ASSESSMENT OF IRON DEFICIENCY IN MALAWIAN CHILDREN LIVING IN AN AREA OF HIGH MALARIA AND BACTERIAL INFECTION MORBIDITY

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of

Doctor of Philosophy

by

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DECLARATION

THIS WORK HAS NOT PREVIOUSLY BEEN ACCEPTED IN SUBSTANCE FOR ANY DEGREE AND IS NOT BEING CURRENTLY SUBMITTED IN CANDIDATURE FOR ANY DEGREE

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ABBREVIATIONS

ABC	Antibodies bound per cell
AI	Anaemia of inflammation
AOR	Adjusted odds ratio
AUC	Area under curve
CRP	C-reactive protein
Dcytb	Duodenal cytochrome *b*1
DMT1	Divalent metal transporter
EPO	Erythropoietin
ESR	erythrocyte sedimentation rate
H/A	Height-for-age
Hb	Haemoglobin
Hct	Haematocrit
HFE	Hereditary haemochromatosis gene
HIV	Human immunodeficiency virus
HJV	Hemojuvelin
IL	Interleukin
IRE	Iron responsive element
IRP	Iron regulatory protein
MCH	Mean cell haemoglobin

MCHC	Mean cell haemoglobin concentration
MCV	Mean cell volume
mTfR	membrane-bound transferrin receptor
Nramp	Natural-resistance-associated macrophage protein
OR	Odds ratio
PE	Phycoerythrin
RI	Reticulocyte index
ROC	Receiver operating characteristic
RR	Relative risk
sTfR	soluble transferrin receptor
TIBC	Total iron binding capacity
TfR-F	Transferrin-ferritin
TGFβ	Transforming growth factor β
TNFα	Tumour necrosis factor α
UTR	untranslated region
W/A	Weight-for-age
W/H	Weight-for-height
WHO	World Health Organisation

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Firstly I would like to thank my supervisors in Liverpool Professor Bernard Brabin and Dr Imelda Bates for their support and advice throughout my PhD. Thank you for helping me up after the many times I stumbled. I wish also to express my gratitude to my sponsors, the Gates Malaria Partnership (GMP) towards my tuition and upkeep, and the Wellcome Trust for funding my work through the Severe Anaemia Project.

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Declaration of work done

My study was integrated in a larger Severe Anaemia study, hence the responsibility of some of the laboratory work was shared among a few individuals. My contribution was as follows:

Activity		Responsibility
Sample collection and processing		shared
Blood collection		shared
Bone marrow aspiration		shared
Processing samples and storing		shared
Bone marrow		
Marrow iron smear	• Slide preparation	shared
	• Staining	sole
	• Reading	sole
Mass spectrometry	• Pilot studies	sole
	• Marrow preparation	sole
	• Reading	Done by others
Flow cytometry	• Cell preparation	shared
	• Reading	sole
	• Calibration with beads	sole
Blood		
Iron markers determination (except sTfR)		Done by others
Inflammation markers determination		Done by others
sTfR determination (ELISA)		Sole
Haemoglobin	• Hemocue©	Shared
	• Full blood Count	Done by others
Microbiological blood culture		Done by others

I was also responsible for day to day running of Chikwawa study site, involved in recruitment and follow-up of patients.

ABSTRACT

Assessment of iron deficiency in Malawian children living in an area of high malaria and bacterial infection morbidity

Kamija Phiri

Introduction and Objectives: In sub-Saharan Africa iron deficiency (ID) and malaria infection are the major causes of anaemia in children under five years of age. Assessment of ID, using biochemical iron markers in areas of high malaria transmission and infection can be difficult as markers are altered by the acute phase response. Results of the 'gold standard' test used to diagnose ID, namely bone marrow (BM) iron microscopy, has occasionally been inconsistent. This study aimed to evaluate methods of assessing ID in the BM and peripheral blood, and investigate the association between ID and infection in children living in a malaria endemic area.

Methodology: This descriptive study was in children (cases Hb<5g/dl, hospital (HC), community (CC) and surgical controls (SC), with Hb level >5g/dl) recruited in Blantyre and Chikwawa districts in southern Malawi. Clinical data, blood and bone marrow samples (cases and SC only) were collected at recruitment. Blood samples were used for biochemical, haematological, microbiological and parasitological analyses. Amount of iron in BM was determined using microscopy, flow cytometry and mass spectrometry.

Results: A total of 1161 children (381 cases, 377 HC, 380 CC and 23 SC) aged between 6-60 months, were recruited between July 2002 and August 2004. A new histological grading for the BM iron smear (assessing iron store and cellular iron status) was tested, in contrast to the conventional method (assessing iron stores status). Among the severely anaemic children, 29.4% had deficient iron stores, 39.6% functional iron deficiency and 31.0% normal iron status. Examination of 8-9 BM fragments was adequate to assess the iron stores status. There was a poor correlation of BM iron content by microscopy with flow cytometry (r=0.04, *p=0.6*), or mass spectrometry (r=0.14, *p=0.2*), and by flow cytometry with mass spectrometry (r=0.09, *p=0.4*). Iron sequestered in hemozoin was identified to be an important confounder of these measurements. Lower levels sTfR were observed in cases with inflammation (difference 27.9µg/ml, *p<0.001*) or malaria parasitaemia (difference 4.3µg/ml, *p=0.02*), than in those without. Modelling (R^2=0.497, F-statistic=14.2, *p<0.001*) predicted that sTfR was influenced by iron status (β=0.42, *p<0.001* for iron stores status, β=0.26, *p=0.001* for cellular iron status), inflammation (β=-0.36, *p<0.001*), HIV infection (β=-0.23, *p=0.001*) and reticulocytosis (β=0.16, *p=0.03*). Using commonly-accepted cut-offs, ferritin had 96% specificity and 21% specificity, and sTfR had 90% sensitivity and 37% specificity for the detection of deficiency of iron stores among cases. Receiver Operating Characteristics (ROC) curves, showed that the TfR-F Index (sTfR ÷ Log ferritin), with AUC^{ROC}=0.79 (*p<0.001*), had 74% sensitivity, 73% specificity and 73% accuracy, and was the optimal iron marker for iron status assessment in children in this population. MCHC, with a lower diagnostic efficiency (67% sensitivity, 64% specificity, 65% accuracy) may be used in tertiary health care in resource-poor settings. Ferritin, sTfR and TfR-F Index were poor at diagnosing functional iron deficiency. Prevalence of bacteraemia was 20% (n=52) in cases and 5% (n=14) in HCs. *Salmonella* species was the commonest isolated micro-organism among cases (73%, n=38) and HC (86%, n=12), despite a high rate of contamination (28% BM and 13% blood samples). Among cases ID was positively, but not significantly associated with reduced risk of bacteraemia (AOR 0.60, 95% CI 0.20-1.79, *p=0.9*), malaria parasitaemia, (AOR 0.67, 95% CI 0.37-1.20, *p=0.4*) or HIV infection (AOR 0.67, 95% CI 0.24-1.87, *p=0.4*). Iron deficiency was associated with a lower proportion of malaria pigmented leukocytes (difference 2.6%, *p=0.01*).

Conclusion: A new histological iron grading method was useful in determining iron status. Only a third of severely anaemic children would require iron therapy. TfR-F Index may be the optimal, although not ideal biochemical iron marker for diagnosing deficiency of iron stores. Ferritin, sTfR and TFR-F Index were less useful in diagnosing functional iron deficiency. ID was not significantly associated with infection.

CHAPTER 1

INTRODUCTION AND AIMS

1.1 BACKGROUND TO THE STUDY

In sub-Saharan Africa iron deficiency and malaria infection are the major causes of anaemia in children below the age of five years. It is not known what role malaria, iron deficiency and other bacterial infections play in the progression to severe anaemia. A plausible pathway is that children are chronically anaemic due to iron deficiency and possibly persistent asymptomatic plasmodia infection and thus easily become severely anaemic with an acute malarial attack. The attributable risk of severe anaemia from malaria or iron deficiency has been poorly estimated. This has been due to the difficulty in diagnosing iron deficiency in areas that have high prevalence of malaria and other inflammatory conditions.

The acute-phase response to infection alters the plasma concentration of most biochemical measures of iron status, rendering assessment of status difficult (Beesley *et al,* 2000). Ferritin, although a sensitive indicator of iron status, rises considerably during malaria infection (Beesley *et al,* 2000). It is still unclear whether the concentration of soluble transferrin receptors (sTfR) is unaffected by inflammation or malaria as reported in some earlier studies (Lipschitz *et al,* 1974; Menendez *et al,* 1997; van den Broek *et al,* 1998; Verhoef *et al,* 2001). Most of these studies were poorly powered, flawed by selection bias (Kuvibidila *et al,* 1995), and limited to individuals who had

asymptomatic malaria (Verhoef *et al,* 2001), or individuals not residing in malaria endemic areas (Beesley *et al,* 2000), or pregnant women (van den Broek *et al,* 1998). Most importantly, very few studies have been done comparing the biochemical measures of iron status in peripheral blood with those assessing iron in bone marrow aspirates (van den Broek *et al,* 1998).

Bone marrow iron has been said to be the gold standard for determining the status of body iron stores. Arbitrary grading systems have been used, mainly based on the amount of iron present and its location (Rath and Finch 1948; Gale *et al,* 1963). Histological iron grading is very subjective, with potential for inter- and intra-observer bias. In a study of bone marrow specimens in which iron was reported as absent, further analysis of the cases showed that 30% had been inaccurately diagnosed (Baron *et al,* 2001). It was also observed that the inter-observer difference was 31%, which was unacceptably high for a 'gold standard' test. The use of membrane-bound expression of transferrin receptor (mTfR) as a measure of cellular iron needs using flow cytometry may not only improve iron status assessment but offer a means of evaluating histological bone marrow iron grading.

1.2 OBJECTIVES

General Objective

To evaluate the assessment of iron deficiency and its association with infection in Malawian children living in a malaria endemic area

Theme 1 (Chapters 5 & 6): To assess iron status determination in bone marrow

Specific objectives for Theme 1:

1. To determine the prevalence of deficiency of iron stores and normal iron store status among cases and surgical controls using the conventional histological grading method
2. To determine the prevalence of iron stores deficiency, normal iron status and functional iron deficiency among cases and surgical controls using a detailed alternative histological grading method
3. To assess, in a malaria endemic population, the number of marrow fragments that should be examined to confidently establish the presence or absence of iron stores and grade the quantity present in a bone marrow aspirate
4. To determine bone marrow iron status using flow cytometry by measuring expression of mTfR
5. To determine bone marrow iron status using mass spectrometry by measuring total elemental iron
6. To compare bone marrow iron microscopy method of determining bone marrow iron status with flow cytometry and mass spectrometry

Theme 2 (Chapters 7 & 8): To assess iron status determination in peripheral blood

Specific objectives for Theme 2:

Among severely anaemic children to:

1. Verify whether biochemical iron markers are altered by inflammation
2. Determine whether biochemical iron markers are altered by malaria
3. Determine the main factors that influence sTfR levels among children with severe anaemia living in a malaria endemic area
4. Determine and compare the ability of different biochemical measures to predict bone marrow iron status and functional iron deficiency
5. Investigate how to improve the performance of biochemical iron measures for predicting bone marrow iron status in a malaria endemic area

Theme 3 (Chapter 9): To determine the association between iron deficiency and infection

1. To determine the prevalence of iron deficiency in cases, and hospital, community, or surgical controls
2. To describe the common micro-organisms causing bacteraemia in cases and hospital controls
3. To determine the association between iron deficiency with bacteraemia, malaria or Human Immunodeficiency virus (HIV) infection

1.3 THESIS STRUCTURE

Chapter 2 is a review of the relevant literature with a focus on sub-Saharan Africa. **Chapter 3** summaries the study methods and **Chapter 4** summarises the general study results and includes a brief discussion. **Chapter 5, 6, 7, 8** and **9** describe the results according to the three outlined

themes. The structure of each result Chapter is designed to include sections on introduction, objectives, study methods, results, discussion and conclusion. **Chapter 10** is a summary of the research findings in **Chapters 5, 6, 7, 8,** and **9,** and presents specific conclusions and research recommendations.

CHAPTER 2

LITERATURE REVIEW

2.1 EPIDEMIOLOGY OF IRON DEFICIENCY AND MALARIA

2.1.1 Epidemiology of iron deficiency

Iron status can be considered as a continuum from iron deficiency with anaemia, to iron deficiency with no anaemia, to normal iron status with varying amounts of stored iron, and finally to iron overload. Iron deficiency is the result of long-term negative iron balance. Iron deficiency is defined as a condition in which there are no mobilizable iron stores and signs of compromised iron supply to the tissues and erythron, is noted. The more severe stages of iron deficiency are associated with anaemia.

Iron deficiency is the most common and widespread nutritional disorder in the world. It affects nearly 2 billion people and results in over 500 million cases of anaemia (DeMaeyer *et al,* 1989). Although a large burden of the disease is found among children in developing countries, iron deficiency is the only nutrient deficiency which is significantly prevalent in virtually all industrialised countries. There are no current global figures for iron deficiency, but using anaemia as an indirect indicator, it can be estimated that most pre-school children and pregnant women in developing countries, and at least 30-40% in developed countries, are iron deficient (WHO 2001).

Few studies have attempted to estimate the prevalence of iron deficiency in Africa. An extensive population survey in Cote d'Ivoire, covering

four regions and 16 locations in each region, observed that iron deficiency was prevalent in 63% of children below 5 years of age. However, the estimate may have been affected by selection bias and the use of iron measures which could have been unreliable in the presence of malaria and inflammation (Asobayire *et al,* 2001). Rather high estimates have also been observed amongst school children in Tanzania, despite the fact that different cut-offs, from those in Cote d'Ivoire, were used to define iron deficiency (Stoltzfus *et al,* 1997). There are even fewer population based studies estimating the prevalence of iron deficiency among infants (Faber and Benade 1999; Nyakeriga *et al,* 2004) (Table 2.1).

Table 2.1: Prevalence of iron deficiency in sub-Saharan Africa

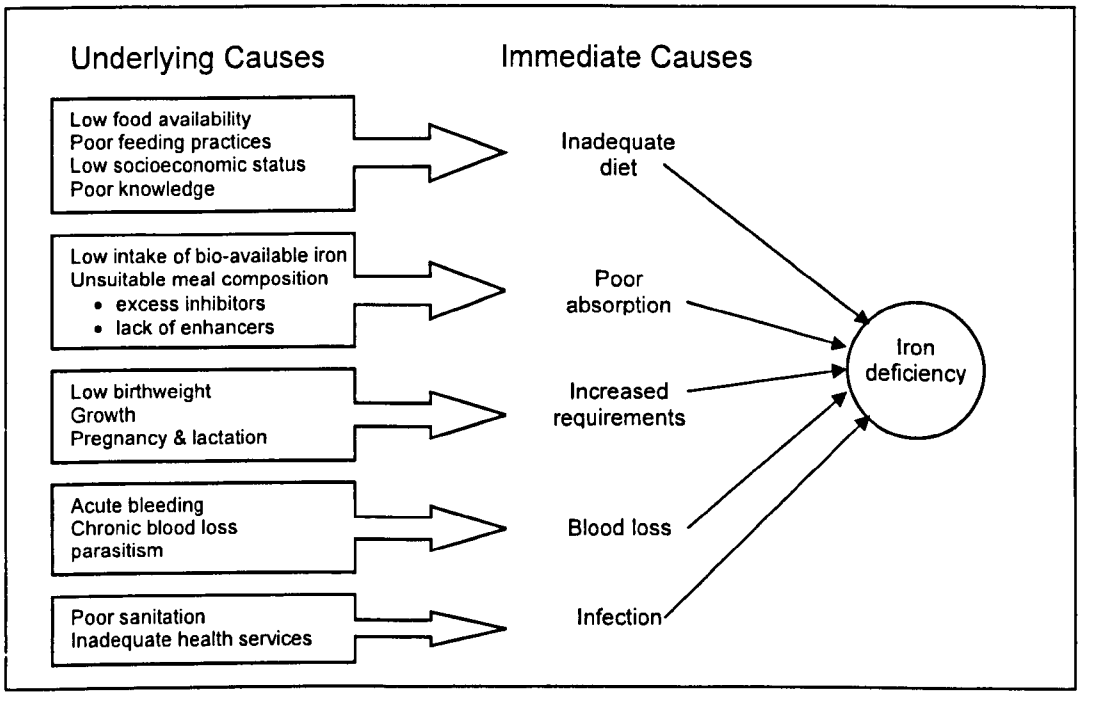
Authors	Study population	Prevalence of Iron deficiency	Definition of Iron deficiency
Stoltzfus et al (1997)	School children	49%	EP >90µmol/mol heme
Nyakeriga et al (2004)	8 months – 8 years	33 – 38%	Ferritin <12µg/ml Trans Sat <10% CRP <10mg/l Negative malaria slide
Asobayire et al (2001)	2 – 5 years	63%	Ferritin <30µg/l sTfR >8.5 mg/l EP >40 µmol/mol haem
Faber et al (1999)	4 – 24 months	43%	Ferritin <10µg/l

EP – erythroprotopophyrin
Trans Sat – transferrin saturation
CRP – C-reactive protein

The high prevalence of iron deficiency in Africa is due to multiple and complex factors that are found in developing countries. These causes of iron

deficiency and iron deficiency anaemia must be viewed beyond the immediate environment of the affected individual and relate to several underlying mechanisms outlined in Figure 2.1.

Figure 2.1: Aetiology of iron deficiency



2.1.2 Epidemiology of malaria

Malaria causes 100 million morbid episodes and over one million deaths every year world-wide. About 90% of all deaths occur in sub-Saharan Africa. In malaria endemic areas, very young children and pregnant women are the population groups most at risk for malaria morbidity and mortality. Many children below the age of five years living in malaria endemic countries are asymptomatically infected with *Plasmodium falciparum*. Ninety percent of all malaria deaths in Africa occur in young children (WHO 2002).

Malaria is a complex disease whose pathogenesis is still not fully understood. Many studies have concentrated on the pathophysiology and management of cerebral malaria, although severe malarial anaemia is an equally important problem which requires further study. The availability of blood transfusion may have reduced the perceived need for further research on malarial anaemia (Molyneux *et al,* 1989; Slutsker *et al,* 1994). The World Health Organisation (1992) defines severe malarial anaemia as a haemoglobin (Hb) concentration <5g/dl or haematocrit (Hct) <15%, in the presence of a normocytic blood film and malaria parasitaemia >10,000 parasites/µl. This definition is limited in that few health providers in malaria-endemic areas have the time or expertise for the adequate examination of red blood cell morphology on a thin film. If this is done a blood film showing abnormalities does not exclude malaria as a contributory cause of anaemia (Bradley-Moore *et al,* 1985). A more practical definition would be a Hb of <5g/dl in association with malaria parasites (Phillips *et al,* 1986). There are potential problems with both definitions as there are many, often interacting, causes of anaemia and the mechanisms underlying these interactions are unclear. Secondly in areas of high intensity of malaria transmission many children are normally parasitaemic. Thirdly the pre-defined cut-off does not allow for variations in age or level of endemicity.

The greatest burden of malarial anaemia is carried by young children and pregnant women in sub-Saharan Africa. Prevalence of anaemia (Hct ≤33%) in communities in malaria-endemic areas of Africa varies between 31%

to 90% in children (Bradley-Moore *et al,* 1985; Premji *et al,* 1995) and between 60% to 80% in pregnant women (van den Broek *et al,* 1998). Malaria attributable anaemia is demonstrated by epidemiological observations of the increased number of hospital admissions with severe anaemia coinciding with the peaks of malaria transmission although this does not rule out seasonal nutritional factors which also contribute (Slutsker *et al,* 1994).

Acquired and congenital protection against malaria

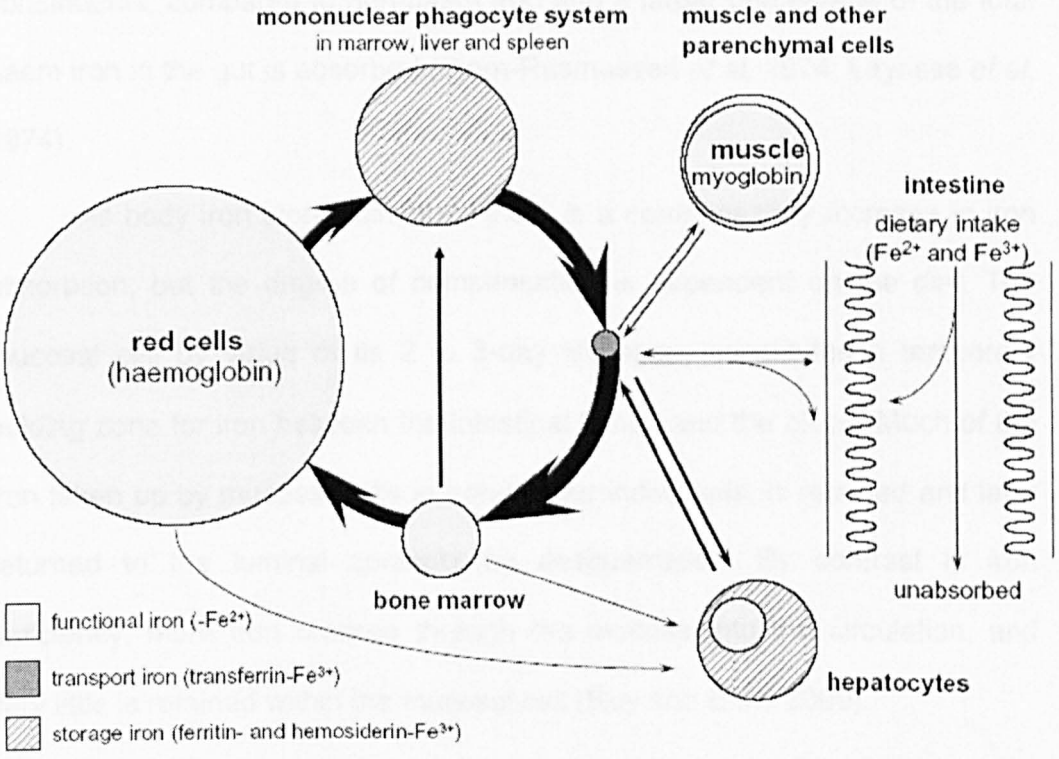
In areas of high malaria transmission repeated exposure leads to the acquisition of specific immunity (Rogier and Trape 1993), which reduces the risk of severe disease in children and symptomatic malaria in older subjects. Several red cell polymorphisms have been associated with altered, usually reduced prevalence of malaria (Allen *et al,* 1997). Their geographical distribution may arise due to a selective effect of malaria on the heterozygote carrier who experiences reduced severity of disease (Flint *et al,* 1998). This 'malaria hypothesis' has been a key insight into the interaction of human genetics and infectious diseases and was first identified when the association of reduced risk of *P. vivax* in Duffy blood group negative individuals or *P. falciparum* in individuals with sickle cell trait (Flint *et al,* 1998).

2.2 IRON METABOLISM

Iron distribution

Iron in the body is present in three main forms. The metabolically active form of iron is ferrous iron (Fe^{2+}). Most iron is incorporated in haemoglobin, while the remainder is present in myoglobin in muscles and in iron-containing, or iron-dependent enzymes, in body cells. The second form is storage iron that can be released as metabolically active iron. This is in the form of ferric iron (Fe^{3+}) and is stored in reticuloendothelial cells, tightly bound to the proteins of ferritin and haemosiderin, and in hepatocytes. The third form of iron exists as transport iron (Fe^{3+}), that is bound to the serum transport protein transferrin. This transports iron released by reticuloendothelial cells through the degradation of haemoglobin, to developing erythrocytes in the bone marrow, for erythropoiesis, or to other iron-requiring cells in the body (Figure 2.2).

Figure 2.2: Distribution of iron in the body



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Iron absorption

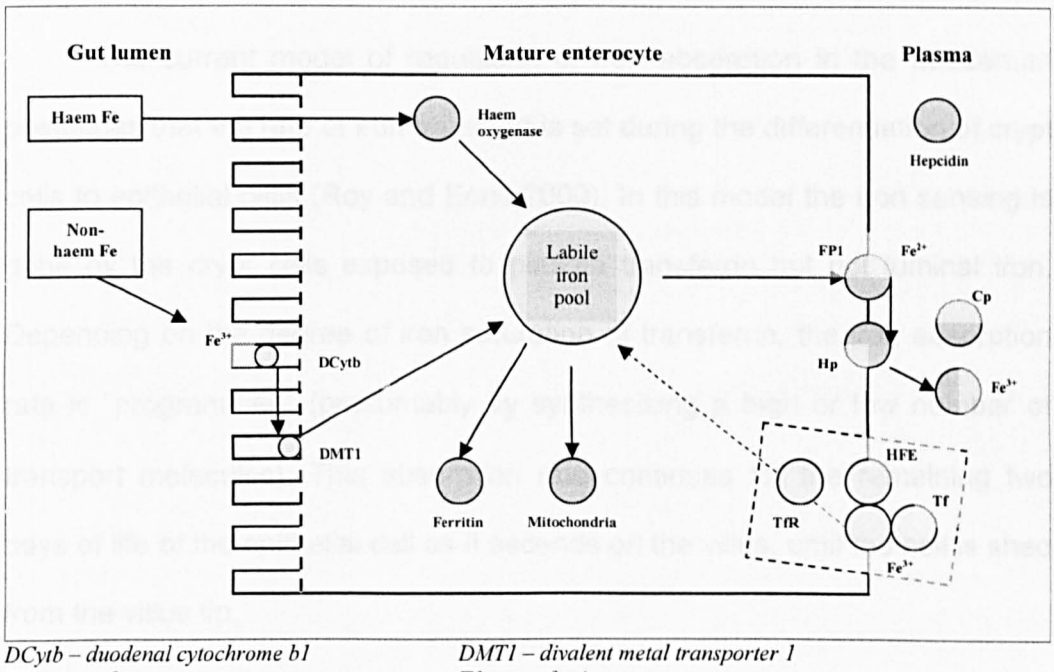
Iron in food is primarily found in the form of ferric complexes, with a smaller amount in the form of haem protein. Ferric iron complexes are partly broken down to the more readily absorbed ferrous form mainly in the stomach. This is facilitated by hydrochloric acid in gastric juice. Assimilation of non-haem iron is enhanced by the formation of readily absorbed complexes with other components of the diet, such as fructose, ascorbic acid and certain amino acids. Conversely, phytic acid can form insoluble complexes with iron in food, which may decrease iron absorption by 50% or more (Jacobs *et al,*

1964). The absorption of haem iron is less affected by other dietary constituents, compared to non-haem iron and a larger percentage of the total haem iron in the gut is absorbed (Bjorn-Rasmussen *et al,* 1974; Layrisse *et al,* 1974).

