation in HEU infants was not associated with increased mortality in the present analysis, it was associated with increased mortality in the main trial (Humphrey *et al*, 2006). Although anemic HEU infants had lower ferritin at 3 mo, it is not clear what the cause of anemia was, as only 10.5% of anemic 3 mo infants had ferritin in the iron-deficient range and there was no evidence of increased inflammation. We cannot exclude the possibility, therefore, that anemia in this group was a marker of ill health, and may be on the causal pathway to mortality. At 6 mo and 12 mo of age, ferritin was in the iron-deficient range in 50-75% of anemic HEU infants, suggesting a high prevalence of iron deficiency anemia in this group with increasing age, similar to our findings in HIV-unexposed infants.

Hepcidin concentrations declined with age in all groups. This is presumably a physiological response to mobilize iron stores and absorb more dietary iron, since endogenous iron becomes depleted between 3-4 months and dietary iron is needed to maintain hemoglobin concentrations (Dallman *et al*, 1980). Girls had higher hepcidin concentrations than boys, as we have previously described for healthy non-anemic infants (Mupfudze *et al*, 2013). Boys have been found to be more prone to anemia (Emond *et al*, 1996; Thorisdottir *et al*, 2011) and lower hepcidin concentrations among male infants might be a physiological response to reduced iron stores (Emond *et al*, 1996; Thorisdottir *et al*, 2011). Sex and age differences in hepcidin levels have also been reported in adults

(Galesloot *et al*, 2011) and higher hepcidin concentrations have been reported in post-menopausal compared to pre-menopausal women, associated with the sharp increase in ferritin as women progress through the menopause (Cook *et al*, 1976; Koziol *et al*, 2001; Galesloot *et al*, 2011).

Anemia had no overall association with hepcidin concentrations during infancy in multivariate analyses. However, among specific subgroups of infants, anemia did appear to be associated with hepcidin concentrations. Elevated hepcidin in anemic 3 mo HIV-unexposed infants may be explained by the elevated CRP levels observed in this group, although the reasons for low-grade inflammation in these apparently healthy infants is unclear. At 6 mo of age, anemia among HIV-unexposed infants appeared to be driven by a combination of inflammation and iron deficiency, but by 12mo anemia was mainly due to iron deficiency and hepcidin levels were low, as has been observed in classical iron deficiency. In the HEU group, both ferritin and hepcidin were lower in anemic compared to non-anemic infants at 3mo of age, suggesting a physiological hormonal response through hepcidin in response to lower iron stores. However, hepcidin was not useful in distinguishing anemic from non-anemic 6 mo and 12 mo HEU infants, or HIV-infected infants at any age. Hepcidin, like ferritin, is an acute phase protein and thus normal or raised hepcidin levels may be observed in anemic individuals in the presence of inflammation, as was characteristic of HEU and HIV-infected infants in this cohort. Hepcidin levels were more strongly correlated with ferritin than with CRP in all groups of infants. Hepcidin and ferritin have been shown to respond similarly to changes in

inflammation and storage of iron and ferritin has been shown to be most strongly associated with hepcidin in other studies (Ganz *et al*, 2008; Galesloot *et al*, 2011).

This is the first study to characterize hepcidin levels in the context of pediatric HIV infection and exposure, and one of the few studies to describe hepcidin in infants. A strength of this study is the unique cohort of African infants who were recruited prior to ART availability and therefore represent a well-characterized natural history cohort. A limitation of this study is the absence of viral load and CD4 data in HIV-infected infants, so it was not possible to ascertain how hepcidin is related to HIV disease progression in infants. Although we assayed samples at 3, 6 and 12 months, the cross-sectional nature of our study limits our ability to make inferences about the trajectory of hepcidin over the first year of life.

In summary, hepcidin concentrations are related to inflammatory status in infants, as in adults, and HIV-infected infants therefore have high levels of hepcidin; HEU infants have higher levels of inflammation and hepcidin than HIV-unexposed infants. Overall, differences in hepcidin did not explain anemia during infancy, except in young HIV-unexposed infants. Hepcidin concentrations progressively declined over the first year of life, and girls had higher levels of hepcidin than boys. Future studies should further explore the role of hepcidin in the pathogenesis of HIV infection, in particular whether sequestration of iron in macrophages may contribute to HIV disease progression, particularly in perinatally infected infants.

The relationship between Infant and Young Child feeding (IYCF) indicators, Water Sanitation and Hygiene (WASH) indictors and hemoglobin levels among young Zimbabwean children

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Abstract

Anemia affects 2 billion people worldwide, particularly young children in developing countries. Substantial research has demonstrated that dietary iron deficiency and subclinical and clinical infections are important causes of pediatric anemia. It has recently been proposed that environmental enteropathy (EE) may cause prevalent systemic inflammation in young children, that EE suppresses iron absorption and erythropoiesis, and that EE is caused by poor water, sanitation and hygiene (WASH). Using the 2010-11 Zimbabwe Demographic Health Survey (ZDHS), we tested the hypothesis that WASH indicators that reflect fecal exposure are associated with lower hemoglobin (Hb) in children 6-24 mo of age (n=1271). We controlled for WHO infant and young child feeding (IYCF) indicators, including the iron-rich food indicator, and hypothesized that these would not be associated with Hb due to lack of accuracy with regard to iron nutrition. Continuous regression analysis with survey weights and survey-specific commands were applied to account for the complex multistage cluster sampling survey design. We found that two WASH indicators (uncontained child stool disposal, and absence of soap at handwashing station) were associated with lower hemoglobin levels, but found no association between any of the WHO IYCF indicators and Hb in multivariate analysis. Our findings are consistent with the hypothesized role of poor WASH as a contributing cause of pediatric anemia in low-income settings. Further studies are needed to elucidate the role of WASH interventions of risk of anemia in infants and young children.

Introduction

Anemia is a serious public health problem affecting 2 billion people worldwide; particularly young children in developing countries, where its causes are multifactorial (WHO.; Lokeshwar *et al*, 2011; Milman, 2011). In Zimbabwe the prevalence of anemia is 71%, 74% and 68% among 6-8 month-olds, 9-17 month-olds, and 18-24 month-olds, respectively (Zimbabwe National Statistics Agency & ICF International Inc., 2012) and has remained relatively constant over the past 5 years (Central Statistics Office (CSO) [Zimbabwe] & Macro International Inc., 2007). Anemia in infancy is associated with impaired cognitive and behavioral development (Walter *et al*, 1989; Walter, 1994; Rao& Georgieff, 2002), impaired oxygen transport(Cullis, 2011) and a poorer prognosis in the context of many chronic diseases, including HIV(Xu *et al*, 2010).

Primary processes implicated in the pathogenesis of anemia in developing countries include: 1) inadequate iron intake; 2) iron deficiency caused by extra-corporeal blood loss due to *Schistosomiasis* and soil-transmitted helminths (STHs): hookworm, and *Trichuris trichiur*a infection and 3) anemia of inflammation resulting from high rates of infectious diseases including HIV, tuberculosis, malaria and *Schistosomiasis*, as well as subclinical infections and inflammation.(Weatherall, 1990; Stoltzfus *et al*, 1997; Friedman *et al*, 2005; Tolentino& Friedman, 2007; Midzi *et al*, 2010). Recently, the prevalent subclinical gut disorder termed environmental enteropathy (EE) has been hypothesized to contribute to anemia of inflammation, even in the absence of clinical disease(Prendergast& Kelly, 2012).

Chronic fecal-oral transmission of microorganisms may be the primary cause of EE, a subclinical intestinal pathology ubiquitous among people living in unhygienic conditions(Humphrey, 2009). EE may contribute to the high rates of anemia among infants and young children in developing countries through impaired digestive/absorptive and barrier functions of the small intestine (Lunn *et al*, 1991; Campbell *et al*, 2003). The most common features described in EE is some degree of villus atrophy (Beaton& Ghassemi, 1982; Martorell, 1995; Stephensen, 1999) including a reduction in villous height, broadening of the villi, and increased crypt depth (Cook *et al*, 1969; Carneiro Chaves *et al*, 1981) and increased inflammation and permeability . The characteristic distorted gut architecture compromises the intestinal barrier, reduces absorptive capacity and increased intestinal permeability, enabling microbial translocation which leads to chronic systemic inflammation (Figure 1) (Ukabam *et al*, 1986; Iqbal *et al*, 1996; Humphrey, 2009; Prendergast& Kelly, 2012). Chronic systemic inflammation may result in anemia of inflammation through mechanisms mediated by inflammatory cytokines, acute phase proteins and hepcidin (Weiss, 2002; Means, 2004; Weiss& Goodnough, 2005; Weiss, 2009).

Schistosomiasis infection (*S*. *haematobium* and *S*. *mansoni*) and STHs: hookworm, *Trichuris trichiur*a and *Ascaris lumbricoides*, are also endemic in developing countries with poor Water Sanitation and Hygiene (WASH) practices (Hotez *et al*, 2005), affecting over 2 billion people, 5–10% of whom are children under 24 months of age (WHO., 2005). Children 12-24 months who are at highest risk for anemia often acquire their first STH infection during the same time period (Stoltzfus *et al*, 2004; Kung'u *et al*, 2009).

Schistosomiasis is among the top 14 out patient treated diseases in Zimbabwe (Midzi *et al*, 2011) with overall mean prevalence of S. *haematobium*, S. *mansion* and STHs of 20.8%, 9.0% and 6.0% respectively and prevalence rates of S. *haematobium*, up to 70% in some regions of the country (Taylor P, 1985; Chandiwana *et al*, 1988; Midzi *et al*, 2011) (available from, the Ministry of Health and Child Welfare (MoHCW) of Zimbabwe). Only 29.6% of Zimbabwean households have access to improved toilet facilities (Zimbabwe National Statistics Agency & ICF International Inc., 2012) and 20.3%, 9.3% and 6.0% of individuals infected with *S*. *haematobium*, *S*. *mansoni* and STHs respectively had a toilet at home (available from MoHCW of Zimbabwe)

Dietary iron deficiency also contributes to pediatric anemia. Dietary iron requirements are extremely high during the second half of infancy when endogenous iron stores become depleted and the infant is no longer self-reliant for iron to meet the needs for erythropoiesis and growth (Lind *et al*, 2004; Baker& Greer, 2010; Domellof, 2011). The Recommended Dietary Allowance (RDA) for iron among 7 to 12 month old infants is 11mg/day (Baker& Greer, 2010). Except for animal liver and organ meat, even the best natural dietary sources of iron are not iron-concentrated enough to provide 11mg elemental iron in the small volumes consumed by infants. For example, to meet this requirement, an infant would need to consume 400 g red meat, 700 g chicken, 11 eggs, or 6 cups of cooked legumes daily.

The median iron consumed by 9-12 and 6-8 month-old infants in formative research among 32 infants from 2 small rural Zimbabwean villages was 3.1 and 1.8 mg/day

respectively (Paul *et al*, 2011). Hence, almost all 9-12 month olds (95%) and a 100% of 6-8 month-old infants did not meet the RDA for iron (Paul *et al*, 2011). However use of lipid based nutrient supplement (LNS) containing iron (9mg/20g) in trials of improved practices (TIPs) in the same infants was associated with increased probability of consuming the RDA from mean (SD), 0.025 (0.661) to 0.854 (0.678) in infants without and with LNS respectively (Paul *et al*, 2012). We hypothesize that the key IYCF indicator "consumption of iron-rich/iron- fortified food for children 6-23 months" is not associated with hemoglobin levels in Zimbabwean children because the iron-rich IYCF indicator primarily counts intake of natural foods and so does not reflect iron consumption at levels that are relevant to infants' iron needs.

The objective of this study was to determine if IYCF and WASH indicators are associated with hemoglobin levels in Zimbabwean children aged 6-24 months. We hypothesized that WASH practices are associated with hemoglobin levels in Zimbabwean children, due to the high prevalence of *Schistosomi*asis, STH and the EE pathway discussed above, but that the WHO IYCF indicators would not be associated with hemoglobin due to lack of specificity with regard to iron consumption.

Methods

Data and sample

We used data from the 2010-2011 Zimbabwe DHS (ZDHS), available on the DHS website (Zimbabwe National Statistics Agency & ICF International Inc., 2012). The ZDHS collected information from a nationally representative probability sample of all 10 Zimbabwean provinces using a stratified, two-stage cluster design yielding 9,756 households and a household response rate of 96%. Detailed information on survey design, management, quality control, and major findings is provided in the 2010-2011 ZDHS report(Zimbabwe National Statistics Agency & ICF International Inc., 2012).

Hemoglobin testing was carried out among children aged 6-59 months and was successfully measured in 80% of eligible children. We analyzed data from children 6-24 months of age for whom age-appropriate IYCF indicators could be constructed from available data. The initial population comprised 1741 children. Our analysis was restricted to the youngest child in a household in this age range with an available hemoglobin measurement, whose mother was interviewed. Hence 51 and 314 children were excluded because they were the older child in a household with more than one child between 6 and 24 months and because they did not have a valid hemoglobin measurement respectively. 105 children were excluded from the multivariate analysis because they were not "*de jure*" household members or lacked information on included variables. Therefore, 1376 children were selected and studied in the univariate analysis and 1271 children were included in the multivariate analysis.

Variables

Outcome: The outcome variable hemoglobin (g/dl) was measured using the HemoCue hemoglobinometer (HemoCue, Mission Viejo, CA; (Sharman, 2000)). Severe, moderate and mild anemia were characterized by hemoglobin levels < 7.0 g/dl; between 7.1g/dl and 9.9g/dl; and between10.0 g/dl and 10.9 g/dl respectively(Zimbabwe National Statistics Agency & ICF International Inc., 2012).

IYCF indicators: The infant and young child feeding indicators were calculated as specified by the WHO Indicators for Assessing Infant and Young Child Feeding Practices (WHO., 2008) (Table 4.1). Early initiation of breast-feeding, (i.e. the child was put to the breast within the first hour of birth) was measured based on maternal recall. Continued breast-feeding at 1 and 2 years of age was assessed in children age 12-15 months and 20-23 months, respectively and introduction of solid, semi-solid or soft foods was assessed in infants aged 6-8 months. The above indicators assessing small subsets of children were not included in multivariate analysis. Consumption of iron-rich foods, bottle-feeding and age-appropriate breastfeeding (the child received breast milk as well as solid or semi-solid foods) in the previous 24 hours was assessed using a 24-hour recall in all children age 6-23 months. Minimum dietary diversity (MDD) was defined as consumption of foods from 4 or more of the following 7 foods groups: i) grains, roots, tubers; ii) legumes and nuts; iii) dairy products; iv) flesh foods (meat, fish, poultry, organ); v) eggs; vi) vitamin A-rich fruits and vegetables, and vii) other fruits and vegetables. The minimum meal frequency (MMF) was estimated by counting the number of times solid, semi-solid, or soft foods were consumed in the previous 24 hours. The MMF for breastfed infants 6–

8 months and 9-23 months old is 2 and 3 meals per day respectively, with 1–2 additional snacks as desired (WHO/PAHO., 2003). The MMF for non-breastfed children 6-23 months is 4 meals and 1-2 cups of "milk feeds" (infant formula, milk or yogurt) per day (WHO., 2008). The minimum acceptable diet (MAD) was estimated from the MDD and the MMF: consumption of 2 and 3 meals per day with 1–2 additional snacks as desired(WHO/PAHO., 2003) by breastfeeding 6–8 months and 9-23 months old infants respectively, and 4 meals and 1-2 cups of "milk feeds" per day for non-breastfeeding children 6-23 months old together with the consumption of 4 or more of the above 7 foods groups (WHO., 2008).