As body iron stores diminish, there is a compensatory increase in iron absorption, but the degree of compensation is dependent on the diet. The mucosal cell by virtue of its 2 to 3-day life span, constitutes a temporary holding zone for iron between the intestinal lumen and the blood. Much of the iron taken up by mucosal cells in iron-loaded individuals, is retained and later returned to the luminal contents by desquamation. By contrast in iron deficiency, more iron crosses through the mucosa into the circulation, and very little is retained within the mucosal cell (Roy and Enns 2000).

Mechanisms for non-haem iron absorption include specific ones which are saturable and receptor-mediated, and at higher doses passive diffusion may occur. Duodenal cytochrome *b*1 (Dcytb) reduces Fe^{3+} released from non-haem iron to the soluble Fe^{2+} form, which is then transported across the brush border membrane by a divalent metal transporter 1 (DMT1) (Gunshin *et al,* 1997). In the mucosal cell the iron enters into the labile pool from which some is incorporated into ferritin. This iron is lost when cells exfoliate. Iron that is retained by the body is actively transported across the serosal membrane by ferroportin 1 and later taken up by transferrin in the non-soluble iron form Fe^{3+} (Figure 2.3).

Figure 2.3: Iron absorption in the gastro-intestinal mucosa



DCytb – duodenal cytochrome b1
TfR – transferrin receptor
HFE – haemochromatosis gene
Hp – Hephaestin
DMT1 – divalent metal transporter 1
Tf – transferrin
FP1 – ferroportin 1
Cp – Ceruloplasmin

Reproduced from Worwood and Hoffbrand (2005)

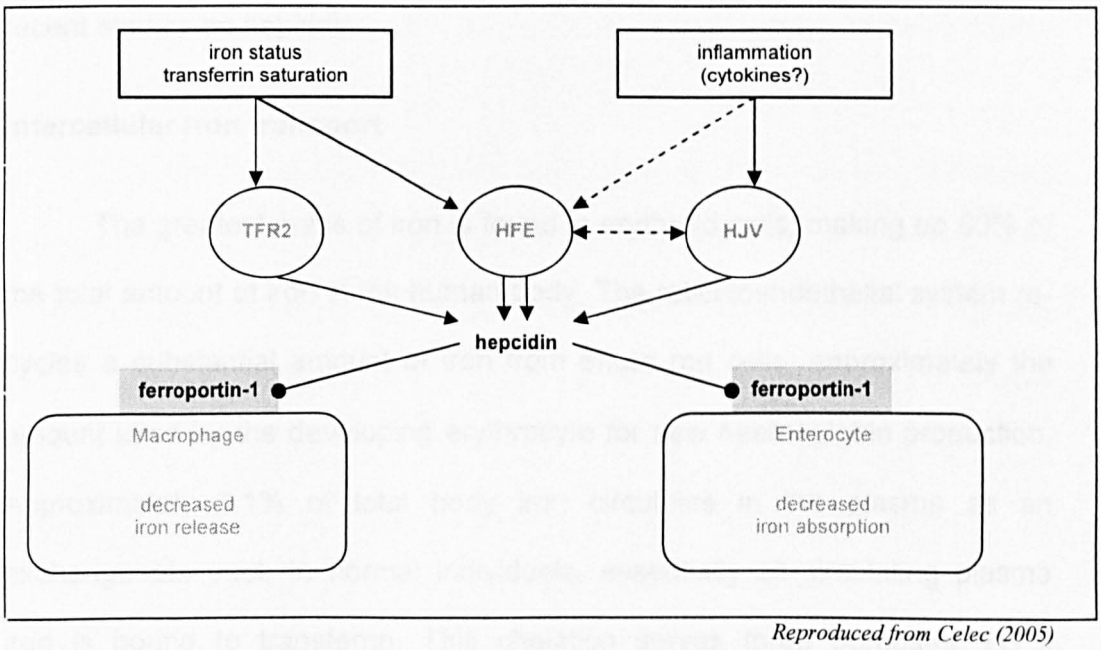
Hephaestin, a ferroxidase expressed predominantly in the villous cells of the duodenum is implicated in the conversion of Fe^{2+} to Fe^{3+} (Levenson and Tassabehji 2004). Haem iron is initially bound by haem receptors at the brush border membrane and released intra-cellularly by haem oxygenase. The iron enters the labile pool and consequently follows the same pathways as non-haem iron (Worwood and Hoffbrand 2005).

Regulation of iron absorption

The current model of regulation of iron absorption in the duodenum postulates that the rate of iron transport is set during the differentiation of crypt cells to epithelial cells (Roy and Enns 2000). In this model the iron sensing is done by the crypt cells exposed to plasma transferrin but not luminal iron. Depending on the degree of iron saturation of transferrin, the iron absorption rate is "programmed" (presumably by synthesizing a high or low number of transport molecules). This absorption rate continues for the remaining two days of life of the epithelial cell as it ascends on the villus, until the cell is shed from the villus tip.

There has recently been evidence that hepcidin (Ganz 2003), a small 20-25 amino acid peptide, plays a crucial role in regulating iron absorption. It is mostly expressed in hepatocytes in the liver. It is down-regulated during iron deficiency (Nicolas *et al,* 2002), and up-regulated during increased iron stores states and inflammation (Nemeth *et al,* 2003). Hereditary haemochromatosis protein (HFE), transferrin receptor 2 (TFR2) and hemojuvelin (HJV) probably effect their roles indirectly in the control of iron absorption through the regulation of hepcidin synthesis (Celec 2005) (Figure 2.4). Hepcidin is the predominant negative regulator of iron absorption in the small intestine, and in iron transport across the placenta and iron release from macrophages. It is not certain how hepcidin regulates iron absorption but it is postulated to be through binding to ferroportin and accelerating its degradation (Ganz 2003).

Figure 2.4: Regulation of iron absorption



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It is unclear how iron regulates hepcidin production, but its been shown that TFR2, HFE, and HJV are each required for sufficient expression of hepcidin in response to iron (Bridle *et al,* 2003; Papanikolaou *et al,* 2004; Nemeth *et al,* 2005). In the simplest model, the hepatocytes would serve as an iron-sensor cell as well as producer of the hepcidin. However, direct exposure of hepatocytes to ferric iron or iron saturated transferrin did not induce hepcidin production (Nemeth *et al,* 2003). This suggests that iron-sensing may take place in other cells. Based on the likely involvement of macrophage-derived interleukin-6 (IL-6) in hepcidin induction in infections, Kupffer cells and perhaps the sinusoidal cells could sense iron and signal hepatocytes in order to regulate hepcidin production. The nature of this signal remains to be determined. The model of iron duodenal transport where crypt

cells are "programmed" may have to be reconsidered in light of these more recent studies on hepcidin.

Intercellular iron transport

The greatest mass of iron is found in erythroid cells, making up 80% of the total amount of iron in the human body. The reticuloendothelial system re-cycles a substantial amount of iron from effete red cells, approximately the amount used by the developing erythrocyte for new haemoglobin production. Approximately 0.1% of total body iron circulates in the plasma as an exchangeable pool. In normal individuals, essentially all circulating plasma iron is bound to transferrin. This chelation serves three purposes: (1) it renders iron soluble under physiologic conditions, (2) it prevents iron-mediated free radical toxicity, and (3) it facilitates transport into cells. Transferrin is the most important physiologic supplier of iron to red blood cells. Other iron transporting proteins include lactoferrin (Metz-Boutigue *et al,* 1984) in extracellular secretions, melanotransferrin (Brown *et al,* 1982), a protein produced by melanoma cells.

The precise mechanism by which iron is loaded on to transferrin as it leaves intestinal epithelial cells, or reticuloendothelial cells, is unknown. There is compelling evidence that the copper-dependent ferroxidase, ceruloplasmin, may be involved in mobilising tissue iron stores to produce diferric transferrin (Harris *et al,* 1995). The sum of all iron-binding sites on transferrin constitutes the total iron-binding capacity of plasma. Under normal circumstances, about

one third of transferrin iron-binding pockets are filled. Consequently, with the exception of iron overload, where all the transferrin binding sites are occupied, non-transferrin-bound iron in the circulation is virtually nonexistent.

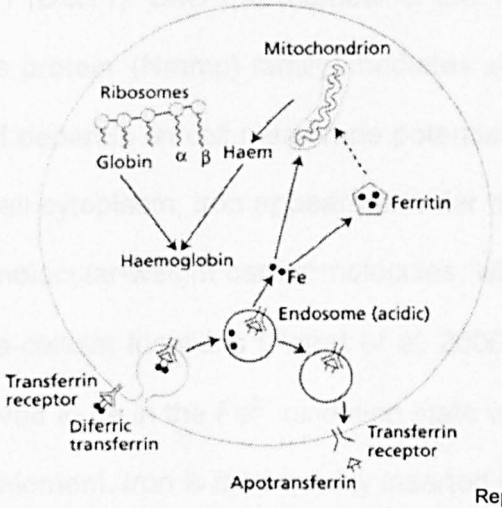
Ferrokinetic studies indicate that at least 80% of the circulating transferrin-bound iron, is delivered to the bone marrow to be incorporated into developing erythrocytes (Finch *et al,* 1982). Other major sites of iron delivery include the liver, which is a primary depot for stored iron, and the spleen. Hepatic iron is found in both reticuloendothelial cells (two-thirds) and hepatocytes (one third). Reticuloendothelial cells acquire iron primarily by phagocytosis and breakdown of aging red cells, extracting it from haem and returning it to the circulation bound to transferrin.

Although compelling evidence exists that the transferrin cycle is important for iron acquisition by the erythron, other tissues can import iron by alternative mechanisms, for example some patients and mutant mice have little or no circulating transferrin (Goya *et al,* 1972; Huggenvik *et al,* 1989). It is considered that iron can also be weakly complexed to albumin, citrate, amino acids, and sugars and behaves differently from iron associated with transferrin (Hershko and Peto 1987; Grootveld *et al,* 1989). Non-haemapoietic tissues, particularly the liver, endocrine organs, kidney and heart preferentially take up this iron.

Intracellular iron metabolism

Iron is taken into cells by receptor-mediated endocytosis of mono-ferric and di-ferric transferrin (Iacopetta and Morgan 1983). The transferrin receptor, a disulfide-linked homodimer consisting of 760 amino acids, binds iron-loaded transferrin with a very high affinity (Zak *et al,* 1994). The haemochromatosis gene, produces a protein that binds to the transferrin receptor (Feder *et al,* 1996). This protein may have a role in determining receptor affinity for ferric transferrin but its full role in iron metabolism is still incompletely understood. On binding of transferrin to its receptor on the cell surface, transferrin-transferrin receptor complex is rapidly internalised by invagination of clathrin-coated pits with the formation of endocytic vesicles. An ATP-dependent pump lowers the pH of the endosome to about 5.5 (Paterson *et al,* 1984) (Figure 2.5).

Figure 2.5: Iron uptake and distribution in the cell



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Three processes occur that aid release of iron from transferrin. Firstly, the acidification of the endosome weakens the association between the iron and transferrin (Yamashiro *et al,* 1984). Secondly, a plasma membrane oxidoreductase reduces transferrin-bound iron from Fe^{3+} state to Fe^{2+}, which directly or indirectly facilitates the removal of the iron from the protein (Nunez *et al,* 1990). Conformational changes in the transferrin receptor also play a role in iron release from the transferrin protein (Bali *et al,* 1991). Intact receptor-bound apotransferrin recycles to the cell surface, where neutral pH promotes detachment into the circulation. Exported apotransferrin binds additional iron and undergoes further rounds of iron delivery to cells. The average transferrin molecule, with a half-life of 8 days, may be used up to 100 times for iron delivery (Hartford and Rouault 1994).

After release of iron from transferrin within the endosome, it traverses the plasma membrane to enter the cytosol proper via the divalent metal transporter 1 (DMT1). DMT1, a member of the 'natural-resistance-associated macrophage protein' (Nramp) family, mediates active transport that is proton-coupled and depends on cell membrane potential (Gunshin *et al,* 1997). Once inside the cell cytoplasm, iron appears to enter this labile iron pool formed by weak, low-molecular-weight carrier molecules, which may assist in delivery to various intra-cellular locations (Petrat *et al,* 2000). This minute pool of transit iron is believed to be in the Fe^{2+} oxidation state which is the biologically active form of the element. Iron is then quickly inserted into iron-containing functional proteins, mitochondrial and non-mitochondrial haem proteins and iron sulphur clusters. Metabolically inactive iron is stored as ferritin and haemosiderin.

Ferritins are complex 24-subunit heteropolymers of protein (Harrison *et al,* 1986). The sub-units of the molecule form a sphere with a central cavity in which up to 4500 atoms of crystalline iron are stored in the form of poly-iron phosphate oxide. A mechanism involving dioxygen converts ferrous to ferric iron, promoting incorporation into ferritin (Levi *et al,* 1988). Ferritin molecules aggregate over time to form clusters, which are engulfed by lysosomes and degraded (Bridges 1987). The end product of this process, haemosiderin, is an amorphous agglomerate of denatured protein and lipid interspaced with iron oxide molecules (Wixom *et al,* 1980).

In cells overloaded with iron, lysosomes accumulate large amounts of haemosiderin, which can be visualised by Prussian blue staining. Although the

iron enmeshed in this insoluble compound constitutes an end-stage product of cellular iron storage, it remains in equilibrium with soluble ferritin. Ferritin iron, in turn, is in equilibrium with iron complexed to low-molecular-weight carrier molecules. Therefore, the introduction into the cell of an effective chelator captures iron from the low-molecular-weight iron pool, draws iron out of ferritin, and eventually depletes iron from haemosiderin. As might be expected, the bioavailability of haemosiderin iron is much lower than that of iron stored in ferritin.

Cellular iron homeostasis

a) Iron responsive elements

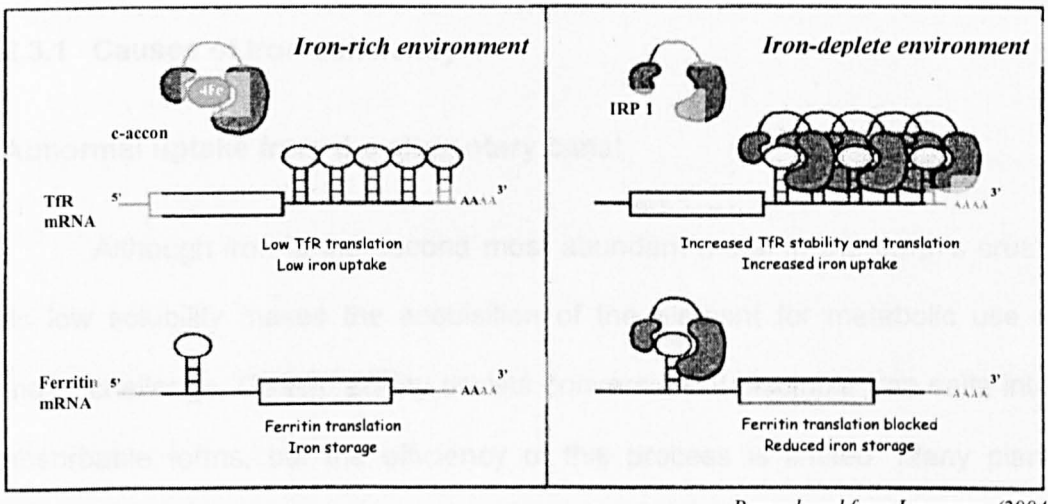
Iron responsive elements (IREs) regulate the stability and translation of a number of mRNAs that code for proteins involved in iron metabolism such as ferritin, transferrin receptor (TfR), DMT1 mitochondrial acconitase, erythroid δ-aminolaevulinic acid synthase (ALAS2), and the iron transporter ferroportin. IREs are found in the 3' or 5'-untranslated region (UTR) of these iron–regulated mRNAs. The location of the IRE, either on 3' or 5' sequence of the mRNA determines the effect of iron on protein translation. IRE function is conferred by the presence of iron regulatory proteins (IRPs) that bind to the hair-pin structures in iron-responsive mRNAs.

Under conditions of low iron availability, IRPs bind to IREs. When an IRP is bound to an IRE in the 5'-UTR of an mRNA, translation is blocked (Figure 2.6). Conversely if the IRE is localised to the 3'-UTR, IRP-binding

enhances mRNA stability and results in increased translation. The 3'-UTR of transferrin receptor mRNA contains five IREs, whereas the 5'-UTR of ferritin mRNA contains a single IRE. Binding of IRP when there are low levels of intracellular iron protects the transferrin receptor mRNA from cytoplasmic degradation but inhibits translation of ferritin mRNA by interfering with the binding of initiation factors.

In contrast, increases in intracellular iron results in the formation of an iron-sulfur cluster that binds the IRPs and prevents IRP binding to IREs. The end result is to decrease iron uptake and increase iron storage under conditions of high iron availability. ALAS2 mRNA also has an IRE in its 5'-UTR, whereas 'housekeeping' ALAS1 mRNA does not. This suggests that the IRP–IRE system may be involved in matching iron supply to haem synthesis. Mitochondrial acconitase which converts citrate to isocitrate, has an IRE on the 5'-UTR of its mRNA. In the liver, acconitase activity decreases during iron deficiency which suggests a role of citrate in iron metabolism as a possible intracellular iron carrier. DMT1 and ferroportin also have IREs on their mRNA, but binding of IRP is relatively weak as compared with the TfR and ferritin IREs (Worwood and Hoffbrand 2005).

Figure 2.6: Regulation of iron in the cell



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b) Iron regulatory proteins

There are currently two known cytosolic RNA-binding proteins that function to regulate iron-responsive mRNA, namely IRP1 and IRP2. IRP1 is a bi-functional protein. It binds to IRE in the absence of iron as previously described. In the presence of iron, the binding of the iron-sulfur cluster to IRP1 results in a protein, namely cytosolic acconitase which has a low affinity for the IRE. IRP2 can also bind to IREs and regulate mRNA stability and translation. It is expressed ubiquitously but is less abundant than IRP1 (Worwood and Hoffbrand 2005). While the sequence of IRP2 is 60% identical to IRP1, its regulation by iron is different. In the presence of high levels of intracellular iron, IRP2 is targeted for degradation by iron-mediated oxidative processes (Iwai *et al,* 1998).

2.3 IRON DEFICIENCY

2.3.1 Causes of Iron deficiency

Abnormal uptake from the alimentary canal

Although iron is the second most abundant metal in the earth's crust, its low solubility makes the acquisition of the element for metabolic use a major challenge. Gastric acidity assists conversion of insoluble iron salts into absorbable forms, but the efficiency of this process is limited. Many plant products contain iron, but absorption frequently is limited both by low solubility and powerful chelators that bind ambient iron (Layrisse *et al,* 1974). The phytates (organic polyphosphates) found in cereal products bind iron with tremendous avidity. Haem, which is primarily derived from animal tissue, is the most readily absorbed form of iron and its uptake occurs independently of gastric pH (Bjorn-Rasmussen *et al,* 1974). Haem absorption, like the uptake of non-haem is increased in patients with high erythroid activity. The scarcity of dietary meat for much of the sub-Saharan population makes iron deficiency one of the most common causes of anaemia. The fact that cultivated cereals, such as maize, are dietary staples for many people in this region, only exacerbates the problem as these plants are a very poor source of iron. On a lesser magnitude of importance, a few metals share the iron absorption mechanisms in the duodenum, such as lead, cobalt and strontium. Competition studies have shown that increased iron absorption also enhances the up-take of these metals. Likewise, increased levels of these metals,

specifically lead, in the diet, can inhibit iron absorption leading iron deficiency (Kwong *et al,* 2004).

Abnormal iron uptake may result from functional disruption in the enteric mucosa and invasion of the submucosa by inflammatory cells thereby hampering iron absorption. Enteropathy of bacterial or viral aetiology resulting in diarrhoea, is common in children in developing countries. Zinc deficiency, manifesting clinically with chronic diarrhoea (Giorgi *et al,* 1984) and iron deficiency often co-exists (Ece *et al,* 1997). Jejunal abnormalities including villous atrophy with crypt hyperplasia and increased numbers of intra-epithelial lymphocytes associated with chronic diarrhoea have been reported in patients with HIV infection (Kotler *et al,* 1984). Hence, in areas where poverty, poor nutrition and a high rate of communicable diseases exist, as in most developing countries, there is a complex interaction of factors that may lead to abnormal iron uptake in the gut.

Blood loss

The enteric parasitic infections, *Ancylostoma* and *Necator* (hookworm) species, *Schistosoma* species, and *Trichuris trichuria* (whipworm) cause blood loss that results in iron deficiency. These infections are highly prevalent in children in sub-Saharan Africa. Studies in Africa have reported hookworm prevalence between 30% to 95% (Stoltzfus *et al,* 1997; Thiong'o *et al,* 2001; Beasley *et al,* 2002). It is more common among children living in rural compared to urban areas (Walsh *et al,* 2000). The magnitude of blood loss is

directly related to the number of adult worms residing in the intestine (Roche and Layrisse 1966). It is estimated that *N. americanus* and *A. duodenale* cause on average 0.03 ml and 0.15 ml per worm per day respectively (Stephenson 2002) and this is sometimes an important cause of severe anaemia in children (Nkhoma *et al,* 2005). Although it is common to find children infected with both species of hookworm, children with a higher proportion of *A. duodenale* eggs experience more severe iron deficiency and anaemia (Albonico *et al,* 1998).

The prevalence of *T. trichuria* in Africa is estimated to range between 7% to 22% (Thiong'o *et al,* 2001; Shapiro *et al,* 2005). Although blood loss is less in children infected with this parasite (0.005ml/worm/day), *T. trichuris* nevertheless contributes to the development of severe anaemia especially in the presence of hookworm, malaria and/or a low dietary iron intake (Stephenson 2002).

2.3.2 Development of iron deficiency

Depletion of iron stores

The first event to occur when the body is in a state of negative iron balance is the depletion of iron stores. Haemosiderin and ferritin which are in equilibrium with intracellular iron are slowly depleted. Iron absorption in the duodenum is increased. At this stage, biochemical measures of iron stores such as ferritin (in the absence of malaria and inflammation) will decrease, but

the haemoglobin (Hb) level will remain normal. This stage is infrequently detected.

Iron deficiency erythropoiesis

Further depletion of body iron leads to a decrease of ferritin below normal levels and a decrease in transferrin saturation. This leads to the development of iron-deficient erythropoiesis which is signified by the rising concentration of soluble transferrin receptor (sTfR) and erythroprotopophyrin (EP). Hb, mean cell volume (MCV) and mean cell haemoglobin (MCH) may still be within the reference range. In the bone marrow the number of sideroblasts (erythroblasts containing cytoplasmic iron granules) is reduced.

Iron deficiency anaemia

If further depletion of iron occurs, frank iron deficiency anaemia develops. At this stage the MCV and MCH are reduced and target cells may be present. There is obvious microcytosis and hypochromasia of red cells and poikilocytosis is more marked. The plasma membranes of iron deficient red cells are abnormally stiff (Tillmann and Schroter 1980). This rigidity may contribute to poikilocytic changes. These small, stiff cells are cleared by the reticuloendothelial system, contributing to the low-grade haemolysis that often accompanies iron deficiency. The cause of this alteration in membrane fluidity is unknown.

Unexplained thrombocytosis may occur in iron deficiency anaemia (Perlman *et al,* 2002). Thrombopoietin, the molecule that stimulates the growth of megakaryocytes and the production of platelets, is structurally homologous to erythropoietin. The high levels of erythropoietin produced in iron deficiency anaemia conceivably could cross-react with megakaryocyte thrombopoietin receptors, modestly raising the platelet count (Bilic and Bilic 2003).

At this stage of development of iron deficiency, the number of sideroblasts in the bone marrow are almost entirely absent. The bone marrow macrophages show absence of iron in their cytoplasm, except where very rapid blood loss outstrips the ability to mobilise the storage iron.

2.3.3 Consequences of iron deficiency

Anaemia

Erythrocyte production is among the first casualties of iron deficiency because iron is mostly directed to haemoglobin synthesis. The anaemia impairs tissue oxygen, producing weakness, fatigue, palpitations and light-headedness.

Epithelial changes

Iron deficiency produces significant gastrointestinal tract abnormalities, reflecting the enormous proliferative capacity of this organ. Some patients develop angular stomatitis and glossitis with painful swelling of the tongue.

The flattened, atrophic lingual papillae make the tongue smooth and shiny. A rare complication of iron deficiency is the Plummer-Vinson syndrome with the formation of a post-cricoid oesophageal web. Long-standing, severe iron deficiency affects the cells that generate the fingernails, producing koilonychia (Worwood and Hoffbrand 2005).

Growth and developmental retardation

Iron deficiency, with or without concomitant anaemia, commonly impairs growth and intellectual development in children. Grantham-McGregor and Ani (2001) have reviewed studies on the effect of iron deficiency on children's cognition and behaviour with the aim of looking for causal evidence. They found that most studies showed associations between iron deficiency anaemia and poor cognitive and motor development and behavioural problems. The longitudinal studies reviewed, consistently indicate that children who are anaemic in infancy continue to have poor cognition, school achievement and more behavioural problems into their middle childhood. However, in all these studies, poor socioeconomic status, an important confounder which limits causal inferences from being made, was not adequately taken into account. Additionally, the effect of iron deficiency on child development is often difficult to separate from overall nutritional deficiency as they often occur simultaneously in poor environments.

The mechanism by which iron deficiency impairs neurological function is unknown. Many enzymes in the neural tissue require iron for normal

function (Beard *et al,* 1994). The cytochromes involved in energy production predominantly are haem proteins. Studies investigating auditory brain stem responses have shown that central conduction time, an indicator of CNS development, was prolonged in children with anaemia as compared to those without (Roncagliolo *et al,* 1998).

2.4 METHODS OF ASSESSING IRON STATUS

Clinical assessment

Clinical assessment of pallor is an important screening method in resource poor countries. For clinically detecting moderate anaemia (Hb 5–8g/dl) in children, sensitivity ranging between 66 – 90% has been reported (Luby *et al,* 1995; Zucker *et al,* 1997). More importantly, the use of clinical pallor assessment for the diagnosis of severe anaemia (Hb <5g/dl) has been associated with sensitivity as high as 93% and a specificity of 85% (Luby *et al,* 1995; Weber *et al,* 1997). In young children, palm pallor is preferred to eyelid pallor as a diagnostic sign, due to the frequency of conjunctivitis which causes redness even in anaemic subjects. In resource-poor settings, where the laboratory haemoglobin tests may be unreliable, clinical assessment of pallor is used to guide blood transfusion practice (Bates *et al,* 2001).

Haemoglobin colour scale

The WHO Hb colour scale has been developed as a simple, inexpensive clinical device for diagnosing anaemia when laboratory based Hb

tests are unavailable. The spectral characteristics of the colour produced by a drop of blood on a tests strip from different Hb standards and identified by a computerised analytical spectrophotometer, were printed on a paper strip. Studies comparing the WHO colour scale to other laboratory Hb determination tests have found the scale to have a sensitivity of 96% and specificity of 86% (Lewis *et al,* 1998; Ingram and Lewis 2000; Montresor *et al,* 2003). Although the diagnostic accuracy of health workers using the scale varies widely, studies have shown that health workers' capacity to identify severely anaemic patients who need referral in settings of no laboratory support, is greatly improved (Montresor *et al,* 2003). Most of the errors or discrepancies have been shown to arise from incorrect use of the scale, and the accuracy improves dramatically when the tests are repeated under supervision (Ingram and Lewis 2000).