The key IYCF indicator in our analysis, "Consumption of iron-rich/iron- fortified food for children 6-23 months" is defined by the WHO as the consumption of an iron-rich food or iron-fortified food that is specially designed for infants and young children, or that is fortified in the home. Iron rich foods include organ meats, meat (beef, pork, lamb, chicken, etc.), fish and seafood or any processed/cured products made from the above (WHO., 2008). Suitable iron-fortified foods include: iron fortified infant/toddler formula, commercially fortified foods specially designed for infants and young children which contain iron, or foods fortified in the home with a micronutrient powder containing iron such as sprinkles or a lipid-based nutrient supplement (LNS) containing iron (WHO., 2008). Iron-fortified foods and products included in the WHO indicator are adapted to reflect iron-fortified foods available in a particular context (WHO., 2008).

There are no national iron supplementation programs for infants and young children.

Hence information on consumption of foods fortified in the home with a micronutrient powder containing iron or a LNS containing iron was not collected, because national use is practically non-existent (Zimbabwe National Statistics Agency & ICF International Inc., 2012). The indicator "Consumption of iron-rich/iron- fortified food for children 6-23 months" was constructed from the following food groups: 1) liver, heart, other organs; 2) meat (beef, pork, lamb, chicken, etc.); 3) fish and shellfish; 4) baby formula and 5) fortified baby food.

WASH Indicators: Sanitary conditions were classified as improved or unimproved depending on the type of toilet facility and whether it was shared with other households (Zimbabwe National Statistics Agency & ICF International Inc., 2012). Toilets that flush or pour flush into a piped sewer system, septic tank, or pit latrine; ventilated improved pit (VIP) latrines or Blair toilets; and pit latrines with a slab were classified as improved, provided only one household used the toilet facility(Zimbabwe National Statistics Agency & ICF International Inc., 2012). Households that shared a toilet facility, had no toilet or used an open pit were classified as non-improved. Disposal of the youngest child's stools was classified as contained if the child used a toilet or latrine, the stool was rinsed into a toilet or latrine or the stool was buried. Stool that was left in the open/not disposed of, thrown into garbage or into a drain or ditch was classified as uncontained(Zimbabwe National Statistics Agency & ICF International Inc., 2012).

Interviewers observed household handwashing stations and gathered information on the availability of soap or detergent and water in households where the handwashing place

was observed. Data were categorized into: soap present at handwashing station; no soap present at handwashing station; no handwashing station; handwashing station not observed because permission was denied or for other reasons.

The main source of drinking water was classified as being from an "improved source" (e.g. piped water source, borehole, protected well or if it was treated regardless of the water source) or from a "non-improved source" (e.g. open well, pond or stream) according to the WHO/UNICEF Joint Monitoring Program (JMP) for Water and Sanitation categorizations(WHO / UNICEF Joint Monitoring Programme (JMP) for Water Supply and Sanitation).

Analyses

Data analyses were conducted using Stata 12 statistical software STATA version 12 (StataCorp, College Station, TX). Survey weights and survey-specific commands (svy in Stata 12) were applied to account for the complex multistage cluster sampling survey design and to appropriately correct standard errors for all analyses. Descriptive analyses were first conducted to present general information on the study population. Continuous regression analysis was used to assess the association between hemoglobin and possible risk factors. Factors that were significant in univariate analysis were included in the multivariate models. In addition, a number of potential confounding factors were included in the multivariate model at the child level (age, sex, birth weight and whether the child had ever received vitamin A supplements) and at the maternal level (maternal hemoglobin), which were likely to influence the hemoglobin status of children.

Covariance matrices were used to assess multi-collinearity among independent variables in the models. The DHS wealth index includes some WASH-related variables as assets(S. O. Rutstein and K. Johnson, 2004) and was collinear with disposal of child's stool; hence it was not added to the multivariate model and maternal education was used as a proxy for wealth in the multivariate model.

Results

Sample Characteristics

The mean (SD) age was 14.0(0.1) months (Table 4.2). The prevalence of low birth weight (< 2500g) and stunting (height-for-age-z-score < -2) was 5.5 and 29.2%, respectively. Mean maternal hemoglobin was 12.9 (SD 1.7) g/dL. In our analytic sample, 29.6% (CI 95%: 26.8; 32.6%) children 6-24 months had mild anemia; 42.2% (CI 95%: 38.9; 44.9%) had moderate anemia and 1.8% (CI 95%: 1.1; 2. 7 %) had severe anemia. More than three-quarters of infants and young children (76.5%) lived in rural areas; two-thirds of mothers (65.0%) had a secondary education or were not currently working (66.9%).

Prevalence of infant and young child feeding practices and WASH indicators

Breastfeeding was common, with the prevalence of ever breastfeeding; continued breastfeeding at one year; early breastfeeding initiation (<1 hour after birth) and age-appropriate breastfeeding (the child received breast milk as well as solid or semi-solid foods in the previous 24 hours among 6-23 months-olds) 98.7%, 88.6%, 75.1% and 69.1%, respectively (Table 4.3). However, the mean percentage of children who consumed food with the MDD, MMF and the MAD from the previous day's 24 hour dietary recall was low: 21.3, 37.1 and 11.2% respectively. Less than half of children (41.1%) had consumed an iron-rich or iron fortified food in the prior 24-hours. Baby formula, fortified baby food, liver and other organ meat, meat and fish accounted for 1.8%; 9.5%; 11.3%; 24.6%; 1.4% respectively (groups not mutually exclusive)

In terms of WASH indicators, 80.5% households had an improved household source of

drinking water; however, only 29.6% households had improved sanitary conditions.

Factors associated with hemoglobin levels in children

Controlling for maternal, child and household characteristics, uncontained disposal of the youngest child's stools and having no soap at the household handwashing station remained associated with 0.22 and 0.37 g/dL lower hemoglobin concentrations, respectively (β=-0.22, p=0.04 and β=-0.37, p=0.003 respectively) after controlling for maternal, child and household characteristics (Table 4.4). However neither the key IYCF "consumption of iron rich/iron fortified food" nor any of the other IYCF indicators was associated with hemoglobin levels. Other factors associated with hemoglobin levels in multivariate analysis were sex, birth weight, maternal hemoglobin, maternal tertiary education and residing in Matabeleland North province.

Discussion

Using nationally representative data, we examined the association between IYCF and WASH indicators and hemoglobin levels among the 6-24 month old children. None of the WHO IYCF indicators were associated with hemoglobin levels in multivariate analysis. However, two WASH indicators (uncontained disposal of the child's stool, and absence of soap at the household handwashing station) were associated with lower hemoglobin levels in multivariate analysis.

Two important barriers to fecal-oral transmission in children are thought to be safe disposal of children's stools and hand washing with soap/detergent after fecal contact (Humphrey, 2009). Hand washing with soap before feeding children and after disposal of child's stools can interrupt the transmission of fecal-oral microbes (Brown *et al*, 2013). Our results are consistent with the guidelines for good hand washing practices that include water and a washing agent such as soap (Kaltenthaler *et al*, 1991). One other study among Ethiopian children also reported an association between unclean hands and anemia (Mahmud *et al*, 2013).

Open defecation by infants and young children is acceptable in many resource-constrained cultures (Yeager *et al*, 1999; Brown *et al*, 2013). For example, less than a quarter of children under five-years in Lima, Peru used a toilet (Yeager *et al*, 1999) which is associated with Schistosomiasis and STH infections (Ziegelbauer *et al*, 2012). However safe disposal of children's stools has received relatively little attention in sanitation programs (Brown *et al*, 2013). A study by Traore *et al* highlights the

importance of disposal of child's stool in diarrheal or dysentery infection (Traore *et al*, 1994). Our finding of an association between uncontained disposal of the youngest child's stool and lower hemoglobin levels provides further evidence for the importance of promoting safe disposal of children's stools.

The creation of population-based measures of IYCF practices allows for research on the scale and distribution of inadequate feeding practices and subsequent consequences for child health and survival by capturing the various dimensions of child feeding that occur during the transition from breastfeeding to family diet between 6 and 24 months. All key infant and young child feeding practices were suboptimal except for two breastfeeding practices (early initiation of breastfeeding, and continued breastfeeding at 1 year), introduction of solid/semi-solid foods at 6-8month and prevalence of bottle-feeding. For example while 21.3 and 37.1% of children 6-23months consumed a diet meeting the MDD and the MMF respectively, very few (11.2%) achieved both of these indicators and consumed the MAD.

The indicator "consumption of iron-rich/iron- fortified food for children 6-23 months" is meant to reflect iron nutriture; hence it was central to our hypothesis. We hypothesized that receipt of iron fortified food or iron supplementation is more likely to be associated with hemoglobin levels in developing countries. A Community Infant and Young Child Feeding (CIYCF) program is run through the Ministry of Health and Child Welfare (MoHCW). However it focuses on counseling mothers on food based approaches through village health workers and health promoters. Hence consumption of home fortified or

LNS is practically non-existent in Zimbabwe. Although baby formula and fortified baby food were included in the key IYCF indicator, only 1.8 and 9.5% of children 6-24 months consumed baby formula or fortified baby food respectively. Consumption of meat accounted for the largest proportion of infants who consumed an iron rich food/ iron fortified food (24.6%). However the iron content is not concentrated enough to meet the RDA for iron in the small volumes consumed by infants. Removing consumption of meat from the IYCF iron indicator in sensitivity analysis resulted in a stronger association with hemoglobin levels that was borderline significant (p=0.069). Hence the wide range of iron content of food included in the IYCF iron indicator may explain the lack of association between the IYCF iron indictor and hemoglobin levels.

This study also identified several other risk factors for anemia, which have been previously reported in the literature, confirming internal validity of the data. Boys had lower hemoglobin levels compared to girls, suggesting a greater vulnerability to anemia in males. This physiologic finding has been established in several studies(Emond *et al*, 1996; Thorisdottir *et al*, 2011). The positive association between maternal hemoglobin and child's hemoglobin status is consistent with earlier findings by several research groups (Colomer *et al*, 1990; De Pee *et al*, 2002) including our own; in a previous study we reported that maternal hemoglobin concentration predicted anemia in the first year of life (Miller *et al*, 2003). Maternal tertiary education is likely to be a distal factor associated with higher hemoglobin levels, as a proxy for higher income and better child caring practices. Residing in Matebeleland North province was associated with lower hemoglobin levels, likely through geographical and economic disadvantages, being an

arid and drought-prone part of the country with historical economic inequalities.

Our study has both strengths and limitations. We used nationally representative data and adjusted for several potential confounders at child, maternal and household levels. However, the cross sectional nature of the data collected in the ZDHS limits inferences to associations rather than causality. Although our results are likely applicable to other developing countries, particularly in sub-Saharan Africa, the ability to generalize our results from a single country study is limited. A multicounty study of anemia using DHS data would test whether our findings are generalizable to other settings. A single 24-hour dietary recall used in the creation of IYCF indicators might not be good proxy for dietary patterns in infancy and young childhood (Arimond& Ruel, 2004).

Conclusion

In this cross-sectional study of Zimbabwean children aged 6-24 months two WASH indictors: disposal of child's stools and presence of soap at handwashing station were associated with hemoglobin levels. However, none of the WHO IYCF indicators, including consumption of iron-rich/iron- fortified food, were associated with hemoglobin levels. Our findings are consistent with the hypothesis that poor WASH conditions may contribute to anemia through infections with *Schistosomiasis* and STHs, EE (Midzi *et al*, 2010; Prendergast& Kelly, 2012) and other clinical infections and inflammation. Further research, including randomized trials of WASH interventions, is needed to further explore the effect of WASH interventions of risk of anemia in infants and young children.

Table 4.1. Definitions of WHO-recommended Infant and Young Child Feeding (IYCF) indicators

Indicators	Definition
Core Indicators	
Early initiation of breast feeding	Proportion of children born in the last 24 months who were put to the breast within one hour of birth
Continued breast-feeding at 1 year	Proportion of children 12–15 months of age who are fed breast milk
Introduction of solid, semi-solid or soft foods	Proportion of infants 6–8 months of age who receive solid, semi-solid or soft foods
Minimum dietary diversity	Proportion of children 6–23 months of age who receive foods from 4 or more food groups
Minimum meal frequency	Proportion of breastfed and non-breastfed children 6–23 months of age, who receive solid, semi-solid, or soft foods (but also including milk feeds for non-breastfed children) the minimum number of times or more
Minimum acceptable diet	Proportion of children 6–23 months of age who receive a minimum acceptable diet (apart from breast milk)
Consumption of iron-rich/iron-	Proportion of children 6–23 months of age who

fortified food for children 6-23 months	receive an iron-rich food or iron-fortified food that is specially designed for infants and young children, or that is fortified in the home.
Optional Indicators	
Children ever breastfed	Proportion of children born in the last 24 months who were ever breastfed.
Continued breast-feeding at 2 year	Proportion of children 20–23 months of age who are fed breast milk
Age-appropriate breastfeeding	Proportion of children 6–23 months of age who received breast milk as well as solid or semi-solid foods during the previous day.
Bottle feeding	Proportion of children 0–23 months of age who are fed with a bottle.

Table 4.2. Sample Characteristics

Child covariates	N	%
Sex		
Male	681	49.6
Female	695	50.4
Age in months mean (SD)	14	0.1
Birth weight		
≥ 2500g[R]	1296	94.5
< 2500g	80	5.5
Height-for-age-Z score		
> - 2.0[R]	967	70.8
≤ -2.0)	409	29.2
Recent diarrhea		
No	1065	75.9
Yes	311	24.1
Recent fever		
No	1196	87.1
Yes	180	12.9
Vitamin A supplementation		
Ever received	1003	74.2

Never received	370	25.8
Maternal Covariates		
Maternal age	25	0.2
Mother took iron supplements during pregnancy		
Did not take iron supplements	615	46.9
Took iron supplements	750	53.1
Number of days mother took iron supplements during pregnancy mean (SD)	33	1.8
Maternal Hemoglobin g/dL (continuous)	130	0.6
Maternal Body Mass Index (BMI)		
< 18.5	126	8.7
≥ 18.5 & <25	929	67.5
≥ 25 & <30	236	17.8
≥ 30	85	6.0
Maternal education		
No education	19	1.2
Primary	449	31.4
Secondary	878	65.0
Tertiary	30	2.3
Maternal work status		
Currently working	421	33.1

Not currently working	955	66.9
Household Characteristics		
Province		
Manicaland	178	15.8
Mashonaland Central	155	11.7
Mashonaland East	174	12.6
Mashonaland West	138	10.6
Matabeleland North	133	5.8
Matabeleland South	148	6.1
Midlands	166	14.1
Masvingo	118	9.7
Harare	92	10.0
Bulawayo	74	3.7
Residence		
Urban	314	23.5
Rural	1062	76.5
Wealth quintile		
Lowest	355	24.4
Second	308	23.0
Middle	284	21.2
Fourth	263	18.9

Highest	166	12.5

Table 4.3. Prevalence of Infant and Young Child Feeding Practices and WASH Indicators among 6-24 month old Zimbabwean children whose mothers were interviewed, ZDHS 2010-2011