Haemoglobin and haematocrit measurement

The Hb and haematocrit (Hct) are the most widely used tests to screen for anaemia and iron deficiency. The concentration of Hb is most reliably measured after accurate dilution of a blood specimen in a solution that converts haemoglobin to cyanmethaemoglobin, which is then quantitated spectrophotometrically. The HemoCue™ system is uniquely suited for rapid field surveys because of the easy one-step blood collection and Hb determination. It gives a satisfactory accurate and precise measurement when evaluated against standard laboratory methods (Johns and Lewis 1989).

Haematocrit is often measured by centrifugation of a minute amount of blood that has been collected in a heparinized capillary tube. The volume of packed cells as a portion of the total volume of blood is measured. A potential source of error in Hb and Hct determination arises from an inadequate technique in obtaining capillary blood. Care must be taken to ensure adequate puncture of the tissue and spontaneous blood flow from the wound. It must be borne in mind that haemoglobin or haematocrit measurement is merely a proxy for iron status and may be less reliable in areas where other causes of anaemia are highly prevalent.

Red cell indices

Among all the red cell indices measured by electronic counters MCV, MCH and mean cell haemoglobin concentration (MCHC) are sensitive indices for determination of iron deficiency. MCV is highly reproducible and is actually less subject to sampling error in skin puncture blood than haemoglobin because dilution by tissue fluid does not affect the red cell size. A low MCV, less than 67fl for 1-2 year olds and 73fl for 2-5 year olds (WHO 2001), favours iron deficiency but may also be characteristic of thalassaemia minor and is sometimes found in the anaemia of inflammation (Bain 1995).

Ferritin

Serum ferritin is considered the most specific biochemical test that correlates with total body iron stores. Low ferritin reflects depleted iron stores which is a pre-condition for iron deficiency in the absence of infection. Serum

ferritin is an acute phase protein and is therefore elevated in response to any infectious and inflammatory process. Consequently, ferritin in the normal range reflects only iron sufficiency in the absence of these conditions. Interpretation of ferritin levels is therefore problematic in populations in which the incidence of infection or inflammation is high. The generally accepted cut-off level for ferritin, below which iron stores are considered to be depleted, is <12µg/l and <30µg/l in presence of infection (WHO 2001).

Transferrin Saturation

Iron deficiency results in a reduction of serum iron level, an elevation in transferrin (total iron-binding capacity [TIBC]) level, hence a net reduction in transferrin saturation (iron/TIBC). A disadvantage of the use of serum iron in diagnosing iron deficiency, is its large biologic variability (Tietz *et al,* 1994). One component of this variability is a pronounced diurnal fluctuation, usually with high values in the morning and low levels at night. Secondly serum iron levels are altered during the acute phase response to infection or inflammation. TIBC is less subject to biological variation and levels greater than 400 µg/dl suggest iron deficiency, where as values less than 200 µg/dl characterise inflammation (Panagiotou and Douros 2004).

Erythrocyte Protopophyrin

There is an accumulation of protopophyrin in red blood cells when insufficient iron is available to combine with it to form haem. In general, elevated erythrocyte protopophyrin correlates well with low serum ferritin, and

can serve as a screen for moderate iron deficiency without anaemia (Yip *et al,* 1983). Another advantage of erythrocyte protopophyrin is that it is unaffected by recent iron medication unlike serum iron and ferritin (Thomas *et al,* 1977).

Iron deficiency, due to the shared mechanism for intestinal absorption with lead, can predispose to lead toxicity. Findings of marked elevations in erythrocyte protopophyrin are most likely due to lead exposure, but moderate increases may be associated with iron deficiency, lead exposure or both (Yip *et al,* 1981). In addition two other commonly encountered conditions can cause significant elevations of erythrocyte protopophyrin, namely infection or inflammation and haemolytic anaemia such as caused by malaria. Therefore, erythrocyte protopophyrin is most useful in non-tropical settings where these conditions are les common (WHO 2001).

Soluble transferrin receptor

Soluble transferrin receptor (sTfR) is a truncated monomer of membrane-bound transferrin receptor (mTfR) which circulates in the form of a complex of transferrin and its receptor. The erythroblasts rather than reticulocytes are the main source of sTfR (R'Zik and Beguin 2001). The bulk of sTfR measured in serum has been shown to be proportional to the mass of mTfR (R'Zik *et al,* 2001). The total mass of mTfR depends both on number of erythroid precursors in the bone marrow (a function of marrow erythropoietic activity), and on the number of mTfR per cell (a function of iron status of the

cell). Iron deficiency elevates sTfR considerably (Beguin *et al,* 1988). Investigators found that sTfR measurements were not adversely affected by the presence of inflammation or malaria (Semba *et al,* 2000; Asobayire *et al,* 2001), however other studies have challenged these findings (Menendez *et al,* 1997; van den Broek *et al,* 1998; Verhoef *et al,* 2001). Nevertheless, even under the presumption of sTfR being a reliable marker of cellular iron deficiency, the interpretation of an individual value may be complex in a patient who has both changes in erythropoietic activity and iron status occurring simultaneously (Mast *et al,* 1998).

There are currently also no reference values for sTfR measurements due to lack of international standardisation. Attempts have been made to develop regression-based reference values, although various commercial assays give disparate values (Suominen *et al,* 2001).

Bone marrow examination

Bone marrow iron assessment is said to be the 'gold standard' test for diagnosis of iron deficiency. Bone marrow aspirates or biopsies are stained with Prussian blue or Perl's stain. Potassium hexacyanoferate reacts with ferric iron to form an insoluble complex that is easily visualised under a microscope. Although this test has been the 'gold standard' for close to 100 years, recent studies have questioned its validity (Barron *et al,* 2001). It has been shown to give high false negative results. This may be due to the uneven distribution of iron in the bone marrow (Bain 2001). The test, which is

semi-quantitative, has also been associated with a high inter-individual variability (Barron *et al,* 2001). Alternative techniques under study include the use of flow cytometry to quantitate mTfR expression on erythroblasts and mass spectrometry measurements of the total elemental iron in the bone marrow.

2.5 MECHANISMS OF INFLAMMATION

Inflammation is a complex highly orchestrated process involving many cell types and molecules, some of which initiate, amplify, or sustain the process, some of which attenuate it, and some of which cause it to resolve. A number of participating molecules are multi-functional and contribute to the dynamics of the inflammatory response at different stages. Changes in the concentrations of acute-phase proteins occur largely due to changes in their production by hepatocytes. Conditions that commonly lead to substantial changes in plasma concentrations of these proteins include bacterial and viral infections, trauma, surgery, tissue infarction, and various immunologically mediated and crystal-induced inflammatory conditions (Kushner 1982).

Cytokines, intracellular signalling polypeptides produced by activated cells are the chief stimulators for the production of acute-phase proteins. These inflammation-associated cytokines include IL-6, IL-1β, tumour necrosis factor α (TNFα), interferon-γ, transforming growth factor β (TGFβ), and IL-8 (Wigmore *et al,* 1997). They are produced by a variety of cell types, but the most important sources are macrophages and monocytes at inflammatory

sites. Although IL-6 is the chief stimulator, its production is dependent on the nature, or site of the inflammatory stimulus (Fattori *et al,* 1994).

Combinations of cytokines have been found to have additive, inhibitory, or synergistic effects. IL-6 enhances the effect of IL-1β in inducing the expression of IL-1–receptor antagonist (Gabay *et al,* 1997), and IL-4 inhibits the induction of some acute-phase proteins by other cytokines (Loyer *et al,* 1993). Soluble IL-6 receptor α molecules increase the effects of the ligand (Mackiewicz *et al,* 1992), whereas other soluble receptors, such as those for TNFα and IL-1, are inhibitory. The clinical symptoms that often accompany inflammation are similarly induced by cytokines, and include anorexia, somnolence, and lethargy.

The acute phase response represents the process whereby new 'set points' are substituted for those which normally maintain internal stability. It is presumed to play a major adaptive and defensive role in the immune response. The assumption that changes in the plasma concentrations of acute-phase proteins are beneficial is based largely on the known functional capabilities of the proteins and on speculation on how these might serve useful purposes in inflammation, healing, or adaptation to noxious stimuli (Trey and Kushner 1995). A number of components of the acute phase response, such as fever, hypercortisolaemia and hypoferraemia, are considered to provide a systemic environment more suited to the defensive and adaptive requirements of coping with significant tissue injury or infection. Fever plays significant roles in host defence (Kluger 1992) and has been

reported to amplify the emergence of T-cell immunity (Hansen *et al,* 1993), while somnolence leads to reduced energy demands. Decreases in serum iron and zinc are thought to have beneficial effects in defence against bacterial or viral infection (Rogers 1992) and in tissue repair. However, the value to the host of decreased erythropoiesis and anaemia which is be discussed in later sections, is less clear. It is possible that certain changes which occur during the acute phase response serve no useful purpose, but may merely represent adverse components of the inflammatory state.

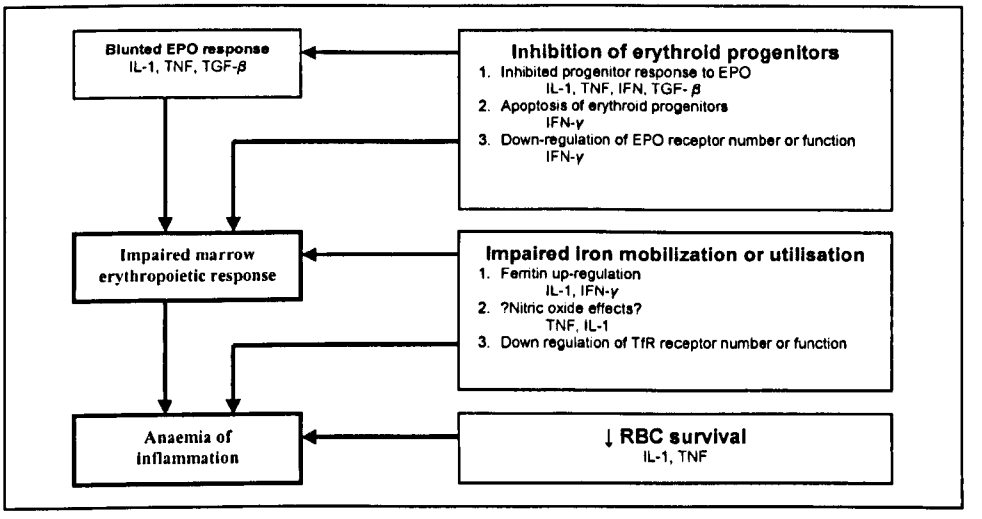
2.6 ALTERED IRON METABOLISM IN ANAEMIA OF INFLAMMATION

The anaemia of inflammation (AI) is an acquired condition that is commonly observed in the clinical settings of infection, arthritis, malignancy, trauma and organ failure. Initially the anaemia is mild, characterised primarily by a decrease in the number of red blood cells. Over time, the anaemia may become more severe, with hypochromic, microcytic erythrocytes (Bain 1995). Although the anaemia increases the morbidity of the underlying illness, it probably results from a host-defense mechanism designed to sequester iron from the invading pathogens.

The clinical manifestations of AI include depressed serum iron levels, in spite of adequate iron stores, decreased TIBC, and increased ferritin. Often, iron-laden macrophages can be observed in bone marrow specimens (Bain 2001). Intestinal absorption of iron is impaired, which will eventually lead to true iron deficiency.

There are three major processes involved in the pathogenesis of AI. Firstly, there is a modest (<10%) shortening of red cell survival which creates a demand for a slight increase in bone marrow red cell production. The bone marrow cannot respond adequately due to impaired erythropoiesis and impaired mobilisation of iron from the reticuloendothelial cells (Means 2004). The impairment of erythropoiesis, in turn, results from two processes; blunting of the expected increment in erythropoietin (EPO) production in response to the degree of anaemia, and a decreased response of the erythroid progenitors to EPO. It is reported that the blunting of bone marrow response to EPO may be mediated by IL-1, TNFα and TGFβ which affect EPO mRNA transcription (Hochberg *et al,* 1988). It has also been demonstrated that IL-1, which is increased during inflammation, inhibits colony forming units–erythryoid (CFU-E) which later develop into erythroblasts (Means and Krantz 1993) (Figure 2.7).

Figure 2.7: Contributions of cytokines to the pathogenesis of AI



Reproduced from Means (2004)

The impaired iron mobilization in AI has been attributed to the effects of inflammatory cytokines. A correlation between the immune activation marker neopterin and increasing ferritin levels in patients with malignancies has been reported, suggesting a role for immune activation in the altered iron metabolism of AI (Denz *et al,* 1990). Other investigators have reported that rodents injected with recombinant TNF develop a hypoferraemic anaemia associated with impaired reticuloendothelial iron release and reduced incorporation into erythrocytes (Alvarez-Hernandez *et al,* 1989). IL-1 increases translation of ferritin mRNA, and it has been proposed that this additional ferritin acts as a trap for iron that might otherwise be available for erythropoiesis (Rogers *et al,* 1994). Nitric oxide has similar effects on ferritin translation and also interferes with translation of transferrin receptor mRNA (Recalcati *et al,* 1998). In addition, inflammatory effects on cellular iron uptake may also directly inhibit erythroid differentiation and proliferation. The acute

phase reacting protein α-antitrypsin appears to inhibit erythropoiesis by impairing transferrin binding to its receptor, and subsequent internalisation of the transferrin-transferrin receptor complex by erythroid cells (Peetre *et al,* 1986). These mechanisms are summarised in Table 2.2.

Table 2.2: Proposed mechanisms of cytokine involvement in iron metabolism in AI

Proposed mechanism	Cytokines implicated
Up-regulation of ferritin message	IL-1, IFN-γ
Nitric oxide induction (IRP/IRE)	TNF, IL-1
Down-regulation of erythroid TfR receptor number/function	Unknown
Hepcidin induction	IL-6, IL-1 via IL-6

During studies of the antimicrobial properties of various human body fluids, Park, Valore *et al* (2001) isolated a new peptide from human urine and named it hepcidin, based on its site of synthesis (the liver, hep-) and antibacterial properties in vitro (-cidin). It is postulated that hepcidin is the key mediator of AI. Hepcidin seems to be a multi-functional molecule. It fits the criteria for an iron stores regulator, erythropoietic regulator and the mediator of iron mucosal block in the gut. It appears to be the major contributor to the hypoferraemia associated with inflammation. Hepcidin regulation is influenced by a number of factors namely, body iron status, infection, inflammation, anaemia and hypoxia (Ganz 2003).

It has been shown that hepcidin is the dominant regulator of dietary iron absorption and release of iron from macrophages (Fleming and Sly

2001). During infection and inflammation, IL-6 is produced which is the main stimulant of hepcidin production in hepatocytes. Pathogenic-specific molecules such as lipopolysaccharide, probably acting on macrophages and Kupffer cells, induce the production of IL-6 which in turn induces the production of hepcidin mRNA in hepatocytes (Nemeth *et al,* 2003). Hepcidin is decreased in response to anaemia and hypoxia. These stimuli decrease hepcidin production and remove the inhibitory effect on iron absorption and iron release from macrophages so that more iron is available for compensatory erythropoiesis (Nicolas *et al,* 2002). Increased EPO levels correlate with decreased hepcidin levels but a direct relationship has not been shown.

2.7 IRON-UPTAKE IN PLASMODIA SPECIES

There is very little known on how protozoan parasites obtain their iron *in vivo*. Although *Plasmodium falciparum* parasites invade and replicate in highly iron-rich cells, the erythrocytes, it appears that this iron may not be readily available to them. Ball *et al* (1948) demonstrated many years ago that in parasitised erythrocytes the haemoglobin continues to be degraded, but the haem concentration remains virtually unchanged. Additionally, hemozoin, the material generated by the parasite during degradation of haemoglobin, does not stain with Prussian blue, suggesting that the haem has not been broken down to release iron. No parasites that degrade haem have been identified (Rodriguez and Jungery 1986). Therefore, it is unclear how *P. falciparum*

obtains its iron. The possibilities include: (i) iron obtained from serum either using a transferrin-related or transferrin-independent system (ii) erythrocyte ferritin; (iii) haemoglobin; (iv) a pool of labile iron within the parasitised erythrocyte.

Serum iron as a source of iron

Early studies showing a protective effect of iron deficiency against human malaria (Murray *et al,* 1978; Oppenheimer *et al,* 1986), were consistent with the possibility that plasma transferrin may be a source of iron for the intra-erythrocytic parasite. This hypothesis was further strengthened when a parasite-derived molecule was identified which appeared to function as a transferrin receptor (Rodriguez and Jungery 1986). However, it was later demonstrated that no transferrin receptors existed on parasitised erythrocytes (Pollack and Schnelle 1988). The possibility that iron non-specifically bound to transferrin is taken up from plasma into parasitised erythrocytes has been proposed (Pollack and Schnelle 1988), but the bulk of evidence does not support this mechanism (Peto and Thompson 1986).

Erythrocyte ferritin as a source of iron

Although the mature erythrocyte cannot synthesize ferritin, it does contain residual ferritin produced during the earlier erythroblast phase (Cazzola *et al,* 1983). The acquisition of iron by the parasite from ferritin present in the cytoplasm of the erythrocyte is a theoretical possibility but remains uninvestigated. The fact that iron deficiency is associated with low

red blood cell ferritin concentrations (Cazzola *et al,* 1983), without inhibition of parasite growth (Hershko and Peto 1988), may be regarded as evidence against ferritin as an iron source. Nevertheless, red cells in iron deficient subjects do contain detectable amounts of ferritin (Cazzola *et al,* 1983), and the possibility remains that the parasite utilises iron from ferritin.

Host haemoglobin as a source of iron

Host haemoglobin does not seem to be a likely source of iron for the parasite. Haem oxygenase activity has not been identified on most *Plasmodia* species (Gabay *et al,* 1994). The fact that haem is metabolised into hemozoin without releasing the iron (Egan *et al,* 2002), and that *P. falciparum* synthesizes its own haem (Surolia and Padmanaban 1992), is very suggestive that host haem would not be necessary for malaria parasite metabolism.

Labile intra-erythrocytic iron as a source of iron

Recent *in vitro* studies appear to confirm the longstanding theory that a small labile pool of iron in erythrocytes (which may be crucially smaller in people with iron deficiency) provides the iron that parasites require (Hershko and Peto 1988; Loyevsky *et al,* 1999). This may provide a mechanism which underlies the clinical observations suggesting that iron deficiency protects from malaria. This labile intracellular pool may also be used by other intra-cellular pathogens, such as *Mycobacterium tuberculosis* and HIV. Although little attention has been given to this labile iron, it may prove to be very

important in exacerbating infections if iron supplementation results in a transitory but significant change of this pool.

2.8 IRON-UPTAKE IN PATHOGENIC BACTERIA

Most of the body's iron is found intracellularly, in ferritin, haemosiderin or haem, while extracellular iron in serum is attached to the high affinity iron-binding transferrin proteins. Although there is normally an abundance of iron present in the body, the amount of free iron is far too small to support bacterial growth (Bullen *et al,* 1978; Bullen 1981). In addition, during infection the host reduces the total amount of iron bound to transferrin, a process called the hypoferraemia of infection (Cartwright *et al,* 1946). Although the triggering signal appears to be IL-1, the subsequent processes that lead to decrease in serum iron levels are not fully understood. In spite of all these mechanisms, pathogenic bacteria clearly multiply *in vivo* to establish an infection. Additionally since all known bacterial pathogens need iron to multiply, it has been argued that they must be able to adapt to the severely iron-restricted extracellular environment and develop alternative mechanisms for acquiring iron. Possible mechanisms for bacterial pathogens to acquire iron (Griffiths and Williams 1999) are summarised in Table 2.3.

Table 2.3: Possible mechanisms used by pathogenic bacteria for acquiring iron bound by transferrin

1. Proteolytic cleavage of transferrin, disruption of the iron-binding site and release of iron (Carlsson *et al,* 1984).
2. Reduction of Fe^{3+} complex to Fe^{2+} complex and the consequent release of Fe^{2+} from transferrin (Cowart and Foster 1985).
3. Production of low-molecular-mass iron chelating compounds, known as siderophores, that are able to remove iron from the iron-transferrin complex and to deliver it to the bacterial cell (Raymond and Carrano 1979).
4. Direct interaction between receptors on the bacterial cell surface and the iron-transferrin complex in a manner analogous to the reaction occurring between transferrin and the reticulocytes (Williams and Griffiths 1992).

There is little evidence that bacterial pathogens in general are able to degrade either transferrin or lactoferrin and thus release iron (Mechanism 1, Table 2.3), apart from some strains of *Bacteroides* species (Carlsson *et al,* 1984). It is suggested that the ability of these organisms to degrade not only transferrin but also haemopexin and haptoglobulin, may contribute to their virulence. There has been only one example to date where it has been suggested that a bacterial pathogen uses a reductant to remove iron from an iron-binding protein (Mechanism 2, Table 2.3). Gram-positive *Listeria monocytogenes* secretes a soluble reductant that effectively removes iron from the iron-transferrin complex (Cowart and Foster 1985).

Although the best understood systems used by pathogens are dependent on the production of siderophores (Mechanism 3, Table 2.3), it does not mean that they are the commonest or the most efficient. *Salmonella, Escherichia* and *Shigella* species secrete the iron-chelator enterobactin under conditions of iron restriction (Pollack and Neilands 1970; Rogers *et al,* 1977;

Perry and San Clemente 1979). Enterobactin is a high affinity iron-chelator, an example of a siderophore, which is able to efficiently extract iron from iron-transferrin complex and deliver it to the bacterial cell. Another known siderophore is aerobactin (Neilands 1995). Epidemiological data suggests that the presence of an aerobactin iron-uptake system promotes the ability of the pathogen to cause septicaemia (Williams 1979; Montgomerie *et al,* 1984), but there is not enough biological evidence to support it. In addition the reason why bacteria produce two siderophores as opposed to one which may confer an advantage, is poorly understood (de Lorenzo and Martinez 1988; Brock *et al,* 1991).

There is an increasing number of pathogens which support a siderophore-independent, receptor-mediated mechanism (Mechanism 4, Table 2.3). This mechanism is highly host specific and resembles that employed by human cells. This system is; (i) dependent on direct contact between the bacterial cell surface and the iron-transferrin complex; (ii) involves specific cell surface receptors; and (iii) exhibits a high degree of preference for the transferrin or lactoferrin of a particular pathogen's natural host. This system seems to be used by a number of important human pathogens such as *Neisseria*, *Hemophilus*, and *Pasteurella* species (Griffiths and Williams 1999).

In addition to obtaining iron from iron-binding proteins, transferrin and lactoferrin, many pathogenic bacteria can obtain iron from cell free

haemoglobin or haem for multiplication *in vivo* e.g *Escehericha*ea and *Yersinia* species (Brubaker 1991; Bullen *et al,* 1991).

2.9 IRON METABOLISM IN HIV INFECTION

2.9.1 Clinical studies

Our understanding of the interaction of iron and Human Immunodeficiency virus (HIV) comes firstly from clinical studies that raise the possibility that 'high' iron status may adversely influence the outcome of HIV infection. Costagliola *et al* (1994) studied the progression to Acquired Immunodeficiency Syndrome–related complex in thalassaemia major patients who were receiving an iron chelator, desferrioxamine. After adjusting for age and splenectomy, the rate of progression of HIV disease decreased significantly as the mean daily dose of desferrioxamine increased.

Two studies compared the use of dapsone versus pentamidine in the treatment of *Pneumocystis carinii* infection in HIV-positive subjects. It was observed in one of the studies, in which the dapsone preparation contained iron, that the CD4 counts and mortality were much higher in the group receiving dapsone compared to those receiving pentamidine (Salmon-Ceron *et al,* 1995). In the other study, where the dapsone preparation without iron was used, there was no difference in morbidity or mortality between the dapsone and pentamidine treatment groups (Bozzette *et al,* 1995). From the results of the two studies, it has been suggested that the administration of the iron may be associated with an excess mortality in HIV infected people.

Survival studies among HIV infected individuals comparing subjects with haptoglobin 2-2 polymorphism which is associated with increased iron stores, and subjects with other haptoglobin polymorphisms, showed a significantly lower median years of survival in the former group (Delanghe *et al,* 1998). Another retrospective study among HIV infected subjects, concluded that high iron stores, as determined by bone marrow iron grade, might be associated with shorter survival times in HIV infected subjects (de Monye *et al,* 1999).

These clinical studies do not provide conclusive evidence but are suggestive that increased iron status may be detrimental in HIV infection.

2.9.2 Laboratory studies

In vitro studies have provided further evidence suggestive of iron accumulation both at tissue and cellular level in HIV infected patients. Immunologically, HIV infection is characterised by progressive, quantitative and qualitative loss of CD4 T-lymphocytes, and activation of the immune system, with expansion of monocytes/macrophages and of activated CD8 T-lymphocytes (Bofill *et al,* 1996). Many of the activities of HIV target cells are iron-dependent (Pattanapanyasat *et al,* 1992), in particular, macrophages play a central role in iron metabolism as they are responsible for phagocytosis of senescent erythrocytes and for iron recycling. In HIV-infected individuals, serum measurements of iron status resemble those seen in AI, which is due to immunologically altered iron metabolism (Fuchs *et al,* 1993; Kreuzer and Rockstroh 1997).

Increased iron stores are often detectable in several tissues of HIV infected individuals, including bone marrow, brain, liver, muscle with iron accumulating in macrophages, microglia, Kupffer cells, endothelial cells, and myocytes (Boelaert *et al,* 1996). It is postulated that HIV directly alters cellular metabolism, probably by increasing the cytosolic iron pool (Savarino *et al,* 1999). Transferrin receptors (TfRs), which can provide information on intracellular iron levels, are down-regulated on T-lymphocytes in HIV infection and this effect can be modulated by iron chelation (Savarino *et al,* 1999). Additionally, it has been shown that HIV infected cells produce lower levels of citrate which is important in the alternative iron metabolism pathway (Pugliese *et al,* 2002). Lower citrate levels are associated with an increased intracellular iron pool (Haile *et al,* 1992).

Other findings suggest that free radicals are likely to contribute to HIV-associated cytopathogenicity. Iron is a well known, potential catalyst of free-radical-mediated reactions, and oxygen-derived radicals can damage lipids, proteins, and nucleic acids (Savarino *et al,* 1999). With regard to HIV, an iron-mediated oxidative stress was shown by Shatrov *et al* (1997) to be induced by HIV Tat protein. Accordingly, inhibitory effects of iron chelation were demonstrated on the viruses cytopathogenicity.

From clinical and laboratory studies there seems to be adequate evidence to suggest that iron loading in HIV infected individuals may have deleterious effects. However at the present time, there have been no studies to investigate the effect of iron supplementation on HIV infection.