Infant and Young Child Feeding Practices	N	%
Core Indicators		
Early initiation of breastfeeding (6-23 months)		
≤ 1 hour of birth	1051	75.1
> 1 hour after birth	325	24.9
Continued breastfeeding at 1 year (12-15 months)		
Breastfeeding	158	88.6
Not breastfeeding	25	11.4
Introduction of solid/ semi solid foods (6-8 months)		
Introduced	204	90.2
Not introduced	26	9.8
Minimum dietary diversity (6-23 months)		
≥ 4 food groups	280	21.3
< 4 food groups	1096	78.7
Minimum meal frequency (6-23 months)		
≥ 2 (6-8 months) ≥ 4 (9-24 months)	469	37.1

< 2 (6-8 months) < 4 (9-24 months)	844	62.9
Minimum acceptable diet (6-23 months)		
Received acceptable diet	134	11.2
Did not receive acceptable diet	1179	88.8
Consumption of iron-rich or iron-fortified foods (6-23 months)		
Yes	528	41.1
No	795	58.9

Optional Indicators

Child ever breastfed (6-23months)		
Ever breastfed	1354	98.7
Never breastfed	22	1.3
Continued breastfeeding at 2 years (20-23 months)		
Breastfeeding	49	17.9
Not breastfeeding	211	82.2
Age appropriate breastfeeding (6-23 months)		
Breastfeeding	935	69.1
Not breastfeeding	441	31.0
Drank from bottle with nipple (6-23 months)		
Child was not bottle fed	1279	93.1
Child was bottle fed	96	6.8

WASH Indicators		
Household source for drinking water		
Improved	1067	80.5
Not Improved	266	19.5
Time to source of drinking water		
On premises	428	33.1
≤ 30minutes	692	53.4
> 30minutes	200	13.5
Sanitary conditions		
Improved	392	29.6
Unimproved	933	69.8
Disposal of child's stool		
Contained	1089	80.1
Uncontained	284	19.7
Soap/detergent and water at hand washing station		
Soap/detergent at hand washing station	298	21.6
No Soap/detergent at hand washing station	453	30.9
No hand washing station	105	7.9

Table 4.4: Univariate and multivariate models of potential risk factors for low hemoglobin among children 6-24 months (ZDHS 2010-2011)

Explanatory variable		Univariate		[1, 2] Multivariate	
	N	β (95% Conf. Interval)	P- value	β (95% Conf. Interval)	P-value
Child covariates					
Sex	1376				
Male[R]					
Female		0.30 (0.15, 0.46)	<0.001	0.35 (0.19, 0.50)	<0.001

Age in months (continuous)	1376	0.01 (-0.002, 0.03)	0.09	0.01 (-0.01, 0.02)	0.32
Birth weight	1376				
≥ 2500g[R]					
< 2500g		-0.53 (-0.95, -0.11)	0.01	-0.68 (-1.12, -0.24)	0.003
Height-for-age-Z score	1376				
> - 2.0[R]					
≤ -2.0)		-0.12 (-0.31, 0.07)	0.22		
Recent diarrhea	1376				

No[R]					
Yes		-0.06 (-0.24, 0.11)	0.49		
Recent fever	1376				
No[R]					
Yes		0.17 (-0.07, 0.40)	0.17		
Vitamin A supplementation	1373				
Ever received[R]					
Never received		-0.23 (-0.44, -0.02)	0.03	-0.07 (-0.28, 0.14)	0.49

Maternal Covariates

Mother took iron supplements during	1365				
Did not take iron supplements[R]					
Took iron supplements		-0.06 (-0.22, 0.10)	0.47		
Number of days mother took iron supplements	697	-0.001 (-0.004, 0.001)	0.36		
Maternal Hemoglobin (g/dL), mean (SD)	1362	0.01 (0.005, 0.02)	<0.001	0.01 (0.003, 0.01)	<0.01
Maternal Body Mass Index (BMI)	1376				
≥ 18.5 & < 25[R]					

< 18.5		-0.07 (-0.37, 0.23)	0.64	0.01 (-0.28, 0.30)	0.95
≥ 25 & <30		0.17 (-0.03, 0.37)	0.10	0.13 (-0.06, 0.32)	0.17
≥ 30		0.35 (0.01, 0.69)	0.04	0.22 (-0.12, 0.56)	0.21
Maternal education	1376				
No education[R]					
Primary		0.11 (-0.5.1, 0.74)	0.72	0.20 (-0.41, 0.80)	0.52
Secondary		0.30 (-0.33, 0.93)	0.35	0.31(-0.30, 0.92)	0.32
Tertiary		1.23 (0.35, 2.1.2)	0.01	1.09 (0.15, 2.0)	0.02

Maternal work status	1376				
Currently working[R]					
Not currently working		-0.09 (-0.26, 0.09)	0.32		
Maternal age at child's birth in years	1376	0.01 (-0.001, 0.03)	0.08		
Household covariates					
Province	1376				
Manicaland[R]					
Mashonaland Central		0.06 (-3.39, 4.50)	0.78	0.05 (-0.33, 0.43)	0.79

Mashonaland East	0.01 (-0.38, 0.39)	0.97	-0.02 (-0.40, 0.36)	0.91
Mashonaland West	0.25 (-0.16, 0.66)	0.23	0.29(-0.09, 0.68)	0.13
Matabeleland North	-0.59 (-0.99, -0.19)	<0.01	-0.44 (-0.83, -0.04)	0.03
Matabeleland South	-0.29 (-0.76, 0.18)	0.22	-0.07(-0.51, 0.38)	0.76
Midlands	-0.01 (-0.38, 0.36)	0.98	-0.03 (-0.38, 0.32)	0.87
Masvingo	0.26 (-0.18, 0.70)	0.25	0.25 (-0.15, 0.64)	0.22
Harare	0.11 (-0.30, 0.53)	0.60	0.003 (-0.39, 0.40)	0.99
Bulawayo	-0.20 (-0.64, 0.25)	0.38	-0.32 (-0.77, 0.13)	0.17

Residence	1376		
Urban[R]			
Rural		0.01 (-0.18, 0.21)	0.89
Wealth quintile	1376		
Lowest[R]			
Second		0.16 (-0.06, 0.38)	0.15
Middle		0.29 (0.06, 0.52)	0.02
Fourth		0.12 (-0.11, 0.35)	0.32

Highest		0.45 (0.18, 0.71)	< 0.01
Infant and young child feeding practices			
Early initiation of breastfeeding	1376		
≤ 1 hour of birth[R]			
> 1 hour after birth		0.01 (-0.19, 0.21)	0.94
Continued breastfeeding at 1 year	183		
Breastfeeding[R]			
Not breastfeeding		-0.13 (-0.94, 0.68)	0.75

Introduction of solid, semi-solid or soft foods	230				
Introduced[R]					
Not introduced		0.38 (-0.38, 1.15)	0.33		
Minimum dietary diversity (MDD)	1376				
≥ 4 food group[R]					
< 4 food groups		-0.19 (-0.36, -0.01)	0.04	-0.11 (-0.30, 0.08)	0.26
Minimum meal frequency (MMF)	1313				
Received MMF[R]					

Did not receive MMF		-0.11 (-0.29, 0.08)	0.26
Minimum acceptable diet	1313		
Received acceptable diet[R]			
Did not receive acceptable diet		-0.30 (-0.53, -0.08)	0.008
Consumption of iron-rich or iron-fortified foods	1323		
Yes[R]			
No		-0.12 (-0.29, 0.04)	0.14
Child ever breastfed	1376		

Ever breastfed[R]					
Never breastfed		-0.90 (-1.74, -0.06)	0.04	-0.46 (-1.42, 0.50)	0.35
Continued breastfeeding at 2 years	260				
Breastfeeding[R]					
Not breastfeeding		-0.01 (-0.57, 0.55)	0.98		
Age-appropriate breastfeeding	1376				
Breastfeeding[R]					
Not breastfeeding		0.03 (-0.14, 0.21)	0.72		

Drank from bottle with nipple	1376		
Child was not bottle fed[R]			
Child was bottle fed		0.21 (-0.09, 0.50)	0.16
WASH Indicators			
Household source of drinking water	1333		
Improved[R]			
Unimproved		0.02 (-0.19, 0.22)	0.86
Time to source of household drinking water	1320		

On premises[R]			
≤ 30minutes[R]		-0.06 (-0.25, 0.13)	0.53
> 30minutes		0.01 (-0.29, 0.31)	0.94
Sanitary conditions	1333		
Improved[R]			
Not Improved		-0.08 (-0.26, 0.09)	0.34
Disposal of child's stool	1376		
Contained[R]			

Not contained		-0.29 (-0.490, -0.08)	0.01	-0.22 (-0.42, -0.01)	0.04
Soap/detergent observed	1376				
Soap/detergent observed at hand					
No soap/detergent observed at hand		-0.39 (-0.63, -0.15)	0.001	-0.37 (-0.61, -0.12)	0.003
No hand washing station		-0.22 (-0.51, 0.07)	0.139	-0.08 (-0.37, 0.21)	0.572
Hand washing station not observed		-0.23 (-0.46, -0.001)	0.049	-0.22(-0.49, 0.05)	0.109

[1.] Wealth status was dropped from the multivariate model because it was correlated with disposal of child's stool.

[2.] 1317 children were included in the multivariate model.

Chapter 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Chapter 2

1. We show for the first time in healthy, non-anemic infants that hepcidin concentrations decrease over infancy, with most of the drop in hepcidin occurring between 3 and 6 months. This is presumably a physiologic response to mobilize iron stores and absorb more dietary iron, thus preventing iron deficiency anemia.
2. Infant values therefore appear lower than those reported in adults (mean (SD) hepcidin concentration: 15.43 (2.11)) (Konopásek *et al*, 2011), supporting the higher metabolic need for iron in infancy compared to adulthood.
3. Hepcidin concentrations were higher in girls than boys in each age group, but sex differences were not significant in univariate regression analysis and after adjustment for age, perhaps because of the small sample size. Boys have been observed to be more vulnerable to anemia (Emond *et al*, 1996; Thorisdottir *et al*, 2011) and the lower hepcidin concentrations among boys might be a physiologic response to inherently lower iron stores in infancy (Emond *et al*, 1996; Thorisdottir *et al*, 2011).

Chapter 3

This study is, to our knowledge, the first to characterize hepcidin concentrations in HIV-infected and HIV-exposed infants, and focused specifically on sub-Saharan African infants, who are at particularly high risk of anemia. We show that:

1. Hepcidin concentrations were markedly elevated in HIV-infected infants from as early as 3 months of age, and remained high throughout infancy. Elevated hepcidin levels may simultaneously halt erythropoiesis while promoting the growth and survival of HIV and intracellular co-pathogens such as *Mycobacterium tuberculosis* (Montaner *et al*, 2006; Boelaert *et al*, 2007; Drakesmith& Prentice, 2012).
2. Hepcidin blocks the activity of the iron exporter ferroportin, leading to iron sequestration in macrophages. Consistent with this, we found that ferritin was higher in HIV-infected infants compared to other infant groups, and did not differ between anemic and non-anemic HIV-infected infants, highlighting both the iron sequestration that occurs during HIV infection and the role of ferritin as an acute phase protein.
3. We observed elevated hepcidin and markers of inflammation (AGP and CRP) in HEU compared to HIV-unexposed infants. This suggests that exposure to maternal immune activation *in utero* may lead to low-grade inflammation in HEU infants and may partially contribute to the poor growth, increased morbidity and mortality consistently reported in HEU infants (Thea *et al*, 1993; Brahmbhatt *et al*, 2006; Otieno *et al*, 2006; Makasa *et al*, 2007; Marinda *et al*, 2007; McNally *et al*, 2007; Shapiro *et al*, 2007). However if inflammation contributes to anemia in

HEU infants, it does not appear to be driven by elevated hepcidin levels in this group.

4. Anemia had no overall association with hepcidin concentrations during infancy in multivariate analyses except in HIV unexposed infants and young HEU infants (3 month-olds). Elevated hepcidin in anemic 3 mo HIV-unexposed infants may be explained by the elevated CRP levels observed in this group, although the reasons for low-grade inflammation in these apparently healthy infants is unclear. At 6 mo of age, anemia among HIV-unexposed infants appeared to be driven by a combination of inflammation and iron deficiency, but by 12mo anemia was mainly due to iron deficiency and hepcidin levels were low, as has been observed in classical iron deficiency. In the HEU group, both ferritin and hepcidin were lower in anemic compared to non-anemic infants at 3mo of age, suggesting a physiological hormonal response through hepcidin in response to lower iron stores.
5. Hepcidin was not useful in distinguishing anemic from non-anemic 6 mo and 12 mo HEU infants, or HIV-infected infants at any age. Hepcidin, like ferritin, is an acute phase protein and thus normal or raised hepcidin levels may be observed in anemic individuals in the presence of inflammation, as was characteristic of HEU and HIV-infected infants in this cohort. Hepcidin levels were more strongly correlated with ferritin than with CRP in all groups of infants. Hepcidin and ferritin have been shown to respond similarly to changes in inflammation and storage of iron and ferritin has been shown to be most strongly associated with hepcidin in other studies (Ganz *et al*, 2008; Galesloot *et al*, 2011).

6. Hepcidin concentrations declined with age in all groups and girls had higher hepcidin concentrations than boys, extending our findings in healthy non-anemic infants from Chapter 2 (Mupfudze *et al*, 2013).

Chapter 4

1. Using nationally representative data, Two WASH indicators (uncontained disposal of the child's stool, and absence of soap at the household handwashing station) were associated with lower hemoglobin levels in multivariate analysis, consistent with the high prevalence of *Schistosomi*asis, STH and the EE pathway hypothesis.
2. However neither the key IYCF indicator "consumption of iron rich/iron fortified foods" or any of the other IYCF indicators were associated and hemoglobin levels in multivariate analysis consistent with the hypothesis that the iron-rich IYCF indicator primarily counts intake of natural foods and so does not reflect iron consumption at levels that are relevant to infants iron needs.

5.2 Recommendations for future research

1. Hepcidin appears to be a useful marker consistent with iron metabolism in infants. However our inferences are limited both by our small sample sizes at 3, 6 and 12 months and the cross sectional nature of our study. There is need for larger studies to establish 'reference values' and longitudinal studies across infancy to characterize the trajectory of normative hepcidin values in infancy.
2. Comparison between studies is limited by the lack of congruence in hepcidin measurements from different hepcidin assays, highlighting the need for further studies harmonizing different assays. Although we also reported hepcidin consensus values, their use in the literature is still limited.
3. Future studies should further explore the role of hepcidin in the pathogenesis of HIV infection, in particular whether sequestration of iron in macrophages may contribute to HIV disease progression, particularly in perinatally infected infants.
4. Future studies should further explore the utility of hepcidin measurements in assessing the best time to supplement infants and young children in areas with high rates of infection.
5. Further research, including randomized trials of WASH interventions measuring intestinal permeability, hepcidin levels and anemia is needed to further explore the hypotheses generated by this study.