2.10 PREVENTION OF IRON DEFICIENCY

Iron deficiency which is a nutritional deficiency of major public health concern, is mainly a consequence of poverty. To reduce iron deficiency, efforts should be targeted to reduce poverty, improve access to diversified diets, health services and sanitation, and promote better care and feeding practices.

2.10.1 DIETARY IMPROVEMENT

Food-based approaches represent the most desirable and sustainable method of preventing micro-nutrient malnutrition. These approaches increase micronutrient intake through the diet. A great advantage of food-based strategies is in their potential to result in multiple nutritional benefits. These benefits are both immediate, have wider-coverage and more sustainable in the long term.

Food-based approaches address initially the production, preservation, processing, marketing, and preparation of food. Secondly, they attempt to address feeding practices, such as intra-family food distribution and care for vulnerable groups. To implement these strategies effectively, knowledge of the local dietary patterns, and food beliefs, preferences and taboos is required, as well as the ability to change attitudes and practices.

For iron deficiency, efforts are directed towards promoting the availability of, and access to, iron-rich foods. Agricultural field fortification strategies (Gibson and Hotz 2001) can be used to increase the content of

certain trace elements in cereal grains by applying fertilizers to the soil and to the leaves to enhance their iron content. Similarly, focus of dietary iron improvement is upon foods which enhance the absorption or utilization of iron. These include those of animal origin, and non-animal foods such as some fruits, vegetables and tubers, that are good source of vitamin A and C, and folic acid. An effective nutritional education program also plays an important role in increasing the demand for and consumption of such foods.

Methods of food preparation and processing influence the bioavailability of iron. Cooking, fermentation, and germination can, by thermal or enzymatic action, reduce the phytic acid and hexa- and penta-inositol phosphate content. All inositol phosphates inhibit iron absorption in proportion to the total number of phosphate groups (Gibson and Hotz 2001). Processing procedures that lower the number of phosphate groups improve bioavailability of non-haem iron.

Food-based strategies are adapted to regional and local variations in diet, the age group concerned, seasonal availability and other factors that cause food intake and meal pattern to vary.

2.10.2 IRON FORTIFICATION

Iron absorption from an iron-fortified food can be considered adequate if the target population is able to maintain or improve iron status while consuming the fortified food as part of a normal diet. This is not always easy to achieve. In fact, iron is the most difficult mineral to add to foods and is also difficult to ensure adequate absorption. This is because the most soluble and absorbable iron compounds often cause unacceptable colour and flavour changes when added to foods.

Essential requirements for implementing fortification strategies include the identification of an appropriate food vehicle that reaches the target population, that is centrally processed, and that is widely available and consumed in relatively predictable amounts by vulnerable population groups. Its is essential that the final product not be significantly changed in terms of its organoleptic quality (adverse sensory changes in terms of taste or smell), shelf life, or price, and that the food as prepared be acceptable to the population.

In subsistence farming areas in most developing countries, a fortified-food approach has limited potential because few households consume commercially processed foods. Instead, a fortified food supplement can be distributed through general distribution programmes e.g. schools, other supplemental or emergency feeding programmes. Use of iron cooking pots is an innovative low-cost and sustainable strategy to provide iron in food. Studies in Malawi showed a reduction of iron deficiency in children after 6

weeks use (Geerligs *et al,* 2003). There is need for further research to improve compliance, and determination of quantity of iron assimilated to avoid excess iron accumulation.

The iron compound used to fortify a specific food should be the compound with the highest relative bioavailability that causes no organoleptic changes. The compound may be chosen from three groups of conventional compounds which differ in their solubility in water or dilute acid, or from a small group of novel compounds.

Conventional iron compounds

a) Freely water-soluble compounds

These compounds have the highest relative bioavailabilty of the conventional iron compounds. In practice, ferrous sulfate is the only water-soluble compound that is commonly added to foods. It can only be added to a small number of food vehicles, commonly infant formula and bread (el Guindi *et al,* 1988; Olivares 2002), because it readily causes sensory changes. Fat oxidation may be a problem with iron fortification of liquid milk. Ferrous sulphate and other compounds in this group have been reported to cause unacceptable colour changes in infant cereals, salt, and metallic taste in fruit drinks (Olivares 2002).

b) Compounds poorly soluble in water but soluble in dilute acid

These compounds cause less organoleptic changes than water-soluble compounds but have similar or slightly lower relative bioavailability

depending on how well they dissolve in the gastric juice during digestion. Ferrous fumarate and ferric saccharate are the preferred compounds in this group.

c) Water-insoluble compounds, also poorly insoluble in dilute acid

These are the least well absorbed and as such are the last choice for food fortification. Their main characteristic is that they dissolve slowly and incompletely in the gastric juice. Their absorption is difficult to predict because the extent to which they dissolve depends on their physical characteristics and the composition of the meal. They consist of two main types, namely the iron phosphate compounds and the elemental iron powders.

Novel iron compounds

a) Sodium iron ethylenediaminetetraacetic acid (NaFeEDTA)

Sodium iron ethylenediaminetetraacetic acid (NaFeEDTA) is a potentially valuable fortificant that has so far had limited use. The major advantage of NaFeEDTA over other compounds is that it prevents iron binding to inhibitors of iron absorption, and to phytic acid in particular. Studies have shown a two- to threefold higher absorption of iron from NaFeEDTA in meals containing considerable amounts of phytic acid, as compared to ferrous sulfate (Bothwell and MacPhail 2004). NaFeEDTA is slowly water-soluble and may cause unacceptable colour changes in some foods, but does not provoke fat oxidation in stored cereals.

Double fortification with NaFeEDTA and vitamin A and with iodide has been tried with promising results (Rao 1994; Viteri *et al,* 1995). Although there is evidence that NaFeEDTA does not inhibit bioavailability of other minerals such as zinc or calcium, further research is required to understand how it reacts with these micronutrients (Bothwell and MacPhail 2004).

b) Ferrous bisglycinate

This compound has been developed commercially and there is very little independent evaluation of its usefulness for food fortification. Its high cost, its tendency to cause unwanted colour reactions, and its tendency to provoke fat oxidation in cereal flours, make it a less suitable fortification compound for many food vehicles (Allen 2002).

c) Haemoglobin

Haemoglobin has been added to food in the form of dried bovine red blood cells. Its main advantage is that absorption is relatively high and predictable (Monsen *et al,* 1978). This is because haem iron is absorbed still bound within the porphyrin ring of the haem molecule and is naturally protected from inhibitors of iron absorption. Nevertheless, its low iron content, intense red colour, and the technical difficulties involved in its collection, drying and storage make it an unattractive iron compound for many applications (Martinez *et al,* 1998; Martinez Gracia *et al,* 2000).

d) Encapsulated iron compounds

Ferrous sulfate and ferrous fumarate are encapsulated in a coating that prevents or retards many of the organoleptic changes that are associated with these compounds. More recently they have been used in developing countries to fortify salt where the high moisture content and impurities make addition of iron compounds difficult (Zimmermann *et al,* 2004). However there is need for more bioavailability and efficacy studies to demonstrate that the capsule is removed during digestion and that the iron is adequately absorbed (Zimmermann 2004).

e) Ascorbic acid addition

Ascorbic acid can considerably increase the absorption of fortification iron. The enhancing effect is related to its reducing power and chelating action, and the magnitude of the effect depends on the amount of ascorbic acid added, the level of iron fortification, and the amount of inhibitors present in the meal. Several studies have shown that progressively increasing the ascorbic acid content progressively increases the fractional iron absorbed (Siegenberg *et al,* 1991).

The main problem with ascorbic acid, is its susceptibility to losses during storage and food preparation. Storage losses can be unacceptably high under hot and humid conditions and although sophisticated packages can largely prevent degradation, they may be too expensive for many applications (Gliguem and Birlouez-Aragon 2005).

f) Sodium EDTA

This compound is an alternative enhancer to ascorbic acid and has the advantage of being stable during storage and food preparation. Its disadvantages are that it is not an essential nutrient, although accepted as a food additive in many countries, and it has only been demonstrated to enhance the absorption of ferrous sulfate (el Guindi *et al,* 1988). The advantage of adding ferrous sulfate plus sodium EDTA rather than NaFeEDTA may relate to lesser sensory effects, cost and easier legislation.

2.10.3 IRON SUPPLEMENTATION

Although a recent study showed iron fortification to be more cost effective than iron supplementation, it remains the most common strategy currently being used to control iron deficiency in developing countries (Baltussen *et al,* 2004). This is likely to remain the case until either significant improvements are made in diets of entire populations or food fortification is achieved. It is important to differentiate between supplementation that aims at preventing anaemia by correcting iron deficiency before iron deficiency anaemia is manifest, and therapeutic supplementation, which aims at correcting established iron deficiency anaemia.

Iron supplementation, parenterally or orally, improves iron status and haemoglobin levels. The World Health Organisation recommends daily iron supplementation (2mg/kg body weight/day) in all children (WHO 2001). In areas where the prevalence of anaemia is above 40%, children aged 6-24 months should receive iron supplementation until 24 months of age, while in

areas of a prevalence of anaemia of less than 40%, children should receive iron until they are 12 months old. In all areas, children between 2 and 5 years should have iron supplements for 3 months. This 'blanket' policy may have important connotations in the light of recent evidence that iron supplementation may be associated with increased severe illness and death (Sazawal *et al,* 2006). A large population-based study in Tanzania had to be stopped after observing an increased rate of serious adverse events (death or severe morbidity leading to hospitalisation) in children receiving iron supplementation (RR 1.12, 95% CI 1.02–1.23, *p=0.02*) (Sazawal *et al,* 2006). This effect may be greater in HIV infected children where 'high' iron status has been shown to be deleterious (Gordeuk *et al,* 2001).

Impact of iron supplementation on infection

There is an unresolved concern related to the interaction between iron status, iron supplementation and susceptibility to infection. One school of thought is that iron deficiency is protective, so-called 'nutritional immunity' hypothesis, and the other is that iron deficiency increases susceptibility to infection, possibly by impairing the body's immunity. Evidence of this interaction shall be briefly reviewed with particular reference to two major reviews by Oppenheimer (2001), and Gera Sachdev (2002).

Observational studies on iron deficiency and infection

It is a challenge to study iron deficiency and its effects on infection in humans using observational studies. Iron deficiency is closely related to poor

nutrition, low socioeconomic status and poverty, making it difficult to control for these factors. It is also unethical to study people known to be iron deficient for prolonged periods while withholding treatment. The prevalence of α-thalassaemia in sub-Saharan Africa is high and the mutation causes anaemia and protects against malaria and other infections, making it an important confounder in iron studies, which is frequently not controlled for in analysis (Mockenhaupt *et al,* 2004; van Rheenen and Brabin 2004). Use of biochemical iron markers as a surrogate for iron status in infectious morbidity studies is clearly another major source of bias. For these reasons evidence from these studies is less conclusve.

Probably the most quoted study is by Murray *et al* (1978) who noted that nomads in Somalia entering a feeding camp had no infections if they were iron deficient as compared to those with normal iron status who had a high rate of infection. Masawe *et al* (1974) also reported fewer bacterial infections in patients admitted with simple iron deficiency anaemia as compared to an inpatient group with a variety of other causes of anaemia. However, they also reported a higher prevalence of malaria in the iron deficient patients. In general, these early observational studies supported the hypothesis that iron deficiency was predominantly protective against infection except with malaria.

Intervention studies on iron deficiency and infection

There have been many iron supplementation studies carried out in malaria endemic areas. It must be noted that the study designs, study

populations, main outcomes and evaluation of these outcomes, differed considerably between studies. Most studies have reported high losses to follow-up which may lead to observational bias. Some important aspects to be considered when evaluating iron supplementation trials that have been carried out are discussed in the following sections.

Use of parenteral iron

Early studies in New Zealand on parenteral iron administration in neonates, were associated with increased incidence rates of sepsis (Forman and Stiehm 1969; Barry and Reeve 1977). This was the first indication that iron supplements may increase an individuals susceptibility to infection. A large placebo-controlled trial on parenteral iron supplementation in infancy was then carried out in Papua New Guinea (Oppenheimer *et al,* 1986; Oppenheimer *et al,* 1986). Although there was no difference in infectious morbidity rates at one week follow-up post injection, at the six month follow-up clinical malaria, severe lower respiratory infection, measles and acute otitis media were more prevalent in the iron treated group. In contrast to intervention studies using oral iron, parenteral iron as a route for community-based iron supplementation programs has been less studied. New iron preparations, namely iron gluconate and iron sucrose that have fewer side effects than iron dextran preparations, remain to be investigated for their potential use in areas with high infection transmission.

Effect of age and malarial immunity

The timing of oral iron supplementation may be important, particularly in relation to risk of malarial infection. It has been reported from studies in Tanzanian children that if infants received a small dose of oral iron between the age of 4 and 6 months, a time of rapid haemoglobin synthesis, there may be no effect on malaria morbidity (Menendez *et al,* 1997). The Tanzanian study observed that iron supplementation in infants had no effect on the frequency of malaria episodes (0.87 vs. 1.00 cases per person-year, protective efficacy 12.8%, 95% CI -12.8%–32.5%). It is considered that any imbalance in body iron compartments resulting from iron supplementation would be confined to the period of passively acquired maternal immunological protection limiting malaria parasitaemia in early infancy. The Tanzanian results have been contrasted with those from a study in The Gambia where children older than 6 months were supplemented with relatively high dose iron during a period of maximal malarial transmission (Smith *et al,* 1989). Clinical malaria was more frequent in the iron supplemented group (1.3 vs. 0 episodes of fever with >50 positive high powered field (hpf)/100 hpf , $p < 0.01$).

Selective anaemia intervention or community-based supplementation

Most studies included in reviews of the effect of iron supplementation on infection, have involved a selected anaemic group (Murray *et al,* 1978; Smith *et al,* 1989), and comparison with an un-selected community-based cohort (Oppenheimer *et al,* 1986; Menendez *et al,* 1997). In these studies, which are effectively anaemia treatment trials, individuals with normal

haemoglobin levels are excluded. This raises questions on how generalisable these findings are to an unselected community and whether such studies should be pooled in a meta-analysis.

Common haemoglobinopathies

Some haemoglobinopathies are important confounders as they provide significant protection against clinical malaria, as well as some other infectious diseases (Allen *et al,* 1997). It was noted that susceptibility to malaria was reduced in children with α-thalassaemia compared to normal children in Papua New Guinea (Oppenheimer *et al,* 1987). A similar effect is well described in individuals with sickle cell trait (Flint *et al,* 1998).

Meta-analyses on effect of iron supplementation on infection

Gera and Sachdev (2002) report a detailed meta-analysis on the effect of iron supplementation on the incidence of infectious diseases in children. They evaluated 28 studies using standard techniques of data collection and extraction. They considered iron interventions in the form of oral or parenteral supplementation, or fortified formula milk or cereals. It must be noted that these iron interventions may individually have differing bioavailability levels, as well as differing ways of interacting with infection. The pooled estimate of the incidence rate ratio for infection (iron vs. placebo) was 1.02 [95% CI 0.96 – 1.08], but studies from malaria endemic areas, where iron status markers may be altered by high rates of infection, were pooled together with studies from non-malaria endemic areas. Therefore, their conclusions that the incidence of

malaria, or other infections, was not influenced by iron supplementation may not be conclusive.

Oppenheimer (2001) reviewed 11 studies in children and adults from malaria endemic areas, 7 studies which were included in the review by Gera and Sachdev (2002). He investigated in detail the importance of the different confounding factors that influence evaluation of iron supplementation studies such as age, route of intervention, common haemoglobinopathies, despite being unable to control for them in his analysis. Due to the diverse nature of study populations, locations and form of interventions, pooled analyses were not considered and individual study results were compared. A significant increase in rates of clinical malaria was found in 4 out of 9 studies (ranging from OR 1.6 [95% CI 1.1 – 2.4] to 14.8 [95% CI 1.9 – 116.6]) and no rate reductions were observed in the remaining studies. A significant increase in the rate of clinical pneumonia was observed in 2 out of 5 studies with no rate reductions (OR 1.5, 95% CI 1.0 – 2.3 and RR 5.2, *p=0.004*). It must be noted that definition of clinical pneumonia varied in the studies reviewed. Contrary the meta-analysis by Peto and Thompson (1986) where iron supplementation was associated with an increased risk of developing diarrhoea (RR 1.1, 95% CI 1.0 – 1.2), Oppenheimer (2001) found no evidence to support this finding. Oppenheimer concluded that because no useful effect of iron supplementation on overall infectious morbidity could be shown and negative effects were shown, these effects have to be balanced in a risk-benefit analysis against any known benefits of iron supplementation in individual populations.

A recent study published after the two meta-analyses mentioned above but worth reviewing was by Sazawal *et al* (2006). This randomised controlled trial where 24 076 children were followed up for a year, had to be stopped after observing an increased rate of serious adverse events (RR 1.12, 95% CI 1.02–1.23, *p=0.02*) and hospital admissions (RR 1.11, 95% CI 1.01–1.23, *p=0.03*) in children receiving iron supplementation. However, due to the premature stopping of the trial, it was poorly powered to analyse cause-specific morbidity.

CHAPTER 3

STUDY METHODS

3.1 STUDY DESIGN

This descriptive study titled '*Assessment of iron deficiency in Malawian children living in an area of high malaria and bacterial infection morbidity*' was nested within a larger study titled 'Severe anaemia in Malawian children: a detailed investigation into the aetiology, pathogenesis and long term outcome'. This large study referred to as the 'Severe Anaemia study', had two main study designs. It was a case-control study of children with severe anaemia and their controls, who were then later followed up as a cohort study. The descriptive iron study presented in this thesis involves only data collected at recruitment of children in the case-control study. The background and aims of the Severe Anaemia study are outlined in the following section.

3.1.1 Background to Severe Anaemia Study

Severe anaemia is a major cause of morbidity and mortality in African children (Lackritz *et al,* 1992; Slutsker *et al,* 1994). An improved understanding of the pathological processes leading to severe anaemia is needed in order to develop more effective management and preventive strategies.

Studies on childhood severe anaemia in Africa have previously focussed on prevention through early interventions with iron and/or antimalarials (Menendez *et al,* 1997; Korenromp *et al,* 2004). Few studies

have tried to unravel the complex pathogenesis of severe anaemia through detailed investigations. The presence of dyserythropoeisis in bone marrow, iron deficiency, and a lack of evidence for folic acid or Vitamin B12 deficiencies have been the main reported findings (Abdalla *et al,* 1984; Phillips *et al,* 1986; Abdalla 1990; Newton *et al,* 1997). At the time of these studies, only limited tools were available for investigation of bone marrow dysfunction. The development of new diagnostic techniques has greatly improved our capacity to study haematopoiesis in detail which has resulted in an improved understanding of the pathogenesis (Menendez *et al,* 2000; Provan and Weatherall 2000). An important recent finding has been the central role of the cytokine network in pathogenesis associated with infection and inflammatory disease (Selleri *et al,* 1996; Voulgari *et al,* 1999; Spivak 2000).

3.1.2 Main aims of Severe Anaemia Study

To investigate in Malawian children with severe anaemia:

1. Aetiological risk factors associated with progression from mild to severe anaemia. *Hypothesis*: severe anaemia is not just one part of the anaemia spectrum, but has a distinctive pattern of causes.
2. The contribution of the bone marrow inflammatory cytokine network to the pathogenesis of severe anaemia, and its relation to genetic determinants.
3. The natural history of severe anaemia through long term follow up after a documented episode. *Hypothesis*: children have differential

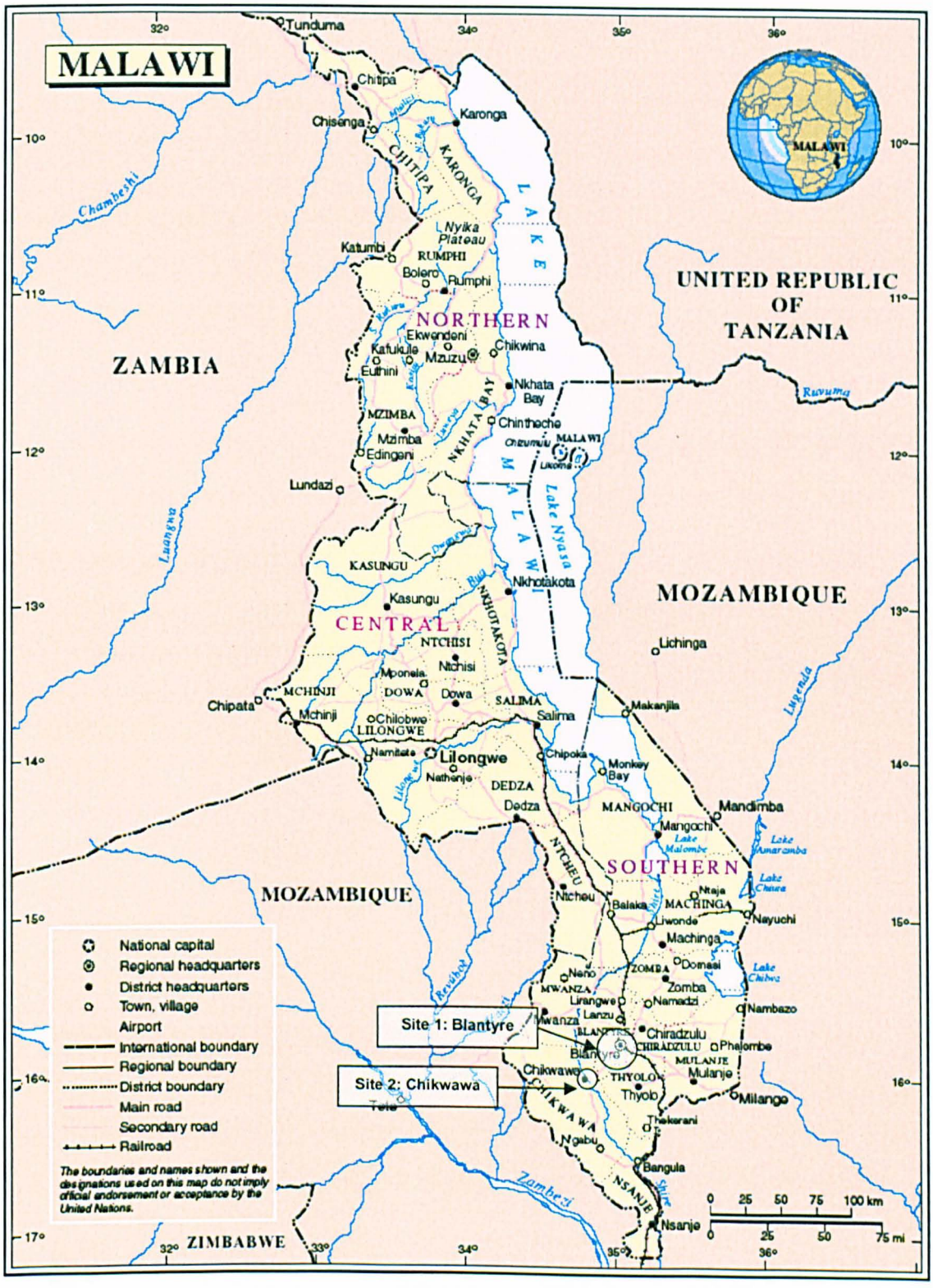
susceptibility to severe anaemia, in relation to either inappropriate or normal bone-marrow cytokine response to infections.

3.2 STUDY LOCATION

3.2.1 Background on Malawi

Malawi is situated in the south-east part of Africa and is bordered on the north by Tanzania, on the west by Zambia, and on the north and east by Mozambique (Figure 3.1). Malawi is approximately 900 kilometres in length and ranges in width from 80 to 160 kilometres. The country has a total area of 118,486 square kilometres, of which 94,276 is land. The remaining area consists mainly of Lake Malawi, which is about 475 kilometres long and runs down Malawi's eastern boundary with Mozambique. The climate is tropical continental with some maritime influences. Temperature and rainfall vary with proximity to Lake Malawi and altitude.

Figure 3.1: Map of Malawi showing Blantyre and Chikwawa Study Sites



Landlocked Malawi ranks among the world's least developed countries. The economy is predominately agricultural, with about 90% of the population living in rural areas and 17% are under five year of age. According to the Malawi Annual and Economic survey (2001) economy depends on substantial inflows of economic assistance from the IMF, the World Bank, and individual donor nations. Agriculture comprises 55% of the gross domestic product (GDP), services comprise 26%, and industry accounts for another 19% of the GDP. Malawi's main exports are tobacco, tea, coffee, peanuts and wood products. The performance of the tobacco sector is key to short-term growth as tobacco accounts for over 50% of exports. Some demographic indicators are presented in Table 3.1.

Table 3.1: Selected demographic indicators from the Malawi Population and Housing Census (1998)

Indicator	1987 Census	1998 Census
Population	7,988,507	9,933,868
Intercensal growth rate	3.2	2.0
Density (pop./sq. km.)	85	105
Women of child bearing age as a percentile of female population	44.2	47.1
Sex ratio (males per 100 females)	94	96
Crude birth rate	41.2	37.9
Total fertility rate	7.6	4.8
Crude death rate	14.1	21.1
Infant mortality rate	151	121
Illiteracy rate (%)	42	58
Male	52	64
Female	32	51

Malawi's health service delivery is based on the National Health Plan 1996 – 2005. The main objectives of this health plan include extension of

peripheral and community-based health services. This health service system has, however, been constrained by lack of financial and human resources.

Blantyre Study Site

Blantyre is the main commercial town of Malawi. Located in the southern region, it has a predominantly urban population of half a million people (Figure 3.1). At an altitude of 800m above sea level, anaemia and malaria is mainly seasonal. Queen Elizabeth Central Hospital in Blantyre, is not only a district and referral hospital, but additionally is the main teaching hospital for the College of Medicine. The hospital has recently renovated its Accidents and Emergency department (A&E) which caters for children presenting to hospital. All children who are admitted to hospital are routinely screened for malaria and anaemia.

Blantyre was selected as a study site because of the large numbers of children with severe anaemia who present to the hospital. The Sevana study established a clinic within the A&E department staffed by two nurses, a clinical officer, research assistant and driver. Study patients were managed in this clinic during recruitment and follow-up visits.

Chikwawa study site

Chikwawa district, located 50km from Blantyre in the Shire valley (altitude 250m above sea level) experiences malaria transmission year round (Figure 3.1). The Chikwawa District Hospital caters for a predominantly rural

population of approximately 400 thousand people and its Under-five clinic treats on average 86 children per day.

Chikwawa was chosen as a study site due to its rural population and contrasting malaria transmission pattern to Blantyre. The Sevana study established a clinic within the Paediatric ward where patients were recruited and followed-up.