APPENDIX

Ethical review

ZVITAMBO
HIV CARE AND PREVENTION FOR MOTHERS AND CHILDREN IN ZIMBABWE

11 December 2012

MRCZ Secretariat
Medical Research Council of Zimbabwe
Josiah Tongogara /Mazowe Street
P.O. Box CY 573
Causeway
HARARE

Dear Sir/Madam,

RE: REQUEST TO CONDUCT A SUB-STUDY UNDER THE ZVITAMBO "VITAMIN A" TRIAL- MRCZ/A/583

We request further permission to:

1. Estimate the reference intervals for plasma hepcidin in (65) non-anemic healthy children at three ages (3, 6 and 12months) and describe the trajectory of plasma hepcidin in the first year of life. The study population will be drawn from children enrolled in the ZVITAMBO Vitamin A trial who had a gestational age >37 weeks, birth weight >2500g and never had abnormal iron indicators (i.e. hemoglobin < 105 g/L at 3 and 6 months and < 100g/L at 12months, serum ferritin < 12µg/l, sTfR > 8.3mg/L), inflammation (i.e. AGP > 1g/L) or an acute illness (diarrhoea, fever, in the prior week or measles in the prior 3 months) at assessment visits at 3, 6 and12 months of age.
2. Evaluate the differences in mean plasma hepcidin associated with HIV exposure, HIV infection and anemia by measuring plasma hepcidin levels in cryopreserved samples from the ZVITAMBO trial collected from five groups of infants (65 infants per group): HIV-unexposed anemic; HIV-exposed uninfected and anemic; HIV-exposed uninfected and non-anemic; HIV-infected and anemic and HIV-infected and non-anemic infants at 3 ages (3, 6, 12months).

REASON FOR SUB-STUDY

The ZVITAMBO archive provides us with a well-characterized population of Zimbabwean infants in which to explore the trajectory of plasma hepcidin in the first year of life. Hepcidin plays an important role in iron metabolism, however reference values for plasma hepcidin have not yet been characterized in infants and young children. It is not clear how hepcidin changes over time in infancy in association with the underlying dynamic iron physiology associated with infancy. The second aim allows us to evaluate the role of hepcidin in HIV and anemia. This study will be the first study to evaluate plasma hepcidin concentrations in HIV exposed and infected infants and will add to the body of literature on hepcidin and iron metabolism in infancy.

Thank you very much for your on-going support of our work.

Yours faithfully

Tatenda Geraldine Mupfudze
PhD Candidate- Johns Hopkins School of Public Health
Zvitambo Project

December 13, 2012

Elizabeth A Skinner
Chair
IRB Office
Johns Hopkins University Bloomberg School of Public Health
615 N. Wolfe Street, E 1100
Baltimore, MD 21205

RE: Amendment to IRB No. H.22.02.06.27.ARI - Vitamin A Supplementation of Breastfeeding Mothers and Their Neonates at Delivery: Impact on Mother-to-Child HIV Transmission during lactation, HIV Infection Among Women During the Post-partum Year and Infant Mortality

Dear Ms. Skinner:

I am writing to request permission to amend Protocol IRB No. H.22.02.06.27.ARI.

Iwould like to carry out the following additional work under the Vitamin A Trial archive:

1. Estimate the reference intervals for plasma hepcidin in (65) non-anemic healthy children at three ages (3, 6 and 12 months) and describe the trajectory of plasma hepcidin in the first year of life. The study population will be drawn from children enrolled in the Zvitambo Vitamin A trial who had a gestational age >37 weeks, birth weight >2500g and never had abnormal iron indicators (i.e. hemoglobin < 105 g/L at 3 and 6 months and < 100g/L at 12 months, plasma ferritin < 12µg/l, sTfR > 8.3mg/L), inflammation (i.e. AGP > 1g/L) or an acute illness (diarrhoea, fever, in the prior week or measles in the prior 3 months) at assessment visits at 3, 6 and12 months of age.

2. Evaluate the differences in mean plasma hepcidin associated with HIV exposure, HIV infection and anemia by measuring plasma hepcidin levels in cryopreserved samples from the Zvitambo trial collected from five groups of infants (65 infants per group): HIV-unexposed anemic; HIV-exposed uninfected and anemic; HIV-exposed uninfected and non-anemic; HIV-infected and anemic and HIV-infected and non-anemic infants at 3 ages (3, 6, 12 months).

The Zvitambo archive provides us with a well-characterized population of Zimbabwean infants in which to explore the trajectory of plasma hepcidin in the first year of life.

Hepcidin plays an important role in iron metabolism, however reference values for plasma hepcidin have not yet been characterized in infants and young children. It is not clear how hepcidin changes over time in infancy in association with the underlying dynamic iron physiology associated with infancy. The second aim allows us to evaluate the role of hepcidin in HIV and anemia. This study will be the first study to evaluate plasma hepcidin concentrations in HIV exposed and infected infants and will add to the body of literature on hepcidin and iron metabolism in infancy.

Finally, we also request permission to add Tatenda G. Mupfudze to the research plan as student investigator.

Enclosed please find the amendment application and the revised protocol including an outline of the additional areas of study proposed. Thank you very much for your continuing support of our work.

Sincerely,

Jean Humphrey, Sc.D.
Director, Zvitambo
1 Borrowdale Road
Borrowdale, Harare
Zimbabwe

ZVITAMBO

HIV CARE AND PREVENTION FOR MOTHERS AND CHILDREN IN ZIMBABWE

14 February 2013

MRCZ Secretariat
Medical Research Council of Zimbabwe
Josiah Tongogara/Mazowe Street
P O Box CY 573
Causeway

HARARE

Dear Sir/Madam

RE: AUTHORISATION TO USE VITAMIN A SAMPLES FOR A SUB-STUDY - PLASMA HEPCIDIN CONCENTRATIONS IN ZIMBABWEAN INFANTS

Zvitambo Project hereby gives Tatenda Mupfudze, a PhD Candidate at Johns Hopkins School of Public Health permission to use samples from the Vitamin A study stored in the Zvitambo archive for her doctoral research. The sub-study title is Plasma Hepcidin Concentrations in Zimbabwean Infants.

The samples can only be used for the purpose of this study and cannot be distributed or used for any other purposes.

For any further information please do not hesitate to contact me.

Yours sincerely

_____

Professor Jean Humphrey
Director - Zvitambo

Telephone: 791792/791193
Telefax: (263) - 4 - 790715
E-mail: mrcz@mrcz.org.zw
Website: http://www.mrcz.org.zw

Medical Research Council of Zimbabwe
Josiah Tongogara / Mazoe Street
P. O. Box CY 573
Causeway
Harare

MRCZ APPROVAL LETTER

Ref: **MRCZ/B/447** **28 March, 2013**

Tatenda Geraldine Mupfudze
Zvitambo Project
1 Borrowdale Road
Borrowdale
Harare
Zimbabwe

RE: PLASMA HEPCIDIN CONCENTRATIONS IN ZIMBABWEAN INFANTS

Thank you for the above titled proposal that you submitted to the Medical Research Council of Zimbabwe (MRCZ) for review. Please be advised that the Medical Research Council of Zimbabwe has **reviewed** and **approved** your application to conduct the above titled study. This is based on the following documents that were submitted to the MRCZ for review:

a) Study proposal.

APPROVAL NUMBER : **MRCZ/B/447**

This number should be used on all correspondence, consent forms and documents as appropriate.

- **APPROVAL DATE** : **28 March, 2013**
- **TYPE OF MEETING** : **FULL BOARD**
- **EXPIRATION DATE** : **27 March, 2014**
  After this date, this project may only continue upon renewal. For purposes of renewal, a progress report on a standard form obtainable from the MRCZ Offices should be submitted one month before the expiration date for continuing review.
- **SERIOUS ADVERSE EVENT REPORTING**: All serious problems having to do with subject safety must be reported to the Institutional Ethical Review Committee (IERC) as well as the MRCZ within 3 working days using standard forms obtainable from the MRCZ Offices.
- **MODIFICATIONS**: Prior MRCZ and IERC approval using standard forms obtainable from the MRCZ Offices is required before implementing any changes in the Protocol (including changes in the consent documents).
- **TERMINATION OF STUDY**: On termination of a study, a report has to be submitted to the MRCZ using standard forms obtainable from the MRCZ Offices.
- **QUESTIONS**: Please contact the MRCZ on Telephone No. (04) 791792, 791193 or by e-mail on mrcz@mrczimshared.co.zw.