3.3 STUDY SUBJECTS

There were four groups of children recruited, aged between 6 and 60 months, namely cases, hospital, community and surgical controls. Recruitment criteria were:

Cases:

- Resident in the study catchment area
- Hb less than 5g/dl on admission
- Not received a blood transfusion within four weeks prior to admission
- Should not have trauma or malignancy as a recognised specific cause of severe anaemia

Hospital Controls:

- Resident in the study catchment area
- Should be presenting to hospital due to an illness
- Hb greater than 5g/dl on recruitment

Community controls:

- Share same residency as a case
- Physically healthy child

Surgical controls:

- Should be presenting to hospital for an elective surgical procedure
- Physically healthy child with no signs of infection or inflammation
- No malaria parasitaemia

The aim of recruiting hospital controls was to control for factors that influence health-seeking behaviour in the population. Community controls, to control for environmental factors that may influence development of severe anaemia. Surgical controls were to provide a healthy bone marrow for comparison with cases.

3.4 SUBJECT RECRUITMENT

Cases, hospital and community controls were recruited in a ratio of 1:1:1.

Case recruitment

In Blantyre, prospective cases were identified by screening all haematocrit recordings in the A&E laboratory and through notification of suspected severe anaemic children (Hb <5g/dl or Hct <15%) by A&E medical staff. In Chikwawa, where children were not routinely screened for anaemia, all children on admission to the Paediatric ward, were reviewed by study staff

and those with clinical signs of severe anaemia had their Hb level determined. Additionally, the laboratory staff and Under-five clinic medical staff notified the study staff of all suspected severely anaemic children. Anaemia in children (6 – 60 months) was defined as Hb <11g/dl or PCV <33% (WHO 2001). Severe anaemia was defined as Hb <5g/dl in accordance with the recommended guidelines for whether a child receives blood transfusion that were used in the hospitals where the study was undertaken.

Informed consent was requested from the guardians of the children if they fulfilled the inclusion criteria for the study (Appendix 3.1). During recruitment, a questionnaire collecting demographic information was administered, followed by a medical history and full physical examination (Appendix 3.2). Prior to administering a blood transfusion, 5ml of venous blood was collected and a bone marrow aspirate was performed. Children in severe cardiac failure (tachypnoiec, tachycardic with 'gallop' rhythm, congested chest signs) or susceptible to anoxia were given local anaesthesia, lignocaine cream, otherwise they were given ketamine intramuscularly. Four millilitres of bone marrow was aspirated using 16-gauge needle and 20ml-volume syringe from the anterior or posterior iliac spine. Determination of the site of aspiration usually depended on the preference of the clinician performing the procedure. A dry tap was usually declared at least after another contra-lateral iliac crest attempt had been unsuccessful. A sample of urine and stool was also collected at recruitment or a day later whilst in hospital.

Hospital control recruitment

A hospital control was recruited the day following when a case was enrolled in the study. The first child at the front of the queue awaiting to be seen by medical staff in A&E (Blantyre) and Under-five clinic (Chikwawa) was selected. After meeting all the inclusion criteria and giving informed consent, a demographic questionnaire, medical history and physical examination similar to that for cases was completed. A blood, urine and stool sample was also collected.

Community control recruitment

On discharge of a case, the child was escorted home by a member of the study team. Within a radius of 100 metres from the compound of the case, the first child fulfilling the selection criteria was selected. After the guardian gave informed consent, the child was taken to the Sevana study clinic were similar recruitment procedures as for a hospital control were undertaken. For cases who died in hospital, a study team member would return to the case's household approximately two weeks later, to recruit a community control.

Surgical control recruitment

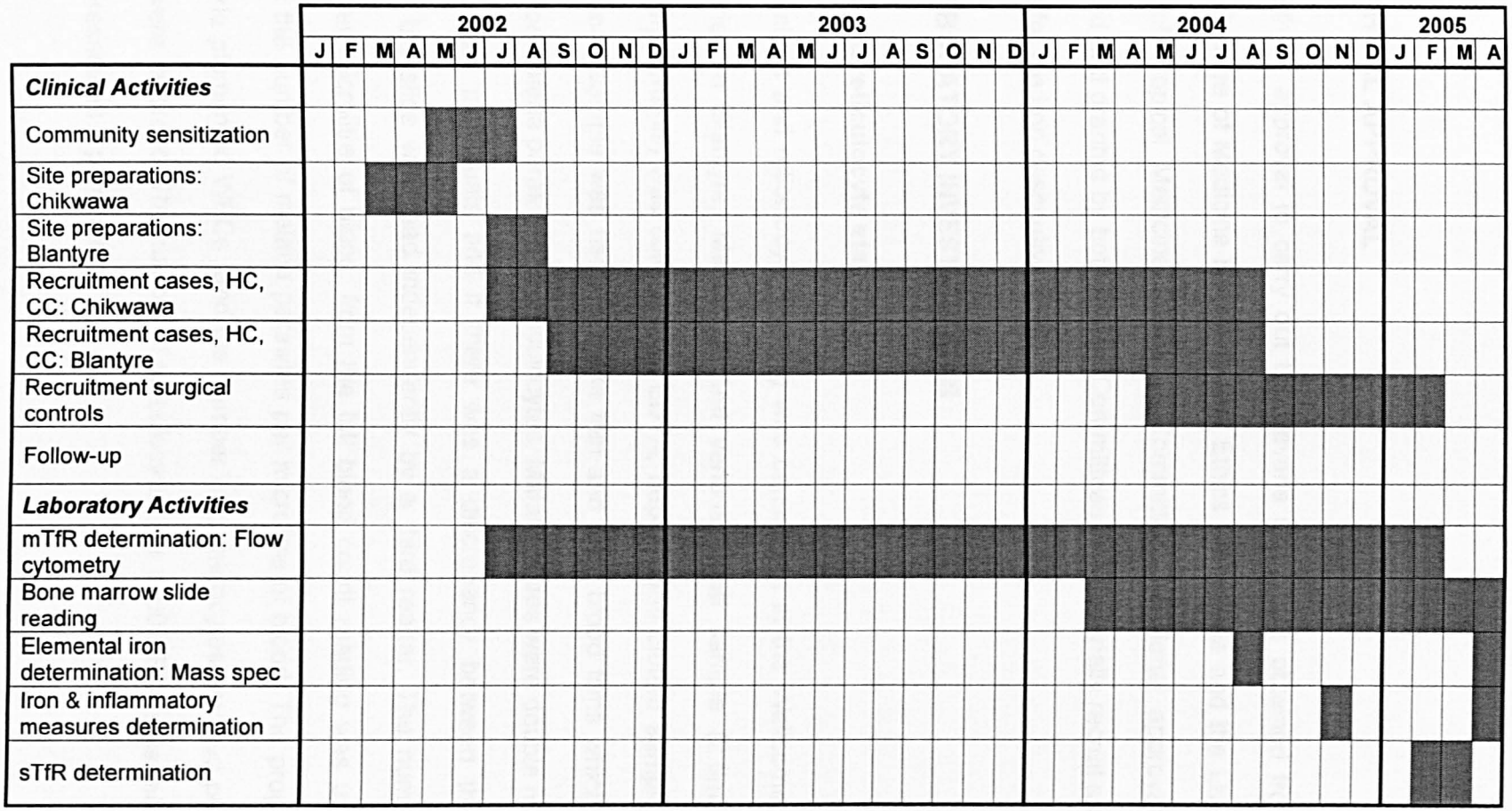
Surgical controls were identified from children attending the surgical clinic and in-patients in the Paediatric Surgical ward in Blantyre. In consultation with surgeons, children who booked for elective surgical procedures, fulfilling selection criteria were recruited, provided their guardians gave informed consent. The children were required not to have received blood

transfusion in the previous 4 weeks, and to have no clinical infection, or inflammatory or malignant pathology. A similar questionnaire as for the other study subjects was completed, collecting information on demographic, medical history and physical examination was completed. In theatre, after the child had been anaesthetised, but prior to the commencement of the surgical procedure, a venous blood sample and bone marrow aspirate was collected. Bone marrow aspirations in these children were performed under general anaesthesia and on the anterior iliac spines only due to their prior positioning on the theatre table in preparation for the surgical procedure.

3.5 SAMPLE SIZE

Sample size for the descriptive iron study, was based on the sample size of the Sevana study. A sample size of 500 cases, and 500 hospital and 500 community controls was initially calculated to enable detecting an odds ratio ≥ 2 for most severe anaemia risk factors (power 90%, 95% confidence interval), unless these were either very common or uncommon i.e. a population prevalence of <10% or >85%. At the commencement of the descriptive iron study, it was expected that 250 of the 500 Sevana study cases will be incorporated into the descriptive iron study. As it was predicted that 25% of these cases would be iron deficient, it was estimated that recruitment of 100 surgical controls would be adequate to provide a comparable equivalent group of iron-replete children.

3.6 STUDY TIMELINE



3.7 ETHICAL APPROVAL

Ethical approval to carry out the Sevana study was obtained from the Malawi College of Medicine Research and Ethics Committee and the Liverpool School of Tropical Medicine Research Committee. Additional approval was requested and granted by both Ethical Committees to additionally recruit surgical controls for the iron descriptive study.

3.8 LABORATORY INVESTIGATIONS

Malaria and reticulocyte staining

Malaria and reticulocyte staining was carried out at the Wellcome Trust laboratories in Blantyre, Malawi. A 5ml venous blood sample (2.5ml from community controls) was collected as EDTA, heparin and clotted sample. The EDTA blood sample was used to make thin and thick blood films which were stained for malaria parasite and reticulocytes. Malaria slides were double read by two separate individuals and if there was a discrepancy between the two readers, the slide was read independently by a third reader. The number of WBCs per microlitre of blood, from the full blood count reading was used to express the number of malaria parasites per microlitre of blood. The proportion of malaria pigmented WBCs and the number of gametocytes counted per 200 WBCs were recorded. The number of reticulocytes per 1000 RBCs was recorded and expressed as a percentage.

Haemoglobin determination

Hb level was determined using three different methods. Firstly using a coulter counter machine (Beckman Coulter, Durban, South Africa) located at the Wellcome Trust laboratories in Blantyre. This also produced output for red cell indices including MCV and MCH. Secondly Hb was determined using the HemoCue B-Haemoglobin Analyser (HemoCue AB, Ängelholm, Sweden) in the study clinics in Chikwawa and Blantyre. The HemoCue analyser consisted of a portable, battery-operated photometer and a supply of treated disposable cuvettes in which blood was collected. This was used in the study clinic to record the initial Hb level at recruitment for all study groups. Lastly the haematocrit, the volume of packed cells as a portion of the total volume of blood, was determined in Wellcome Trust laboratories in Blantyre and the study clinic laboratory in Chikwawa. Hb determination using the HemoCue system was used as the principle method, however if this was missing, the coulter counter Hb, then Hct result were used respectively. Samples from United Kingdom National External Quality Assessment Service (NEQAS) were used for quality control.

Biochemical iron and inflammatory marker determination

Plasma and serum was separated from whole blood within the first hour after collection. They were stored 200µl aliquots at -20 °C and shipped to University of Amsterdam laboratories, Netherlands, for biochemical iron and inflammatory marker determination. Ferritin was determined using electro-chemiluminescence immunoassay (Modular Analytics E170, Roche Diagnostics, Switzerland, detection limit 0.5µg/l) and serum iron using colorimetric assay

(Modular P800, Roche Diagnostics, Switzerland, detection limit 0.9µmol/l). Immunoturbidimetric assay (Modular P800, Roche Diagnostics, Switzerland) was used to determine serum transferrin (detection limit 0.1g/l), haptoglobulin (detection limit 0.20g/l) and CRP (detection limit 1.0mg/l). TIBC was calculated from serum transferrin and transferrin saturation from serum iron and TIBC. sTfR was measured using an enzyme immunoassay (Ramco Laboratories, TX, USA, detection limit 1.0µg/l) at Wellcome Trust laboratories, Blantyre.

Bone marrow iron determination

A bone marrow aspirate was collected from cases and surgical controls. An EDTA bone marrow sample was used to make bone marrow microscopy smears for iron determination and for quantitation of mTfR using flow cytometry both done at the Wellcome Trust laboratories, Blantyre (Section 6.3). Lastly after centrifuging and pipetting out serum and plasma from the aspirate, the bone marrow cells and stroma were stored at -20 °C for elemental iron determination using mass spectrometry at the Clinical Chemistry laboratories, University of Liverpool, United Kingdom (Section 5.3).

HIV testing

Guardians of children enrolled in the study were counselled for HIV testing according to the Ministry of Health HIV counselling guidelines. HIV testing was performed according to WHO recommendations using two rapid tests, (Determine, Abbott Diagnostics and Unigold, Trinity Biotech) at Wellcome Trust laboratories in Blantyre and study clinic laboratory in Chikwawa. Children aged

less than 18 months who still may possess maternal antibodies hence giving a false positive result, were either re-tested at 18 months of age or HIV PCR was performed.

3.9 DATA ANALYSIS

Clinical data from the two study sites was double entered onto a database using Microsoft Access© software. Laboratory data was entered using Microsoft Access© (Hb, red cell indices, and malaria results), Microsoft Excel© (elemental bone marrow iron, biochemical iron and inflammatory measures, and HIV results), Cell Quest© (mTfR results), and Epi Info© software (bone marrow iron microscopy and sTfR results). All data was exported to SPSS for Windows© for analysis. After cleaning, continuous data was categorised using commonly accepted cut-offs, and skewed variables were logarithmically transformed. Univariate followed by multivariate analysis was undertaken with the use of parametric tests for normally distributed data and non-parametric tests for other data. 2-tailed, 95% significance level was employed in all analyses.

Difference in means was evaluated using Student's T-test and difference in proportions, using Chi-square test. Correlation coefficients were calculated to measure linear association between continuous variables and odds ratios (OR) and their 95% confidence interval (CI) were used to measure association between categorical variables. Adjusted odds ratios (AOR) were calculated using Logistic Regression. Linear Regression was used to model for predictors of a variable.

CHAPTER 4

GENERAL STUDY RESULTS AND DISCUSSION

4.1 OVERVIEW

This chapter gives a brief overview of the general results of the study. The demographic characteristics and nutritional status indicators of children recruited in the study were compared between study groups and differences identified. Basic laboratory investigations of haemoglobin concentration and malaria parasitaemia are presented. At the end, there is a discussion on the study design, potential limitations and a brief interpretation of the general study findings.

4.2 GENERAL STUDY RESULTS

A total of 1161 children were recruited into the study. There were 381 cases, 377 hospital controls, 380 community controls and 23 surgical controls (Table 4.1). Nearly half of the children (46%) were recruited in Chikwawa while the rest in Blantyre. There was drought during the second year of recruitment. It was associated with a prolonged dry season and reduced patients with severe anaemia presenting to hospital especially in Chikwawa.

Table 4.1: Recruitment by study group

Study site	Cases	Hospital Controls	Community Controls	Surgical Controls
Chikwawa site	176 (46%)	176 (47%)	177 (47%)	0
Blantyre site	205 (54%)	201 (53%)	203 (53%)	23 (100%)
Total	381 (100%)	377 (100%)	380 (100%)	23 (100%)

Only 23 children were recruited as surgical controls due to fewer numbers of children fulfilling selection criteria than expected. Additionally, industrial action at the hospital in Blantyre and cancelling of operating lists due to scarcity of resources in theatre contributed to the reduced numbers of surgical controls recruited. The sex ratio of children was comparable among cases, hospital and community controls. There was one female recruited for every five males for surgical controls.

The largest proportion of mothers had primary school education, followed by the proportion of mothers who could not read or write (Table 4.2).

Table 4.2: Demographic characteristics by study group

Demographic characteristics	Cases	Hospital Controls	Community Controls	Surgical Controls
Ratio male: female	1:1.1	1:0.9	1:1.0	1:0.2[3,5,6]
Mean age in years (SD)	1.7 (1.1)	1.9 (1.0)[1]	2.1 (1.1)[2,4]	1.8 (0.9)
Mean mothers age in years (SD)	25.3 (6.1)	24.6 (4.8)	25.7 (5.6)[4]	28.7 (6.0)[3,5,6]
Mother's education level (% within study group)				
Cannot read/write	37.4	21.0[1]	27.7[2,4]	27.3
Primary	50.8	47.5	51.1	59.1
Secondary	11.2	26.0[1]	18.6[2,4]	13.6
Tertiary	0.5	5.6[1]	2.7[4]	0
Father's occupation (% within study group)				
Unemployed	31.8	24.5[1]	32.6[4]	52.4[5]
Self-employed	24.0	22.2	25.4	23.8
Wage employed	40.4	37.3	33.7	19.0
White-collar job	3.9	16.0[1]	8.4[2,4]	4.8
Average property number[7]	2.0	2.2[1]	2.1	2.0
Mean siblings alive (SD)	1.8 (1.8)	1.3 (1.3)[1]	1.8 (1.7)[4]	1.9 (1.3)[5]
Mean siblings dead (SD)	0.6 (1.1)	0.3 (0.6)[1]	0.5 (1.0)[4]	0.8 (1.3)

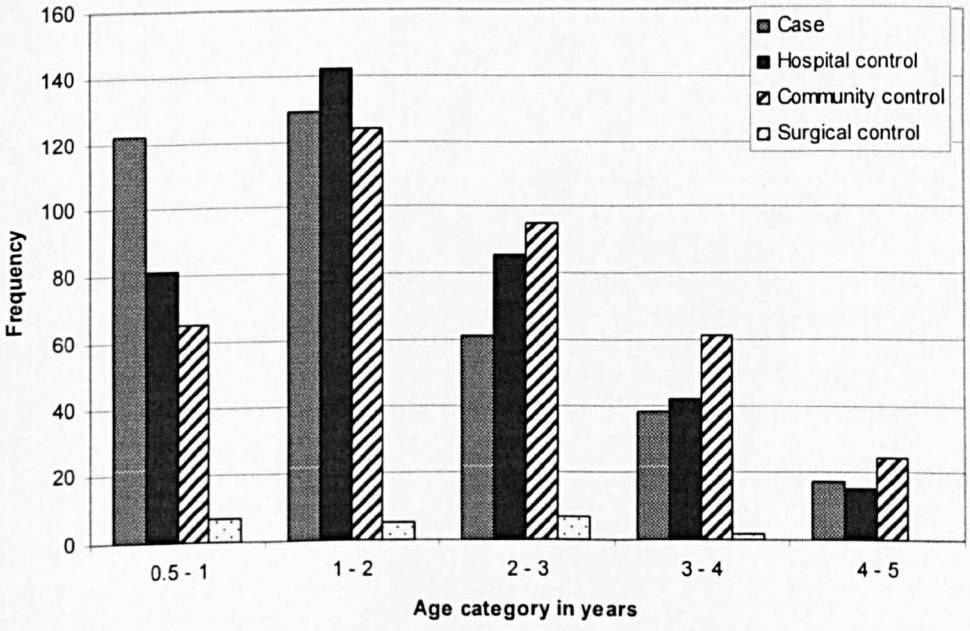
[1] $p<0.05$ between Cases and Hospital controls
[2] $p<0.05$ between Cases and Community controls
[3] $p<0.05$ between Cases and Surgical controls
[4] $p<0.05$ between Hospital and Community controls
[5] $p<0.05$ between Hospital and Surgical controls
[6] $p<0.05$ between Community and Surgical controls
[7] refer to Appendix 3.2

Most of the fathers for cases, hospital, and community controls were wage employed apart from surgical controls were they were mainly unemployed. The

average property number (Appendix 3.2), a marker of socio-economic status was comparable in all four study groups. However, families of hospital controls appeared to be smaller and experienced the fewest number of sibling deaths.

Figure 4.1 shows the age distribution to be skewed towards the younger ages in all study groups. According to Malawi Population and Housing Census (1998) the general population distribution is also skewed towards younger ages. The mean age for study groups were comparable apart from community controls where the children were significantly older (Table 4.2).

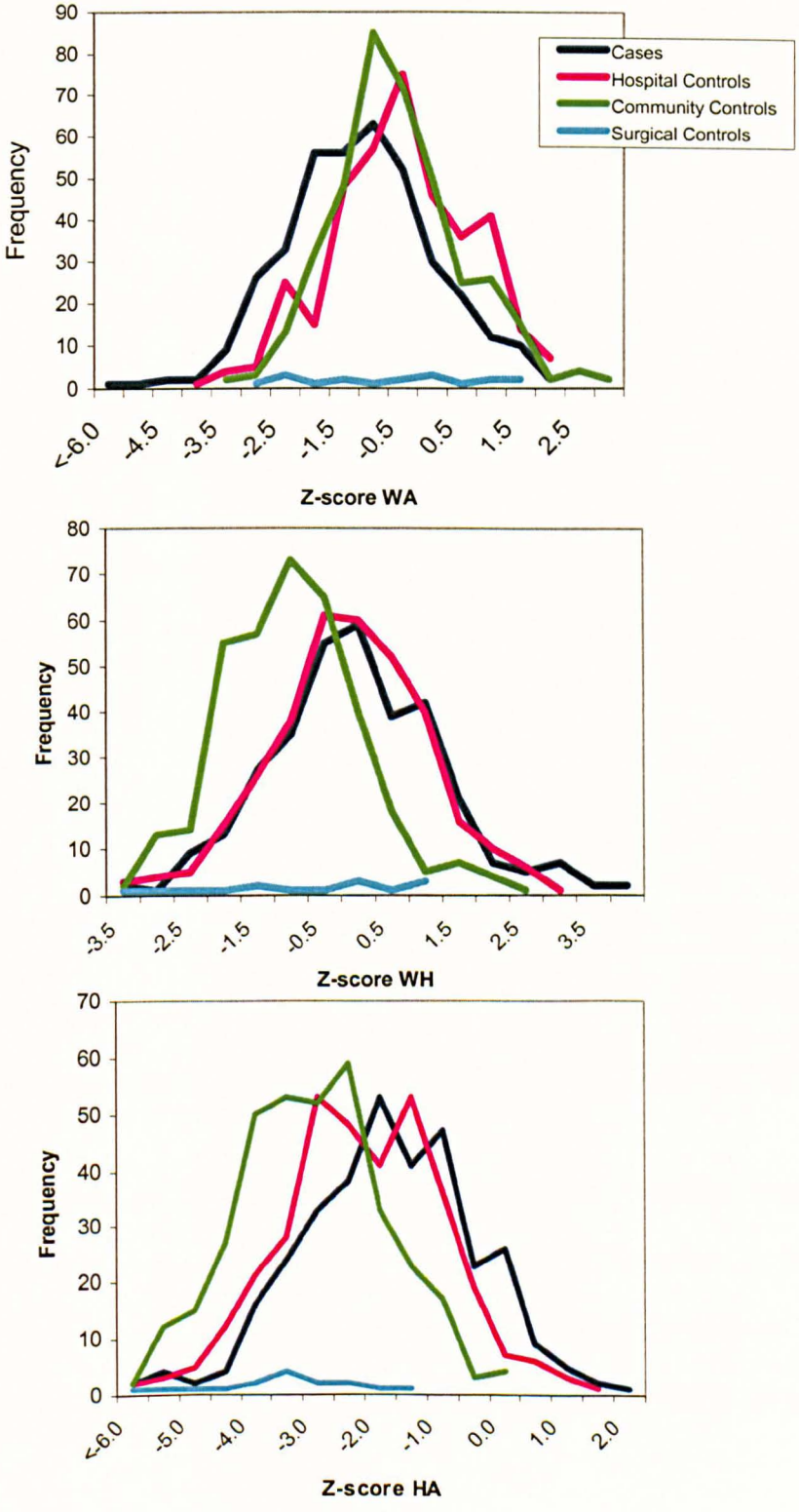
Figure 4.1: Age distribution by study group



Anthropometric indices were used to determine nutritional status of the children. Decreased weight-for-age (W/A) reflected short-term growth faltering (wasting) while decreased height-for-age (H/A) indicated long term growth failure (stunting). Weight-for-height (W/H) reflected both short and long term growth

disturbances (underweight). An international cut-off of less than -2 standard deviations of Z-score of a reference healthy population, was defined as malnutrition i.e. wasting, stunting or being underweight (WHO 1995). According to Figure 4.2, cases were more wasted, while community controls were more underweight. A large proportion of children in all study groups were stunted (<2.0 SD) with their normal distribution pattern shifted to the left.

Figure 4.2: Distribution of Z-scores of weight-for-age, weight-for-height and height-for-age by study group



As expected, Hb level in cases was lower than in the other study groups (Figure 4.3) due to the inclusion/exclusion criteria for cases. The mean Hb for cases (3.6 g/dl, 0.8SD) was significantly different ($p<0.05$) from hospital controls (9.6 g/dl, 2.2SD), community controls (9.9 g/dl, 1.9SD) and surgical controls (9.9 g/dl, 1.8SD).

Prevalence of malaria parasitaemia was significantly greater in cases than in hospital and community controls, and it was commoner for cases to have increased parasite densities (Table 4.3).

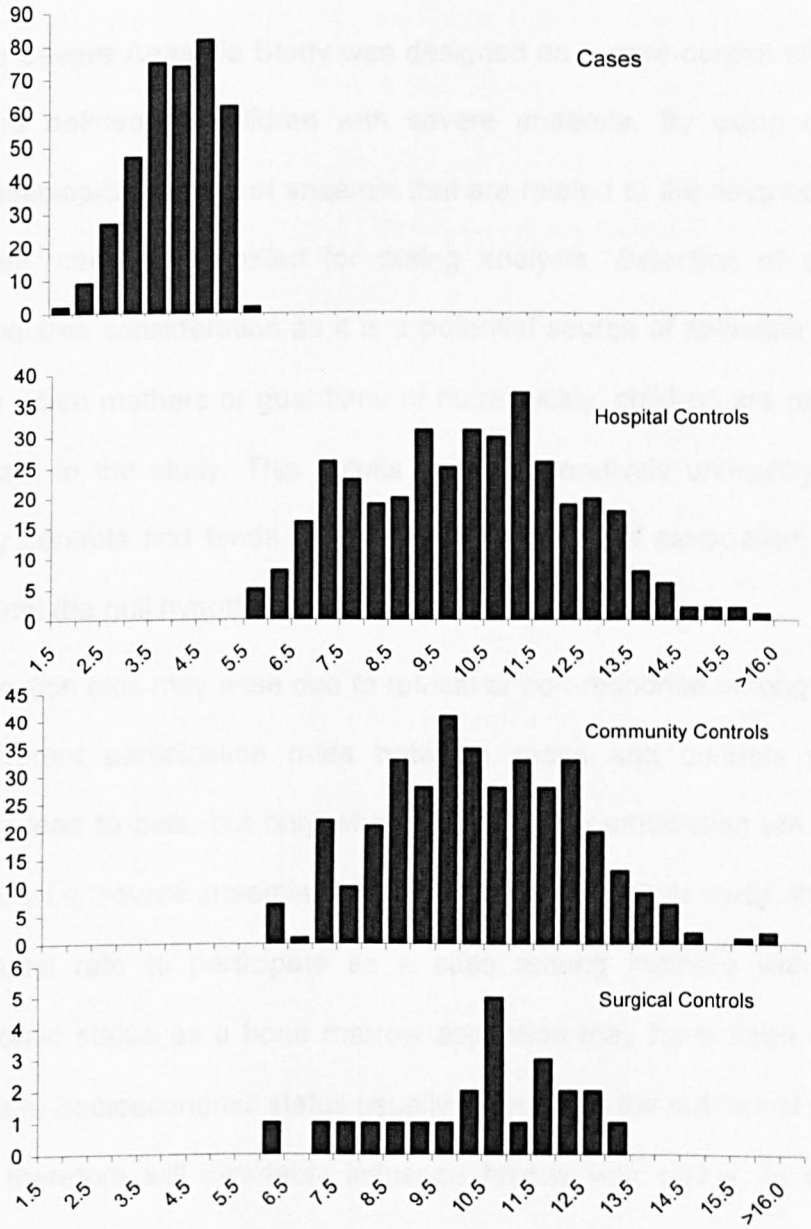
Table 4.3: Malaria results in cases, hospital and community controls

	Cases	Hospital Controls	Community Controls
Malaria parasite count (n)			
No parasites	51% (195)	65% (243)[1]	61% (230)[2]
1 – 5,000 /µl	18% (69)	13% (49)	23% (87)[3]
5001 – 10,000 /µl	4% (14)	2% (9)	6% (21)
>10,000 /µl	27% (102)	20% (75)[1]	10% (36)[2,3]
Mean Log count /µl (SD)	4.04 (1.04)	3.93 (0.92)	3.52 (0.77)[2,3]
Mean percentage malaria pigmented leucocytes (SD)	2.04% (0.85)	1.45% (0.72)[1]	1.38% (0.66)[2]
Mean gametocyte count /µl (SD)	0.77 (3.17)	0.59 (2.46)	0.81 (3.02)

[1] $p<0.05$ between Cases and Hospital controls
[2] $p<0.05$ between Cases and Community controls
[3] $p<0.05$ between Hospital and Community controls

The proportion of white blood cells with malaria pigment was also significantly increased in cases (2.0%) compared to hospital (1.5%) or community controls (1.4%). All surgical controls had no parasitaemia as it was an exclusion criteria for surgical procedures requiring administration of general anaesthesia.

Figure 4.3: Distribution of haemoglobin by study group



4.3 GENERAL STUDY DISCUSSION

The Severe Anaemia Study was designed as a case-control study where cases were defined as children with severe anaemia. By using community controls, aetiological factors of anaemia that are related to the neighbourhood or environment, can be controlled for during analysis. Selection of community controls requires consideration as it is a potential source of selection bias. This may occur when mothers or guardians of more 'sickly' children are more willing to participate in the study. This results in a comparatively unhealthy group of community controls and tends to lessen the measure of association e.g. odds ratio, towards the null hypothesis.

Selection bias may arise due to refusal or non-response among any study group. Different participation rates between cases and controls would not necessarily lead to bias, but only when the rates of participation are related to the exposure i.e. severe anaemia. It is a possibility that in this study, there was a higher refusal rate to participate as a case among mothers with a higher socioeconomic status as a bone marrow aspiration may have been viewed as very invasive. Socioeconomic status usually determines the nutritional status of a child and therefore will invariably influence his/her iron status. In this study, information on children who were not recruited but met the study inclusion criteria, was not collected, therefore, bias cannot be completely excluded.

Surgical controls were recruited with the aim of providing a comparative group of healthy children for bone marrow investigations. 'Healthy' was defined as a child having no signs or symptoms of infection on history and physical

examination and absence of malaria parasites in the blood. A more comprehensive infection and nutritional status screen may have been ideal to select truly healthy controls. There was also an increased predominance of male children among the surgical controls recruited. This may be due to the fact that hernias, which accounted for most of children seeking elective surgery, have an increased incidence in male as compared to female children of 11:1 (Carneiro and Rwanyuma 2004).

Descriptive studies of iron deficiency and its effect on infection are challenging to conduct. This is because iron deficiency is part of a cluster of nutrient and social deprivations, ultimately resulting from poverty that are inevitably interrelated. Socio-economic and nutritional status are important confounders in investigating the association between iron deficiency and infection. A large proportion of children in the four study groups were stunted which is in agreement with other studies from Malawi where 70% of children were stunted by the end of their first year of life (Maleta *et al,* 2003).

Assuming that community controls were a good representation of the general population of children, a mean Hb level of 10g/dl among community controls reflected the average general population Hb level among under-five children in Chikwawa and Blantyre. Studies have described a difference in mean Hb levels between black and white adult populations (Perry *et al,* 1992; Kent 1997) which has varied between 1.0g/dl in earlier reports to 0.2g/dl in later studies (Jackson 1990). Many researchers have attributed this difference to genetic traits, however others believe it is due to a higher prevalence of

infectious and inflammatory diseases among blacks resulting in anaemia of inflammation. This has been substantiated by the finding of higher levels of ESR and ferritin in the black population (Kent 1997).

Malaria is endemic in Malawi with a large proportion of children in the community being asymptomatically infected with *P. falciparum*. This was reflected in the increased prevalence of parasitaemia in community controls of forty percent. The likelihood that malarial parasites are responsible for fever in an individual patient increases in parallel with an increase in density of parasitaemia (Greenwood 1997). Therefore, clinical malaria defined by presence or history of fever and parasitaemia above 5000/µl, was more common in cases as compared to hospital or community controls. The contribution of malaria as a cause of anaemia is difficult to quantify as anaemia is often multi-factorial, but epidemiological studies have demonstrated increased hospital admissions with severe anaemia coinciding with the peaks of malaria transmission (Menendez *et al*, 2000).

Previous clinical studies have consistently demonstrated that elevated proportions of malaria pigmented leukocytes are associated with increased severity of *P. falciparum* infection, and with a worse disease outcome (Lell *et al*, 2005). Cases had a significantly higher percentage of pigmented leucocytes. Malaria was an important cause of anaemia among the cases, but it is uncertain whether the observed increase was clinically relevant as clinically relevant increases have shown to vary in different populations (Nguyen *et al*, 1995).

4.4 CONCLUSION

A large group of children were recruited in this case-control study as cases, hospital, community and surgical controls. These study groups had largely comparable demographic characteristics apart from the sex ratio among surgical controls and the family size among hospital controls. Most children were stunted, and a high proportion of cases were underweight.

Malaria parasitaemia was highly prevalent with increased parasite densities found in cases. The study design and potential sources of bias were discussed.

CHAPTER 5

ASSESSMENT OF IRON STATUS USING BONE MARROW MICROSCOPY

5.1 INTRODUCTION

Iron deficiency results from long-term negative iron balance in the body. It is a state in which there is insufficient iron to maintain the normal physiological function of tissues such as blood, brain and muscles. *Storage iron*, in the form of ferritin and haemosiderin, is the iron pool in the body that is not being used directly by tissues. Healthy children usually have some iron stores to act as a buffer against iron deficiency during periods when dietary intake may be temporarily insufficient. Deficiency of iron stores occurs when these stores are depleted. *Iron depletion* is the state in which storage iron is absent or nearly absent but the tissues that require iron are able to maintain normal physiological functions. *Functional iron deficiency* develops when normal physiological systems for transporting iron to target tissues are impaired in the presence of adequate iron stores (WHO/CDC 2004). This is commonly caused by cytokines released during an acute phase response to infection, leading to impaired erythropoiesis and later anaemia, usually termed 'anaemia of inflammation' (Trey and Kushner 1995).

Bone marrow smear microscopy has been used mainly to assess iron stores status. The examination of the Prussian-blue-stained bone marrow aspirate for the presence or absence of iron granules has been considered the

"gold standard" for determining iron depleted states. In a bone marrow aspirate smear, there are three areas one could assess for iron. These are the marrow fragments, 'loose' reticular cells around fragments, and erythroblasts.

Conventionally, iron has been primarily assessed in marrow fragments. These are areas of a dense cellular network consisting of a meshwork of usually undistinguishable reticular, erythroid, fat and bone cells. These fragments are commonly found towards the tail-end of a smear. Iron visualised in fragments represent iron stores in the form of haemosiderin.

Over the past 50 years various grading methods have been developed, based on the amount of iron present and its location in bone marrow. Rath and Finch (1948) first described six different histological grades of iron in a marrow smear. These were subjective and arbitrarily described as None; Very slight; Slight; Moderate; Moderate heavy; Heavy; Very heavy. Gale *et al* (1963) further described each of these histological grades as shown in Table 5.1. Studies showed that there was a reasonable correlation between histological iron grading and chemical iron concentration in bone marrows. Johansen *et al* (1970) found a correlation coefficient of 0.75 and Gale *et al* (1963) were able to show an increasing trend of chemical iron concentration with increasing histological iron grades. However, others have found a poor relationship between the two methods, raising questions on the validity of the histological grading (Trubowitz *et al,* 1970). Nonetheless, most studies have based their assessment of iron status on this conventional histological grading developed by Gale *et al* (1963) or

a comparable grading method which only takes into account iron in marrow fragments.

Table 5.1: Conventional histological grading (Gale *et al*, 1963)

Grade 0	**None**	No visible iron under high power magnification (x1000)
Grade 1	**Very slight**	Small iron particles just visible in few reticulum cells under high power magnification (x1000)
Grade 2	**Slight**	Small, sparsely distributed iron particles just visible under low power magnification (x100)
Grade 3	**Moderate**	Numerous small iron particles present in reticulum cells throughout the marrow fragment (x100)
Grade 4	**Moderate heavy**	Larger iron particles throughout the fragment with tendency to aggregate into clumps (x100)
Grade 5	**Heavy**	Dense, large clumps of iron throughout the fragment (x100)
Grade 6	**Very heavy**	Very large deposits of iron, both intra- and extra-cellular, obscuring cellular detail in the fragment (x100)

In malaria endemic areas interpretation of iron status may be confounded by malaria pigment, hemozoin. During its pathogenic intra-erythrocyte stage, the malaria parasite ingests host haemoglobin which is converted into hemozoin. Hemozoin which contains denatured ferri-haem, is deposited extensively in body organs including the bone marrow (Rodriguez and Jungery 1986). It is unclear how much and how long the iron sequestered in hemozoin is unavailable for erythropoiesis. It is therefore unknown how representative conventional histological iron grades are of the body iron stores in populations where malaria is highly endemic.

The conventional iron grading method detects the presence or absence of iron in marrow fragments and hence represents a measure of iron stores. However, iron is utilised by developing erythrocytes. It is important to measure

the iron that is being delivered to the cell and metabolically available for cellular processes, as inflammation and malaria may affect delivery of iron to erythroblasts. Erythroblast iron represents a measure of cellular iron utilisation. If there is low cellular iron in the presence of adequate iron stores then there is functional iron deficiency. However there has been inadequate research on use of erythroblast iron as a marker of cellular iron availability (Hanif *et al,* 2005). Low erythroblast iron is associated with iron deficiency and has been shown to increase after iron therapy (Douglas and Dacie 1953). Conventional iron grading of fragment iron alone may therefore not be informative of an individual's iron status in areas of high malaria transmission intensities and inflammatory conditions, where there is a high prevalence of functional iron deficiency.

A few researchers have used an alternative histological grading method that takes into account erythroblast iron. Hanif *et al* (2005) classified individuals with anaemia of inflammation as those with low erythroblast iron and adequate iron stores in the marrow (determined using conventional histological grading method). However, the reason for using erythrocyte iron in combination with erythroblast iron to determine anaemia of inflammation was not clear. Additionally, erythrocyte and erythroblast iron cut-offs were not specified.

Iron visualised in marrow fragments is from a meshwork of reticular cells (monocytes, macrophages) and erythroblasts which are usually undistinguishable. During smear preparation and staining, some cells may rupture, releasing their cytoplasm. On inspection of a fragment it is often not possible to distinguish extra-cellular from intra-cellular iron. Additionally, artefacts

tend to collect in the same area of the smear as the fragments. It is difficult to distinguish these artefacts from iron especially for an inexperienced microscopist as the background cell architecture in a fragment is often absent.

Single reticular cells or those surrounded by developing red cells may be inspected for iron. They tend to be located in the tail end of the smear. Iron in these 'loose' reticular cells represents iron stores. It is hypothesized that the presence of iron in these cells is particularly important when iron in fragments is absent and may signify the lowest level of iron stores depletion.

It is not clear how extensive a slide examination should be performed to distinguish between iron stores deficiency and normal iron stores. Although it has been suggested that two slides be examined, few studies have assessed the number of bone marrow fragments to be examined. Hughes *et al* (2004) showed that in bone marrow aspirations among patients with various haematological conditions in a developed country, a minimum of seven fragments must be examined to establish iron stores deficiency, and a minimum of nine fragments to determine the maximum iron grade. There is a need to validate these findings in different populations.

5.2 OBJECTIVES

1. To determine the prevalence of deficiency of iron stores or normal iron store status among cases or surgical controls using the conventional histological grading method

2. To determine the prevalence of iron stores deficiency, normal iron status and functional iron deficiency among cases and surgical controls using a detailed alternative histological grading method
3. To assess, in a malaria endemic population, the number of marrow fragments that should be examined to confidently establish the presence or absence of iron stores and grade the quantity in a bone marrow aspirate

5.3 METHODS

Marrow preparation and staining

Bone marrow smear preparation

A bone marrow aspirate was collected in an EDTA tube for slide preparation and staining at the Wellcome Trust laboratories, Blantyre. The first few drops of marrow collected in the first syringe were reserved for marrow smear preparation. This maximised marrow fragment collection for later iron determination and also decreased dilution of the marrow with blood which is common with larger aspiration volumes. Additionally, if few fragments were seen during smear preparation, the marrow sample was placed in a watch glass, to enable fragment visualisation and collection of an aspirate richer in fragments.

After smear preparation, all slides were air-dried before being fixed in methanol for 5 minutes. Caution was taken to always keep methanol in air-sealed containers during and after fixing to prevent water artefact on the slides.

Staining

Principle: Bone marrow iron staining was done using a commercial kit and according to methods recommended by the manufacturer (HematoGnost Fe, Darmstadt, Germany). Perl's reaction is based on the formation of ferric ferrocyanide (Prussian Blue) when a ferric ion, released from iron-containing compounds by HCl, reacts with potassium ferrocyanide. Therefore iron from ferritin and haemosiderin react to produce an insoluble precipitate, thus localising cellular iron.

Procedure: Five percent potassium hexacyanoferrate and 5% hydrochloric acid mixed in equal volumes was flooded on slides for 20 minutes before being washed with distilled water. Slides were counter-stained with Nuclear fast red solution for 5 minutes before washing and air-drying. Slides with highly positive iron stores (ascertained by more than one reader) were included in each batch of slides stained as positive controls.

Conventional grading method

Marrow smears were assessed by the author according to the conventional histological grading method (Gale *et al,* 1963) which classifies iron status into iron stores deficiency, normal iron stores and high iron stores. In order to obtain maximum objectivity, the description of each grade was pre-defined and sample illustrations of each grade[1] (Figure 5.1 to 5.6) agreed upon by the author and two consultant haematologists, were used to grade fragments of all

[1] Kindly supplied by Barbara Bain

marrow smears. Iron is unevenly distributed in the bone marrow (Barron *et al,* 2001). It is necessary to evaluate a significant number of marrow smears to ascertain the maximum positivity of a smear which is determined by the highest grade of marrow fragment assessed (Hughes *et al,* 2004). Therefore, up to 15 fragments were graded (Hughes *et al,* 2004) and the highest fragment grade was taken as the overall bone marrow smear score in the present study. Iron stores deficiency was defined as a bone marrow smear score of 0 or 1 i.e. none or very slight (Rath and Finch 1948).

Figure 5.1: Grade 0 (No iron) of conventional grading method (x400 magnification)

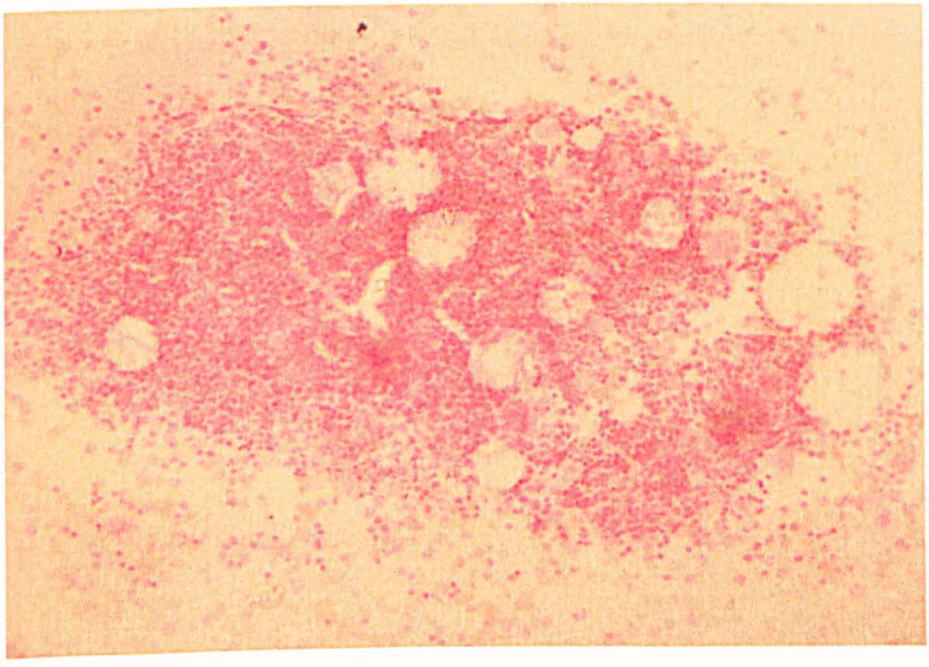


Figure 5.2: Grade 1 (Very slight iron) of conventional grading method (x400 magnification)

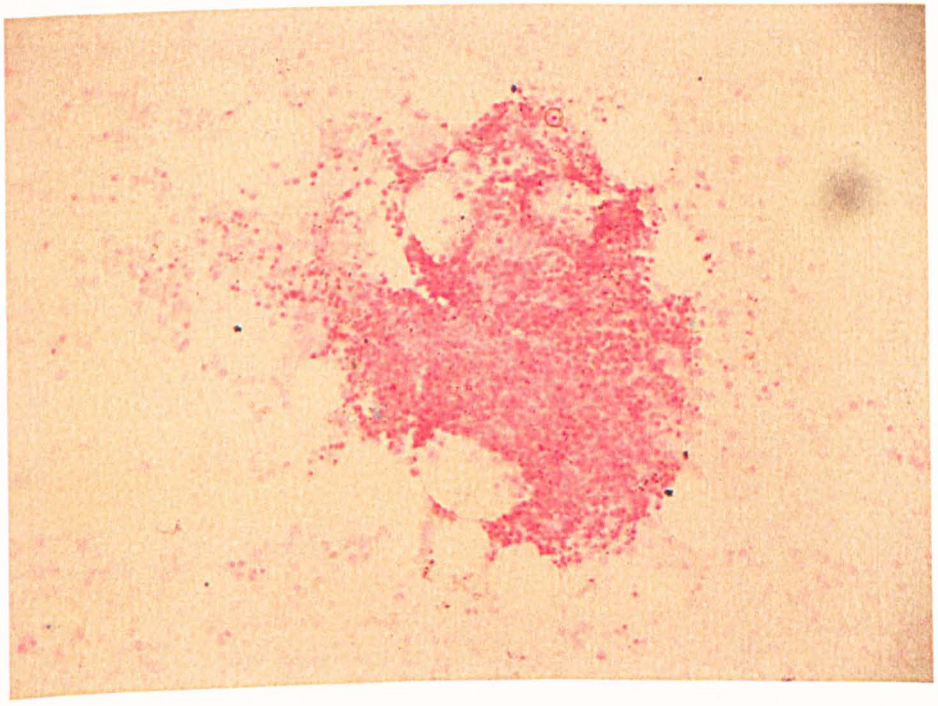


Figure 5.3: Grade 2 (Slight iron) of conventional grading method (x400 magnification)

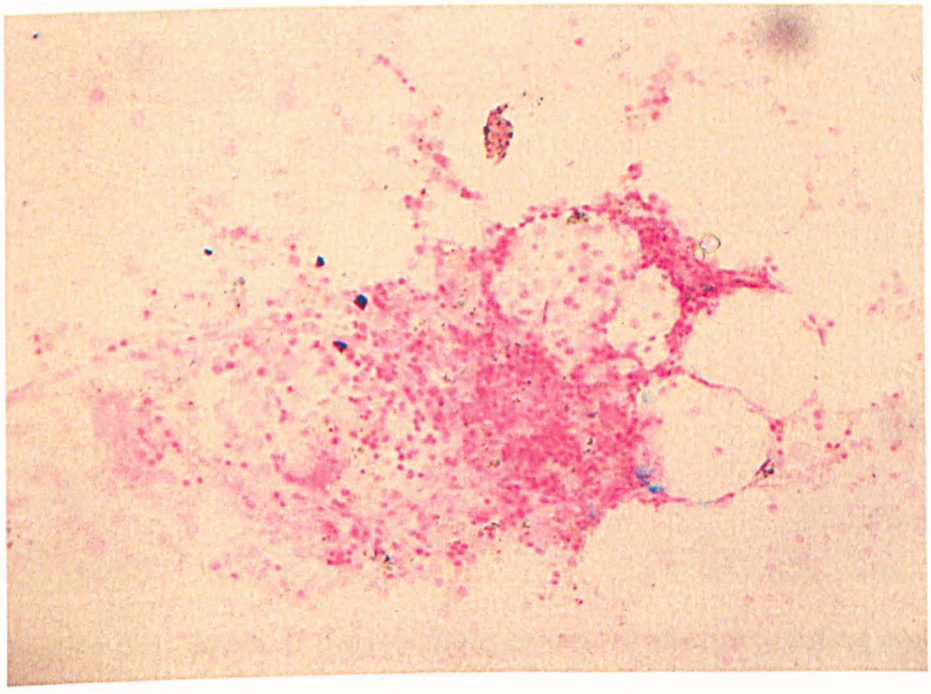


Figure 5.4: Grade 3 (Moderate iron) of conventional grading method (x400 magnification)

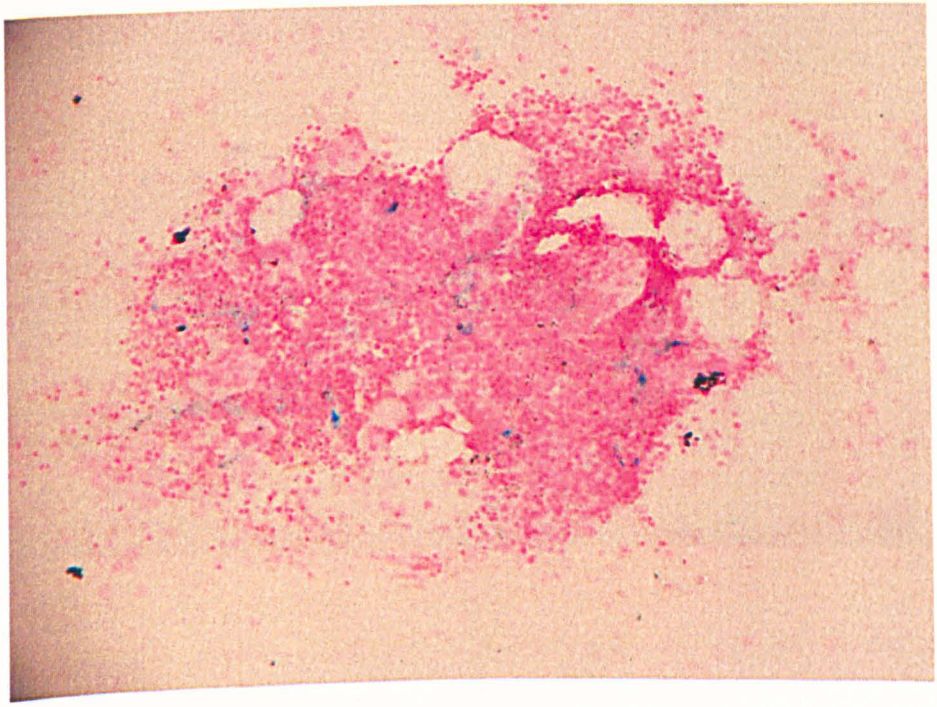


Figure 5.5: Grade 4 (Moderate heavy iron) of conventional grading method (x400 magnification)

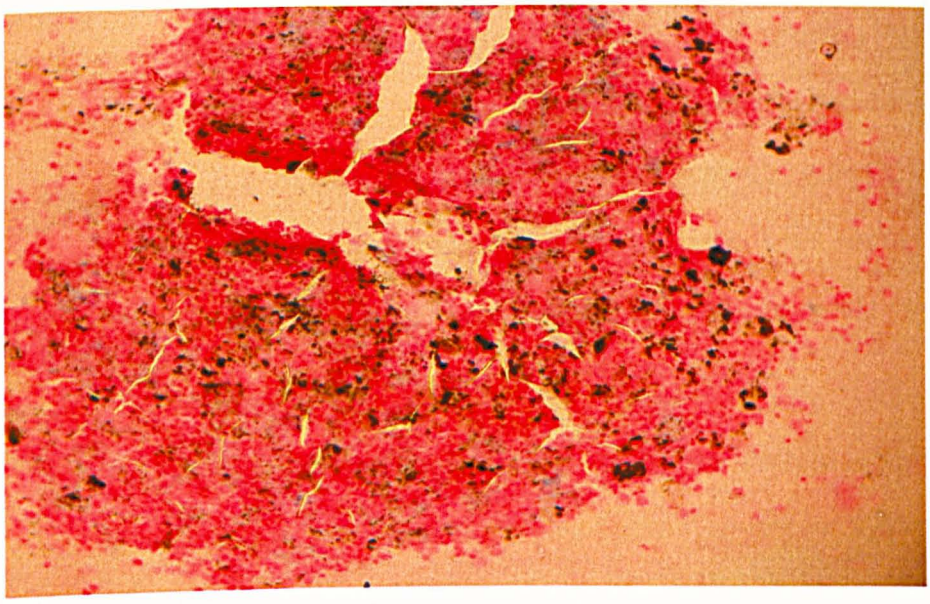
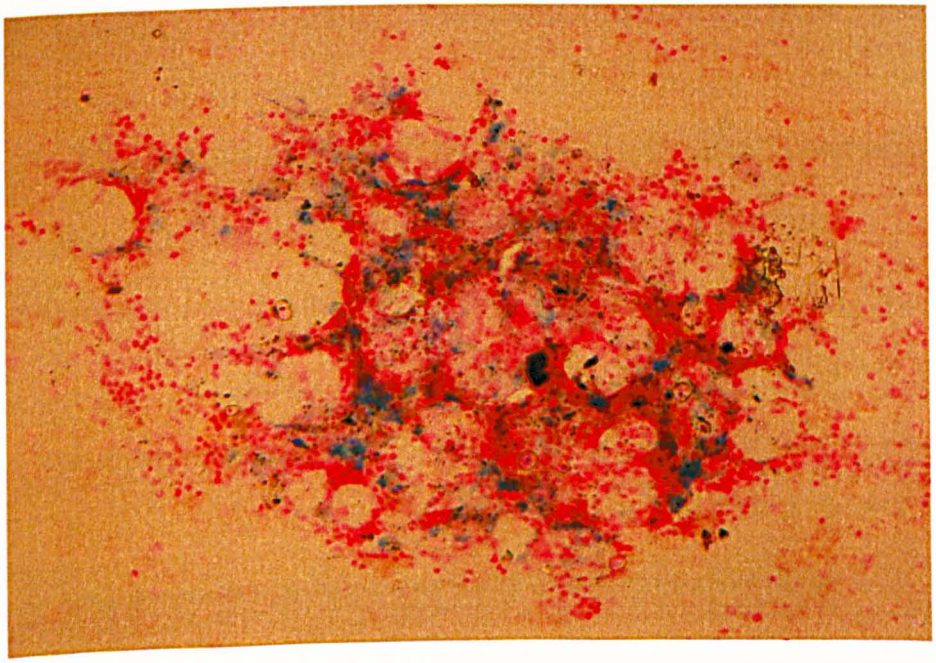


Figure 5.6: Grade 5 (Heavy iron) of conventional grading method (x400 magnification)



Alternative grading method

In contrast to the conventional histological grading method that only assesses iron in marrow fragments, the alternative histological grading method assesses iron in three areas of the bone marrow smear, namely fragment, 'loose' reticular cells around fragments and erythroblasts. Iron assessed in fragment and 'loose' reticular cells represented iron stores while erythroblast iron represented functional iron.