Other

- Please be reminded to send in copies of your research results for our records as well as for Health Research Database.
- You're also encouraged to submit electronic copies of your publications in peer-reviewed journals that may emanate from this study.

Yours Faithfully

.....

MRCZ SECRETARIAT
FOR CHAIRPERSON
MEDICAL RESEARCH COUNCIL OF ZIMBABWE

MEDICAL RESEARCH COUNCIL OF ZIMBABWE
2013 -03- 28
APPROVED
P.O. BOX CY 573 CAUSEWAY, HARARE

PROMOTING THE ETHICAL CONDUCT OF HEALTH RESEARCH

JOHNS HOPKINS BLOOMBERG SCHOOL of PUBLIC HEALTH

JHSPH Institutional Review Board Office

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FWA #00000287

AMENDMENT APPROVAL NOTICE

Date: January 11, 2013

To: Jean Humphrey, ScD
Department of International Health

From: Elizabeth A. Skinner, MSW
Chair, IRB-X

Re: **Study Title**: "Vitamin A Supplementation of Breastfeeding Mothers and Their Neonates at Delivery: Impact on Mother-to-Child HIV Transmission during Lactation, HIV Infection among Women during the Post-partum Year and Infant Mortality"
IRB No: H.22.02.06.27.AR1

The JHSPH IRB-X reviewed and approved the amendment request described below, and received by the JHSPH IRB Office on **December 13, 2012**, at its meeting on **December 20, 2012**.

Single Reviewer ☒ Convened ☐ DHHS 46.110 . . ☒ DHHS..... ☐ FDA 56.110 . . . ☐ FDA..... ☐ Category: 2, 3, & 5 GWAS ..... ☐	Consent/Parental Permission Required From: Adult Participant ..... ☒ LAR ..... ☐ One Parent ..... ☒ Two Parents..... ☐ Legal Guardian ..... ☐ (Foster Care Children)	Form of Consent/Permission: Written Consent..... ☐ Waiver of Signature..... ☒ (Oral Script) Waiver of Informed Consent.... ☐ HIPAA Authorization..... ☐ HIPAA Waiver..... ☐ No Longer Enrolling..... ☐	Study Site(s): U.S. ☐ International ☒ List Country(ies): Zimbabwe
Vulnerable Populations: Children ..... ☒ Foster Care Children ..... ☐ DHHS / FDA 46.404 . . . . ☒ 50.51 ..... ☐ 46.405. . . . ☐ 50.52 ..... ☐ 46.406 . . . . ☐ 50.53 ..... ☐	Assent Required From: No children (waived) . ... ☒ Children aged:_____ ☐ Form of Assent: Written ..... ☐ Oral..... ☐ Assent Statement in Parent Permission..... ☐	Pregnant Women/Fetuses 46.204 ..... ☐ Neonates 46.205 ..... ☐ Prisoners 46.305 ..... ☐ 46.306 ..... ☐ Epidemiological Research.... ☐	Sample Size: (screened plus enrolled) Final Enrollment: Secondary Data Analysis: (# specimens/participants) 28,357

This amendment approval is for the following revisions to the above referenced study:

1. **To estimate the reference intervals for plasma hepcidin in (65) non-anemic healthy children at three ages (3, 6 and 12months) and describe the trajectory of plasma hepcidin in the first year of life. The study population will be drawn from children enrolled in the Zvitambo Vitamin A trial who had a gestational age >37 weeks, birth weight >2500g and never had abnormal iron indicators (i.e. hemoglobin < 105 g/L at 3 and 6 months and < 100g/L at 12months, serum ferritin < 12µg/l, sTfR > 8.3mg/L), inflammation (i.e. AGP > 1g/L) or an acute illness (diarrhoea, fever, in the prior week or measles in the prior 3 months) at assessment visits at 3, 6 and12 months of age.**

2. **To evaluate the differences in mean plasma hepcidin associated with HIV exposure, HIV infection and anemia by measuring plasma hepcidin levels in cryopreserved samples from the Zvitambo trial collected from five groups of infants (65 infants per group): HIV-unexposed anemic; HIV-exposed uninfected and anemic; HIV-exposed uninfected and non-anemic; HIV-infected and anemic and HIV-infected and non-anemic infants at 3 ages (3, 6, 12months).**

3. **To change the sample size from 28,292 to 28,357.**

4. **To add Tatenda Geraldine Mupfudze as a student investigator to the study.**

and is inclusive of the following revised or newly submitted documentation:

Research Plan (Version #7, 12-13-12)

As a reminder, no other changes to this study may be implemented without prior JHSPH IRB review and approval.

The action taken on this study does not change the IRB expiration date, which remains **August 15, 2013**.

If your research involves international travel, please don't forget to register with the International Travel Registry https://apps4.jhsph.edu/ITR/Default.aspx so that the School may locate you in the event of an emergency.

If you have any questions regarding this action, please contact the JHSPH IRB Office at (410) 955-3193 or via email at irboffice@jhsph.edu

EAS/sro

Reference:

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EDUCATION

2009-2014 PhD Student, Program in Human Nutrition, Department of International Health. The Johns Hopkins University Bloomberg School of Public Health, Baltimore, MD.

2008 BS, Human Nutrition, University of St. Catherine, MN.

PROFESSIONAL EXPERIENCE

2009-2011 Graduate Research Assistant, Center for Immunization Research, Johns Hopkins- Baltimore, MD.
Responsibilities: Screened and recruited healthy volunteers for early phase 1 clinical trials. Data entry, data management and report generation.

2008-2009 Sr. Community Health Worker, Women Infants and Children (WIC), Minneapolis, MN.
Responsibilities: Conducted dietary assessments, and took anthropometric and hematological measurements. Provided nutrition education and counseling to pre- and post-natal women, infants and children below 5years.

2009 Community Health Worker, Portico Healthnet, Minneapolis, MN.
Responsibilities: Connected uninsured patients with appropriate health insurance coverage.

2006-2008 Research Assistant - College of St. Catherine, St. Paul, MN.
Responsibilities: Conducted literature reviews. Edited articles for Dr Julie Jones' nutrition columns in Cereal World Foods and Lipid Technology magazines

HONORS/AWARDS

2010 Johns Hopkins, Harry D. Kruse Fellowship in Nutrition
2007 The College of St. Catherine, Senate Leadership Scholarship
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MEMBERSHIP

2010 American Society for Nutrition

Presentations

2008 "Efficacy of Vitamin A supplementation in reducing infant mortality in Zimbabwe," Meeting of the Twin Cities District Diet Association, St. Paul MN. Speaker.

2008 "Efficacy of Vitamin A supplementation in reducing infant mortality in Zimbabwe," 91st Annual Meeting of the Minnesota Dietetics Association. Rochester MN. Poster Presentation.

ADDITIONAL INFORMATION

Personal Statement of Research

My research interests center on maternal and infant health and the interaction between nutrition, and infectious diseases. My current work is aimed at understanding the risk factors and the mechanisms underlying anemia in infants and young children particularly the role of hepcidin in anemia in areas with a high burden of infection.
Specific areas of interest include:

1. Maternal and infant health
2. Anemia
3. Iron
4. Hepcidin
5. HIV/AIDS

Keywords
Maternal and infant health
Anemia
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HIV/AIDS