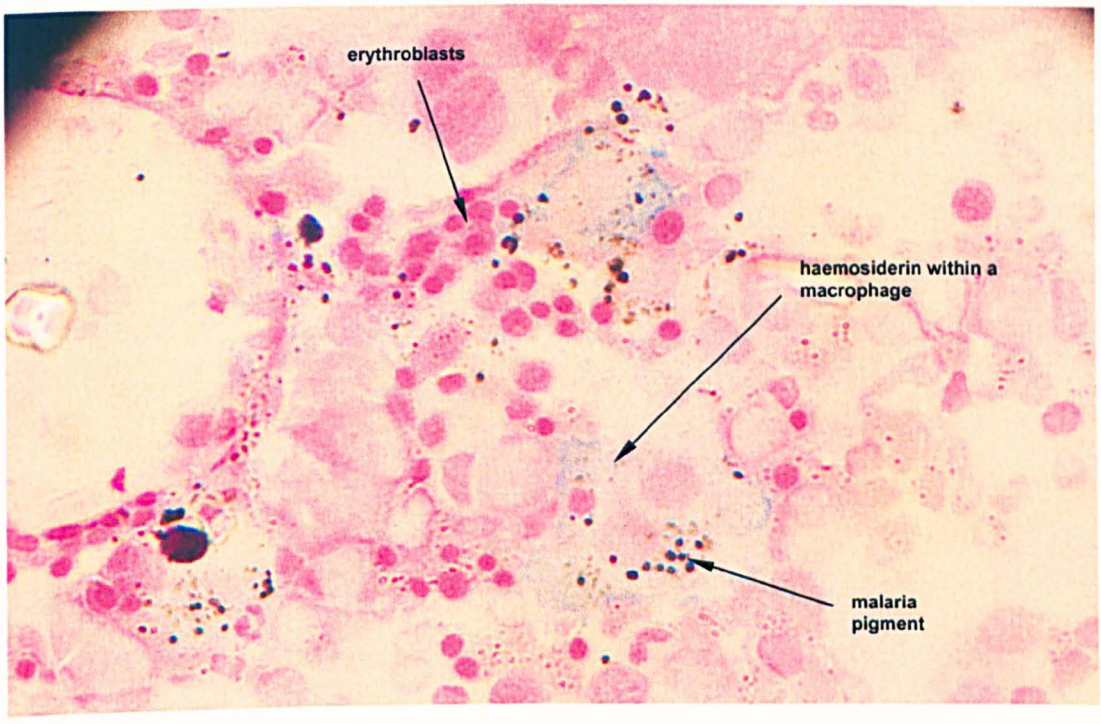
The marrow fragment consists of dense cellular networks of usually undistinguishable cell types. The assessment of iron in fragments using this alternative grading method was exactly the same as the overall conventional grading method. Therefore the same routine of examining and grading up to 15 fragments was employed. The overall fragment iron smear grade (as compared to iron smear score in the conventional grading method) was taken as the highest fragment grade among the fragments assessed. Fragment iron deficiency was defined as a fragment iron smear score of 0 or 1, i.e. none or very slight.

The second area for iron assessment was in 'loose' reticular cells, in particular macrophages around fragments. Twenty high-powered magnification fields (x1000) were inspected for macrophages. If observed, the macrophage was examined for the presence or absence of iron (Figure 5.7).

The third area for iron assessment was in erythroblasts. At high power magnification (x1000), 100 erythroblasts were examined and the percentage containing iron granules in their cytoplasm, also known as sideroblasts, were

enumerated. Erythroblast iron deficiency was defined when <30% of erythroblasts had iron granules (Hastka, Lasserre *et al* 1993).

Figure 5.7: Iron and malaria pigment deposition in macrophage (x1000 magnification)



Results of iron smear assessment using the alternative histological grading method were interpreted as follows (Table 5.2):

Normal status: Normal iron stores and normal cellular iron

Functional iron deficiency: Normal iron stores and deficient cellular iron

Iron stores deficiency: Depleted/deficient iron stores and normal/deficient cellular iron

Combined functional iron and iron stores deficiency: Depleted iron stores and deficient cellular iron

Table 5.2: Classification of iron status using the alternative grading method

Iron detected in:			Iron status category
Iron stores		*Cellular iron*	
Fragment[1]	*Macrophage*[2]	*Erythroblast*[3]	
+	+	+	**Normal**
+	–	+	
+	+	–	**Functional iron deficiency**
+	–	–	
–	+	+	**Iron stores deficiency**
–	–	+	
–	–	–	
–	+	–	**Functional and stores deficiency**

[1] positive fragment iron = fragment grade >2
[2] positive macrophage iron = iron present in macrophage
[3] positive erythroblast iron = iron present in >30%erythroblasts

Minimum fragment number for iron stores deficiency and grade determination

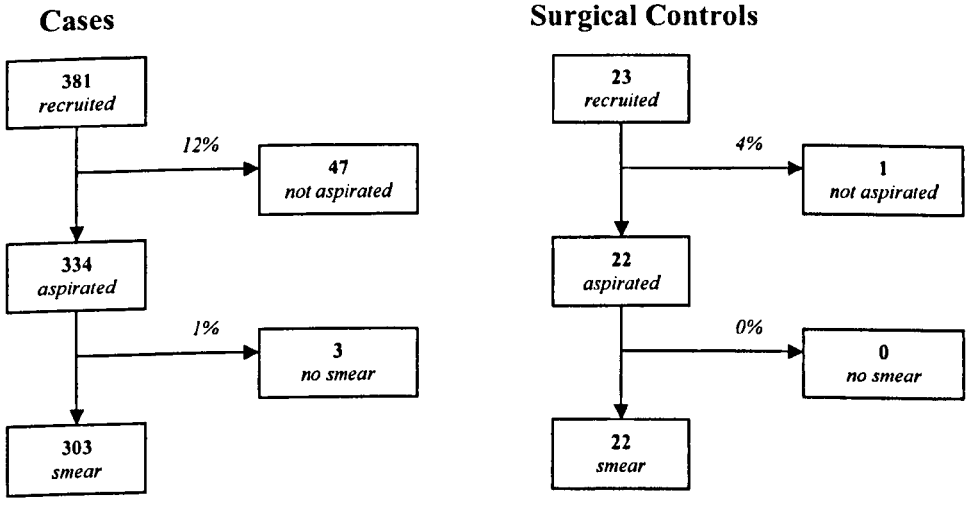
Consecutive fragments were graded for iron as described for the conventional histological grading method with the aim of determining the minimum number of bone marrow fragments that are needed to be examined to confidently establish both the presence or absence of iron stores and the overall score for a smear. Each fragment was scored separately. The smear was systematically examined to prevent reading any fragment more than once. For each smear, both the reading order and score of each fragment was recorded.

5.4 RESULTS

Conventional grading method

Out of a total of 381 cases, 334 had bone marrow aspirations performed, from which 303 marrow smears were prepared (Figure 5.8). Forty-seven (12%) cases did not have a bone marrow aspiration for the following reasons: refusal by guardian, child being too sick, unsuccessful aspiration ('dry tap'). Twenty-two out of 23 (96%) surgical controls had a bone marrow aspirate collected and smear prepared.

Figure 5.8: Flow chart showing the proportion of cases and surgical controls that had a marrow aspiration and smear performed

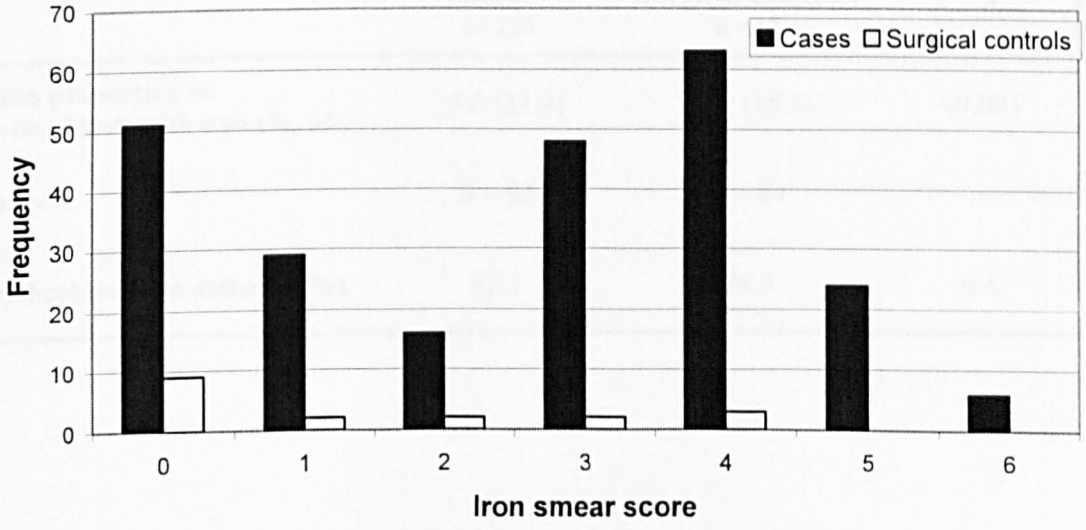


Among 66 of 303 cases (22%) and 4 out of 22 surgical controls (18%), no bone marrow fragments were seen on the smear and these were therefore excluded from further iron assessment using conventional grading method.

Figure 5.9 shows the distribution of iron scores amongst cases. From this distribution, there are apparently two different groups of children, namely, an iron

stores deficient and iron stores replete group. This pattern was also present among surgical controls although the numbers for surgical controls were small. According to the conventional grading method, iron stores deficiency, defined as an iron grade of none or very slight, was higher among the surgical controls (61%) compared to the cases (34%), however this did not represent a significantly increased relative risk of iron stores deficiency among the surgical controls compared to cases (OR = 1.8, 95% CI 0.8 – 4.25).

Figure 5.9: Distribution of iron smear score for cases and surgical controls using the conventional grading method



Alternative grading method

Seventy-eight (26%) cases and 5 (23%) surgical controls smears were too poorly stained for macrophage and erythroblast iron grading. They were therefore not included in further analysis. Macrophages were not identified in a further 8% (*n=25*) of cases, and 14% (*n=3*) of surgical control smears, and these

were not included in macrophage iron grading. Among these remaining cases, 151 out of 197 (77%) had iron in macrophages, compared to 9 out of 14 (64%) among surgical controls. This difference was not statistical significant ($p= 0.3$).

The proportions of children that were iron deficient according to erythroblast iron scores among cases and surgical controls (63% and 77% respectively) were comparable, although the mean proportion of erythroblasts with iron, was significantly higher among cases compared to surgical controls (Table 5.3).

Table 5.3: Results of erythroblast iron using the alternative grading method

	Cases n= 220	**Surgical Controls** n = 17	p-value
Mean proportion of erythroblasts with iron (%, SD)	25.6 (23.4)	13.2 (15.4)	<0.001
Range	0 – 91	0 – 44	–
Erythroblast iron deficient (%)	62.7	76.5	0.4

Cases and controls were classified into different iron status categories depending on their marrow smear examination using the alternative histological grading method as shown in Table 5.4. A large proportion of cases and surgical controls had functional iron deficiency (39.6% and 46.2% respectively). Surgical controls appeared to have a greater deficiency of iron stores, but this difference was not statistically significant.

Table 5.4: Bone marrow iron status category results using the alternative grading method

Iron status category	Cases (n=187)	Surgical Controls (n=13)	OR (95% CI)	p-value
Functional iron deficiency (%)	39.6	46.2	0.7 (0.2 – 2.9)	0.9
Normal iron (%)	31.0	7.7	5.4 (0.8 – 234.6)	0.1
Iron stores deficiency (%)	21.9	38.5	0.5 (0.1 – 1.9)	0.2
Functional and stores deficiency (%)	7.5	7.7	1.0 (0.1 – 44.4)	0.9

OR = [Odds in cases] / [Odds in controls]

To evaluate this classification of iron status using the alternative grading method, mean levels of common peripheral blood markers of iron store levels, namely ferritin and sTfR, and of inflammation, CRP, where determined for each iron category. Low levels of ferritin and high levels of sTfR signify deficiency of iron stores in the absence of inflammation. Mean level of Log ferritin was observed to be significantly lower in the children with deficiency of iron stores than in those with normal iron status and functional iron deficiency (Figure 5.10). Similarly, a higher mean level of sTfR in the

Figure 5.10: Error bar graph for mean Log ferritin levels for different iron status categories

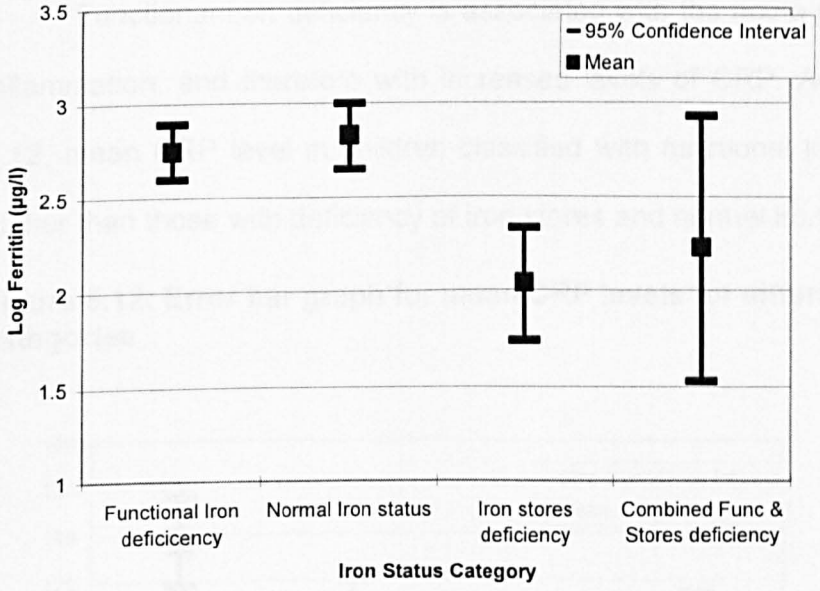
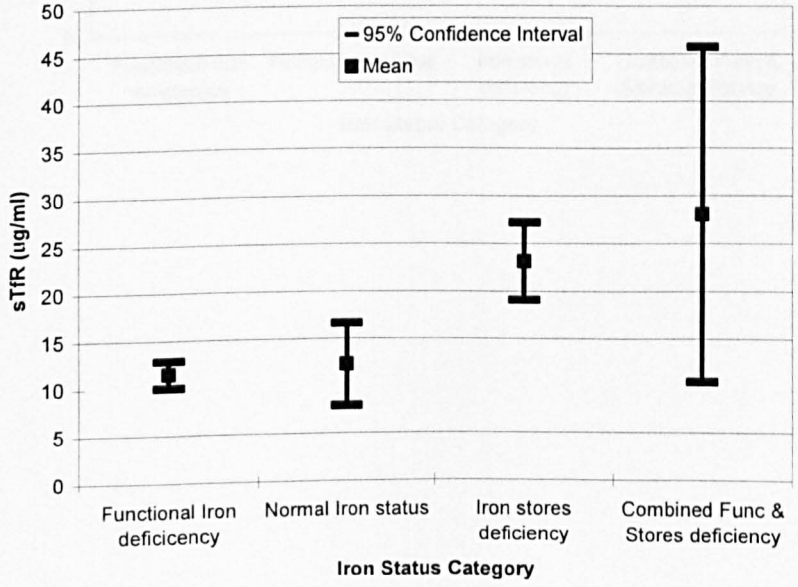


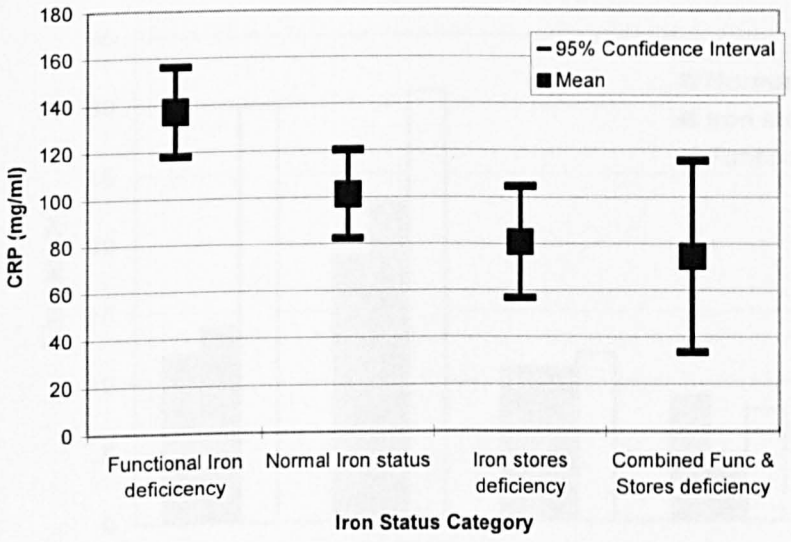
Figure 5.11: Error bar graph for mean sTfR levels for different iron status categories



children with deficiency of iron stores was demonstrated as compared to children with normal iron stores, or functional iron deficiency (Figure 5.11).

Functional iron deficiency is associated with the acute phase response to inflammation, and therefore with increased levels of CRP. According to Figure 5.12, mean CRP level in children classified with functional iron deficiency was higher than those with deficiency of iron stores and normal iron status.

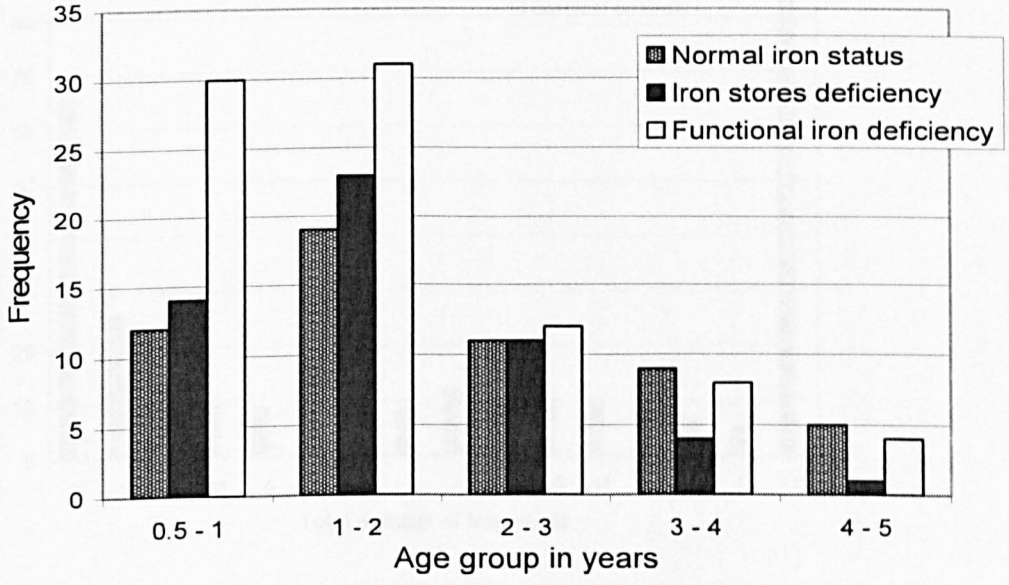
Figure 5.12: Error bar graph for mean CRP levels for different iron status categories



Age-specific iron status

There was an increase in frequency of cases with normal iron status, deficiency of iron stores and functional iron deficiency which peaked in the 1-2 year age group, and then gradually decreased in older children (Figure 5.13). The proportion of cases with functional iron deficiency was higher in the first two age groups (54% and 42% in 0.5-1 and 1-2 years age groups respectively).

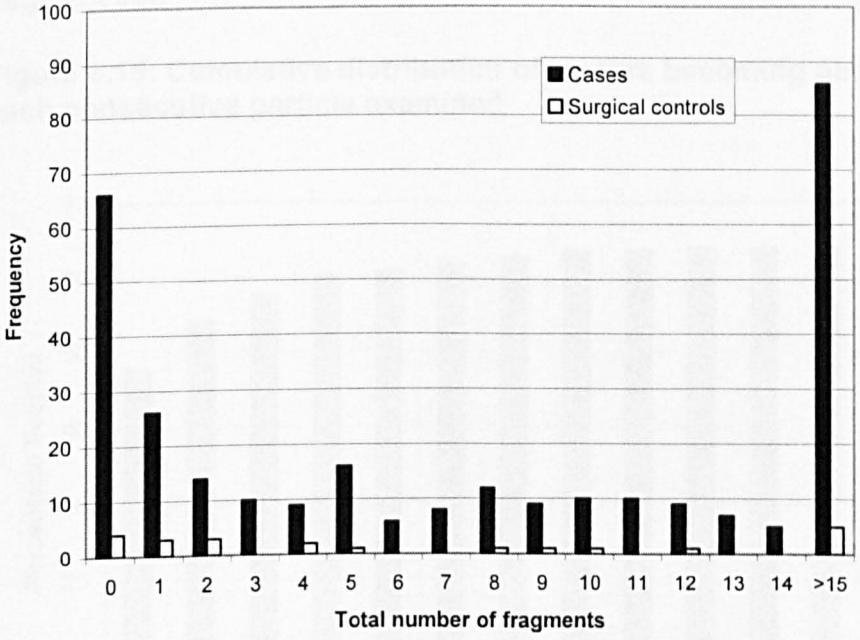
Figure 5.13: Distribution of cases with different iron status using the alternative grading method by age group



Minimum fragment number for deficiency of iron stores and grade determination

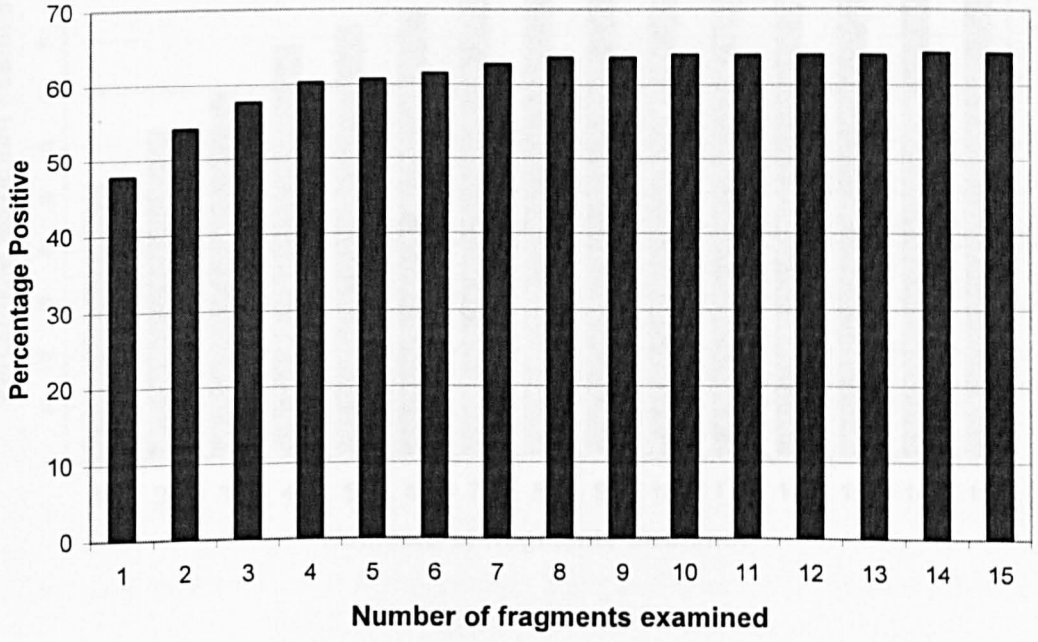
The distribution of the number of fragments per smear is shown in Figure 5.14. Forty-nine percent of samples had 8 or more fragments for iron examination.

Figure 5.14: Distribution of total number of bone marrow fragments per smear among cases and surgical controls



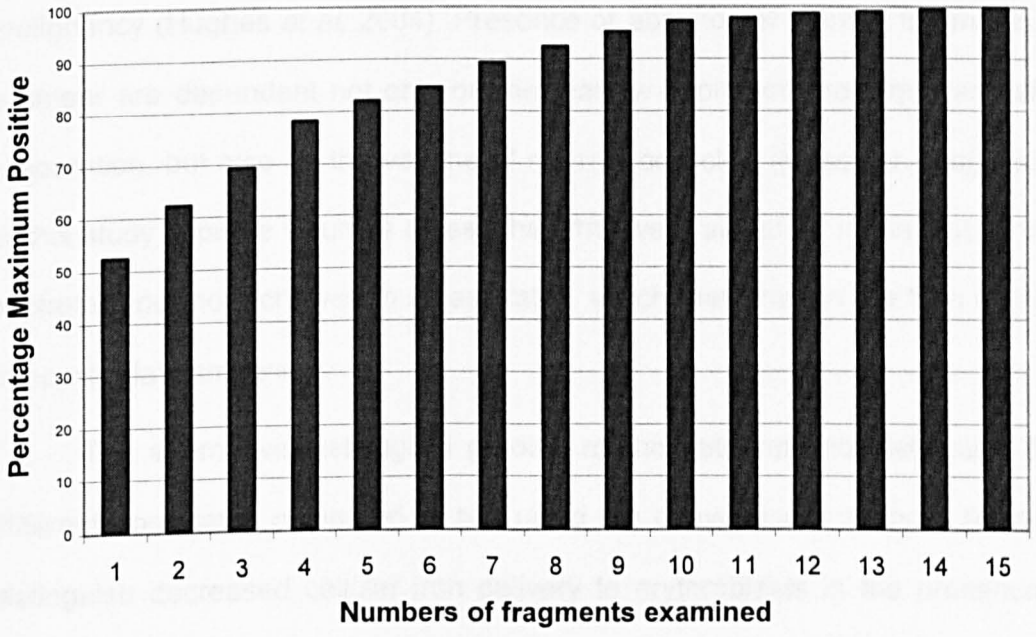
The cumulative proportion of bone marrow smears which became positive for fragment iron (i.e. smear grade 2 or more) with each consecutive fragment examined are shown in Figure 5.15. There was not a significant change in the percentage of positive smears when more than 8 fragments were examined. By examination of the 8th fragment 63.5% were positive versus 64.3% by 15th fragment. This showed that examination of at least eight fragments should allow a valid conclusion that a smear is either positive or negative for bone marrow fragment iron.

Figure 5.15: Cumulative distribution of smears becoming positive with each consecutive particle examined



The cumulative proportion of smears reaching their maximum positivity i.e. their highest smear score (using the conventional grading method), or highest fragment iron score (using the alternative grading method), with each consecutive fragment examined is shown in Figure 5.16. This shows that to be able to confidently determine the highest bone marrow smear score for fragments in over 95% of smears, it would be necessary to examine at least nine fragments.

Figure 5.16: Cumulative distribution of samples reaching maximum positivity with each consecutive fragment examined



5.5 DISCUSSION

Failure to obtain bone marrow on attempted marrow aspiration, or 'dry tap', may be due to an underlying bone marrow pathology. Studies have shown

that although some of these dry taps have normal marrow biopsies, the majority show significant marrow pathology which is frequently associated with fibrosis, or hypercellularity, or both (Humphries 1990). As marrow biopsies were not performed in the present study, fibrotic and hypercellular marrows could not be excluded as a cause for a dry tap, especially considering that such pathologies may be associated with severe anaemia.

There was a high proportion of marrow smears without fragments in this study, although similar findings have been previously reported in a study of patients with a range of haematological conditions including anaemia and malignancy (Hughes *et al,* 2004). Presence or absence of marrow fragments on a smear are dependent not only on the marrow aspiration technique and slide preparation, but also on the volume of marrow collected (Muschler *et al,* 1997). In this study aspirate volumes of less than 1ml were aimed for in the first syringe aspirated, but not achieved in all aspirates, which may explain the high number of aparticulate smears.

The alternative histological grading method attempts to distinguish four different iron states compared to two using the conventional method. Ability to distinguish decreased cellular iron delivery to erythroblasts in the presence of adequate iron stores, termed *functional iron deficiency*, as opposed to mere availability of iron in the reticular endothelial system, *iron stores*, may be particularly important in areas of high malaria transmission and infection. Functional iron deficiency is the altered iron metabolism pattern associated with anaemia of inflammation. It is important to differentiate anaemia of inflammation

from iron deficiency anaemia as management of the two conditions are different. Iron deficiency will respond to iron therapy while in functional iron deficiency there is need to treat the underlying source of infection, or inflammation.

Functional iron deficiency using the alternative histological grading was defined using marrow findings alone. Although the levels of CRP were mostly increased in all groups, the finding of significantly elevated levels among children classified as having functional iron deficiency by the alternative grading method supports the hypothesis that these children had anaemia of inflammation. Additionally, this group of children appeared to have adequate iron stores based on their similar levels of ferritin and sTfR compared to children with normal iron status. It must be noted that although ferritin and sTfR have been used as markers of level of iron stores, they may be affected by inflammation (detailed discussion in Chapter 9).

Nonetheless, the diagnosis of functional iron deficiency as employed in the alternative grading method using erythroblast iron alone, which has been used in some other studies (Baumgartner-Staubli and Beck 1977; Hastka *et al,* 1993), has limitations. Marrow smears were counter-stained with Safranin, giving a uniform pink background colour, which makes visualisation of different types of cells difficult, and hence may affect erythroblast iron assessment. The use of hematoxylin, or May-Grünwald-Giemsa, for counter-staining smears has been recommended as this provides more cellular detail (Hastka *et al,* 1993). Tham and Macon (1990) demonstrated that use of a silver stain to visualise erythroblast iron was more sensitive than Perl's stain, especially when the

marrow iron stores were low. However, the precise chemical basis for the silver staining is still unclear.

Some researchers have attempted to further describe erythroblast iron assessment compared to simply counting erythroblasts with iron. Baumgartner-Staubli and Beck (1977) developed a 'sideroblast score' which assigned an arbitrary value, ranging from 1 to 4 depending on the amount and morphology of iron granules, to each erythroblast with iron. However, there was a poor correlation between the sideroblast score and the marrow iron stores assessed using the conventional grading method.

The Malawi Ministry of Health guidelines are silent on whether to give iron as part of management of children with severe anaemia. In practise in Malawian hospitals, most children with severe anaemia will be routinely prescribed iron for one month post-discharge. Not all children receive or complete iron therapy due to poor availability of iron in health establishments and poor compliance to treatment. According to the present results, approximately 30% of severely anaemic children had deficiency of iron stores (Table 5.4). This implies that only a third of severely anaemic children presenting to hospital would benefit from iron therapy. Further research is needed to try to identify this sub-group of severely anaemic children with deficiency of iron stores by clinical, or with less invasive and costly laboratory investigations that could be utilised in resource-poor settings.

Figure 5.13 shows the contribution of iron depleted states as a cause of severe anaemia in different age groups of children. This represents the relative

importance of inflammatory versus non-inflammatory causes of severe anaemia as they vary with age. The risk of low iron stores as well as functional iron deficiency was highest in children aged 1-2 years. The rapid growth rate in infants especially in preterm and small-for-gestational age babies, increases iron requirements and tends to deplete body iron stores. By the end of the first year of life, a high proportion of children in developing countries are deficient in iron stores (Miller *et al,* 2003). The present study confirms the importance of targeting nutritional interventions to children in the first year of life (Menendez *et al,* 1997), and pregnant mothers (Miller *et al,* 2003; van Rheenen and Brabin 2004) in order to maintain fetal iron stores, before infants become iron deficient and anaemic.

Examination of marrow fragments for iron plays an important role in grading a marrow smear, but it is not clear how extensive this examination should be to distinguish between different fragment iron grades. Hughes *et al* (2004) carried out a detailed study of fragment iron among pathological and normal bone marrow aspirations from patients in the United Kingdom. They showed that a minimum of seven fragments was required for examination in order to establish the presence or absence of fragment iron, and a minimum of nine fragments to establish the maximum fragment iron score, which represents the overall marrow smear grade using the conventional iron grading method. Results of the present study, using a larger sample size and set in a malaria endemic area are in concordant with these earlier findings, therefore providing supporting evidence for its use in paediatric populations.

5.6 CONCLUSION

Results presented in this chapter show that the use of a detailed alternative bone marrow smear examination for iron can be more informative than the conventional grading method. This alternative histological grading method combines assessment of iron stores in fragments and reticular cells and cellular iron in red cell precursors. This enables classification of iron status into normal iron status, deficiency of iron stores and functional iron deficiency but has certain limitations.

The prevalence of deficiency of iron stores of 30% among severely anaemic children has important implications in relation to the role of 'blanket' iron treatment policies in children in Malawi and other malaria endemic countries. Iron intervention strategies should target the unborn child, and the first year of life, in order to prevent early onset of iron deficiency and anaemia in early childhood.

CHAPTER 6

ASSESSMENT OF IRON STATUS COMPARING BONE MARROW FLOW CYTOMETRY, MASS SPECTROMETRY AND MICROSCOPY

6.1 INTRODUCTION

Examination of the bone marrow for haemosiderin is regarded as the most sensitive and reliable diagnostic test for iron deficiency (Bain 2001). Absence of stainable iron in the marrow is considered to be the ultimate proof of decreased total body iron. Very few studies have addressed the accuracy of the test, and the diagnostic efficiency of other tests have been compared against this 'standard' (van den Broek *et al,* 1998; Means *et al,* 1999).

There have been some studies reporting that a finding of absent iron stores on microscopy was not always diagnostic of iron deficiency (Barron *et al,* 2001). Barron *et al* (2001) in a detailed study, reviewed pathological reports of absent stainable iron results and found that this was inaccurate in 30% of the reports. This represented the inter-observer difference associated with reading marrow iron smear. Secondly, after careful review of the 70% 'accurately' reported absent stainable iron results using extensive historical information and laboratory investigations, they found that approximately half (46%) were not iron deficient. However, the study was a carried out in patients with various haematological conditions, information was collected in retrospect and the exact

criteria used to establish iron deficiency after the review of the historical and laboratory information, was not mentioned.

Further evidence supporting the poor validity of bone marrow iron microscopy has come from repeated needle biopsy studies. Significantly different amounts of iron may be demonstrated in corresponding needle-biopsy specimen (Bain 2001). This suggests that iron in the bone marrow is unevenly distributed and therefore a negative marrow aspirate for iron, may not be representative of the true marrow iron content.

Due to these shortcomings there is need to re-evaluate the use of bone marrow iron microscopy and possibly explore other methods or tests to improve the accuracy of determining bone marrow iron content.

Recent advances in flow cytometry have allowed for the quantitation of various receptors on cell membranes. Flow cytometry is a technique used to study the expression of antigens on cell membranes such as the presence of receptors and proteins. Monoclonal antibodies raised against these antigens are coupled to fluorochromes. Incubation of these monoclonal antibodies with the cells of interest, result in the binding of the monoclonal antibody to its given antigen giving a fluorescent signal which can be detected by a flow cytometer.

A number of recent studies have suggested the importance of measuring the amount or density of antigen expression in addition to the frequency of cells expressing the antigen (Iyer *et al,* 1997). Fluorescence quantitation, in terms of the absolute numbers of fluorophore molecules per cell, can provide a more meaningful classification of various staining patterns and intensities and can be

used to establish standards for comparison in different laboratories and instruments.

Virtually all cells have transferrin receptors on their surface, but in the normal adult 80% are on the erythroid marrow. Density of these membrane-bound transferrin receptors (mTfR) on proliferating cells is related to the availability of iron, as deprivation of iron results in prompt induction of mTfR synthesis, and excess iron suppresses them (Beguin 2003). Therefore, quantitation of expression of mTfR on erythroid progenitor cells offers an opportunity to assess cellular iron status.

Recent technological advances in the field of chemical analysis has enabled wider use of mass spectrometry in mainstream chemistry and biochemistry laboratories with the introduction of commercially available, high performance instruments. Mass spectrometry, also called mass spectroscopy, is an instrumental approach that allows for the mass measurement of molecules. A mass spectrometer determines the molecular weight of chemical compounds by ionizing, separating, and measuring molecular ions according to their mass-to-charge ratio. The ions are generated by inducing either the loss or the gain of a charge. Once the ions are formed in the gas phase, they can be electrostatically directed into a mass analyzer, separated according to mass and detected as a mass spectrum according to molecular weight.

The use of mass spectrometry to determine bone marrow iron content has been previously reported (van Dieijen-Visser *et al,* 1991). Different methods of sample preparation and type of mass spectrometer were used compared to

those used in the present study. There is need to develop and evaluate bone marrow mass spectrometry as a potential test to measure bone marrow iron content.

In areas of high malaria transmission, there is an increased rate of malaria pigment deposition in tissues, including the bone marrow. Host haem, ingested by malaria parasites into an acidic food vacuole, undergoes digestion by a series of protease enzymes and is oxidised and incorporated into hemozoin (Egan *et al,* 2002). Hemozoin is phagocytosed by monocytes in the blood and therefore ends up in macrophages in tissue. It has long been known that hemozoin contains ferri-haem in a highly insoluble form (Egan *et al,* 2002). However, it still remains unclear the amount of iron sequestered in hemozoin and the duration it is unavailable for erythropoiesis.

6.2 OBJECTIVES

1. To determine bone marrow iron status using flow cytometry by measuring the expression of mTfR
2. To determine bone marrow iron status using mass spectrometry by measuring the total elemental iron
3. To compare bone marrow iron microscopy method of determining bone marrow iron status with flow cytometry and mass spectrometry

6.3 METHODS

Cases and surgical controls had a bone marrow aspirate performed. Approximately 4ml of bone marrow aspirate was collected for various bone marrow tests, including for flow cytometry, mass spectrometry and microscopy.

Flow cytometry

A FACSCalibur™ flow cytometer (BD Biosciences, Franklin Lakes, USA) with a 488-nm argon ion laser at the Wellcome Trust laboratories, Blantyre, was used to determine mTfR expression on erythroid progenitor cells. Laser Dye Styril-751 (LDS) was purchased from Applied Laser Technology Ltd (Maarheeze, The Netherlands). Monoclonal antibodies, CD71-PE (RVS10) and CD235a-FITC (CLB-AME-1) were purchased from Sanquin Reagents (Amsterdam, The Netherlands). Ficoll-Paque was purchased from Pharmacia Biotech (Uppsala, Sweden). Washing solution (500ml) was constituted from 450ml distilled water, 50ml phosphate-buffered saline (PBS), 0.2% bovine serum albumin (BSA) and 3ml 0.5molar EDTA. PBS, BSA and EDTA were purchased from BD Biosciences (Franklin Lakes, USA).

About 0.5-1ml of bone marrow sample, collected into an EDTA tube for flow cytometry analysis, was processed within 24 hours of aspiration. An array of various other monoclonal antibodies were used to study other cells, hence the volumes of bone marrow sample used, but CD71, CD235a and LDS were the specific monoclonal antibodies of interest in this study.

Bone marrow sample was added on 2.5ml Ficoll-Paque and centrifuged at 1500rpm for 40 minutes at 20°C. The interface was carefully collected and washed using Washing solution. The reconstituted sample was then added to a test tube with pre-added 5µl of CD71-PE and 5µl of CD235a-FITC, and incubated for 30 minutes in the dark before washing. Cells were reconstituted in washing solution and incubated for 5 minutes with 200µl of 1µg/ml LDS-751, before being read by the flow cytometer.

Cell Quest Pro™ software (BD Biosciences, Franklin Lakes, USA), run on a Macintosh computer, was used for cell acquisition and analysis. Cell acquisition was stopped after 50,000 events were detected within a pre-determined gate for mononuclear cells (Gate A) and information on cells was collected for analysis (Figure 6.1). During analysis, these mononuclear cells were plotted in a scattergram of LDS, which detects nucleated cells, vs. forward scatter (FW-SC). Populations of nucleated cells were gated as shown in Figure 6.2 (Gate B). These nucleated cells were further analysed in a scattergram of side scatter (SSC) vs. CD235a-FITC (Figure 6.3). CD235a is an erythroid cell lineage specific marker. CD235a positive cells were gated (Gate C) and analysed using a histogram plot of CD71-FITC in linear values (Figure 6.4). Events with fluorescent intensity less than 10^1 were taken as background noise (auto-fluorescence). The geometric mean fluorescence of the remaining events was recorded. All gates were adjusted manually for each patient by a reader who was blinded for patient characteristics.

Figure 6.1: Scattergram of Side scatter vs. Forward scatter

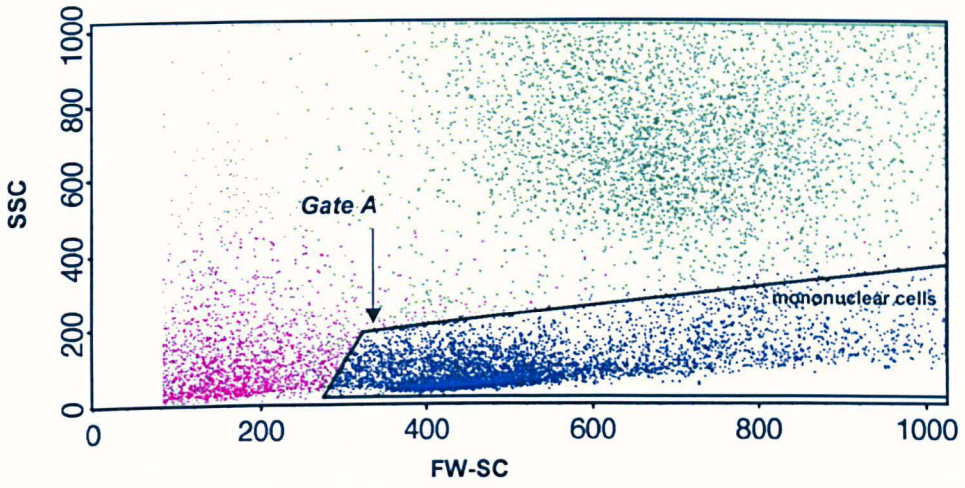


Figure 6.2: Scattergram of LDS vs. Forward scatter

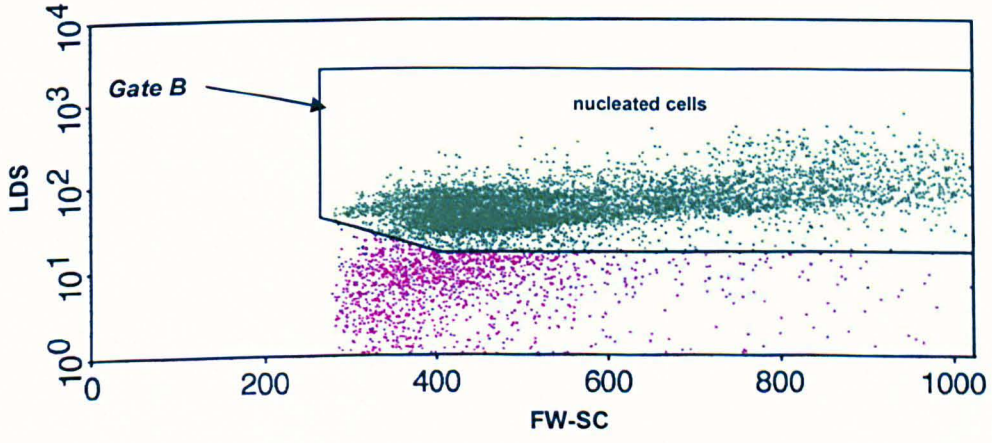


Figure 6.3: Scattergram of CD71-PE vs. CD235a-FITC

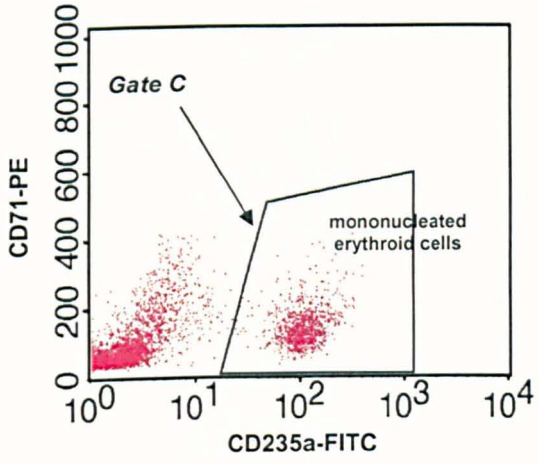
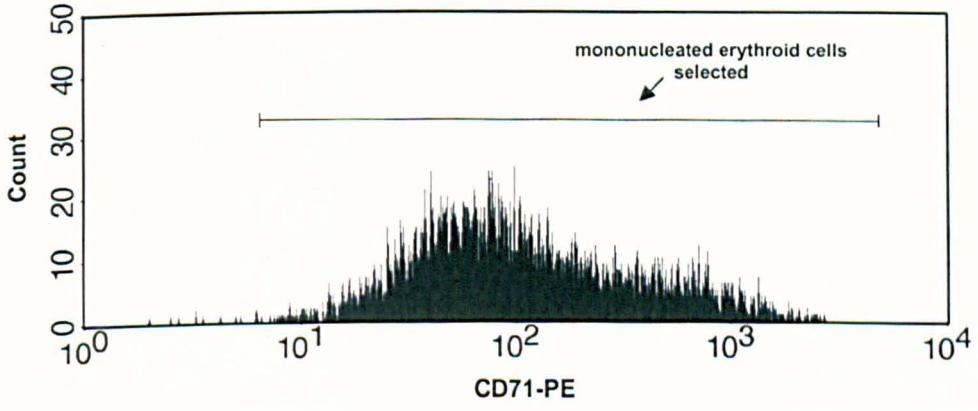


Figure 6.4: Histogram of CD71-PE on mononucleated erythroid cells



QuantriBRITE™ PE (BD Biosciences, Franklin Lakes, USA), a phycoerythrin fluorescence quantitation kit, was used to convert fluorescence intensity into actual numbers of CD71 molecules bound per cell. The kit contains a pellet of beads that have been conjugated with four known levels of phycoerythrin (PE). By running these beads at the same flow cytometer instrument settings as the assay, the FL2 axis can be converted into the number

of PE molecules bound per cell. Using known ratios of PE to antibodies ratio, the number of PE molecules bound per cell can be converted to antibodies per cell. A 1:1 conjugate-PE – monoclonal antibody ratio was used.

Mass spectrometry

Approximately 3 ml of marrow was centrifuged for 3 minutes at 2000rpm and plasma or serum was separated and stored for other tests, while the remaining bone marrow cells and stroma were stored at -80°C until sample preparation for mass spectrometry which was done at the Clinical Chemistry laboratories, University of Liverpool, United Kingdom.

The bone marrow sample preparation method for mass spectrometry analysis was adapted from D'Haese, Van der Vyer *et al* (1985) and piloted using bovine bone marrow. To avoid extraneous iron contamination, all glass and propylene ware instruments were soaked in 10% nitric acid and rinsed in de-ionised water before use. Sample preparation was carried out according to the following procedure:

1. At room temperature, the sample of bone marrow cells and stroma was transferred into a glass tube and placed in an electric oven at 100 to 110 °C for 12 hours (overnight) to dry the sample.
2. The sample was placed in silicate gel dessicator for 3-4 hours to avoid water absorption during the cooling phase. Sample was weighed using a balance (Mettler AC100, Mettler Instrumente AG, Zurich) accurate to 0.1mg.
3. 1ml of nitric acid was added to the sample before putting it in a hot water bath at 65°C. Sample was removed when a clear digest was observed.

4. The solution was transferred to a polymethyl pentane volumetric flask and adjusted to 50ml with de-ionised water. The concentration of elemental iron in this solution was determined using a Thermal-elemental PQ ExCELL Inductively-coupled plasma mass spectrometer (ICP-MS) (Thermo Electron Corporation, Windsford, UK). The results were expressed as weight of iron per weight of dried bone marrow.

Iron microscopy

A few drops of the 1ml bone marrow aspirate were used to make two marrow smears. Five percent potassium hexacyanoferrate and 5% hydrochloric acid mixed in equal volumes, was flooded on slides for 20 minutes before being washed with distilled water. Slides were counter-stained with Nuclear fast red solution for 5 minutes before washing and air-drying. Slides with positive iron stores (fragment iron grade ≥2), were included in each batch of stained slides as positive controls. Staining was done at the Wellcome Trust laboratories, Blantyre.

Bone marrow iron content was graded according to the alternative histological grading method which consists of grading iron stores (fragment and macrophage iron) and cellular iron (erythroblast iron) (Chapter 5). For purposes of comparison analysis in this chapter, which required continuous variables, fragment iron representing iron stores status and erythroblast iron representing cellular iron status were used.

Statistical analysis

Mean antibodies bound per cell, mean elemental iron concentration, mean fragment iron grade and mean erythroblast iron were compared between cases

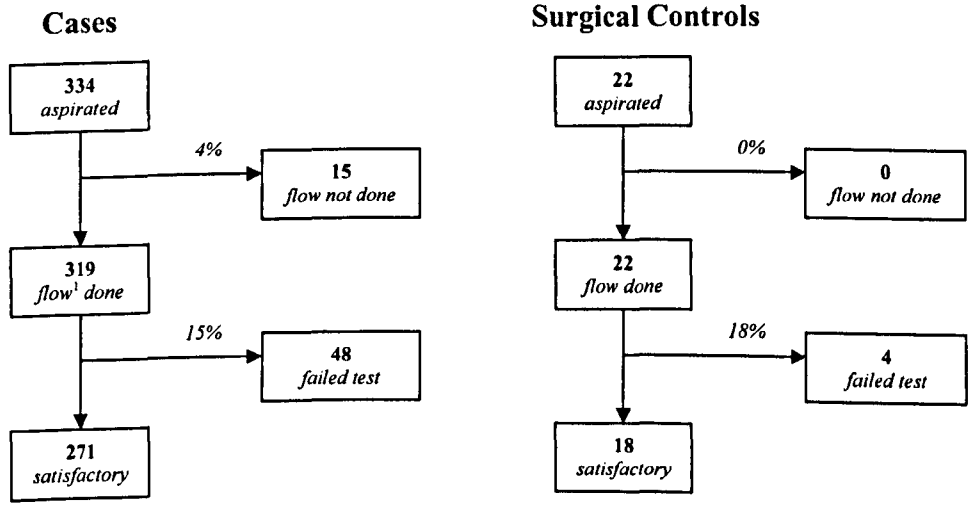
and surgical controls using Student T-test. The three bone marrow iron determination methods, namely flow cytometry, mass spectrometry and microscopy, were compared using Spearman's correlation coefficient.

6.4 RESULTS

Flow cytometry

Out of 334 cases that had a bone marrow done, 15 (4%) were not prepared for flow analysis (Figure 6.5). This was due to either insufficient or missing sample, or temporary unavailability of study staff to perform the flow cytometry analysis. Out of the samples that were prepared for flow cytometry, 48 (15%) were failed tests, due to either failure to add CD71, CD235a, or LDS, malfunctioning of flow cytometer, or missing tube.

Figure 6.5 : Flow chart showing the proportion of cases and surgical controls that were processed for flow cytometry



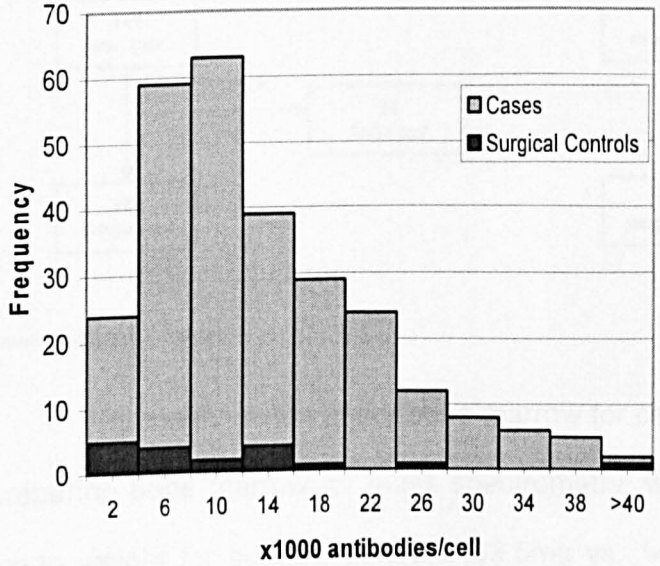
[1] flow cytometry analysis

The mean antibodies bound per cell (ABCs) for cases (12.5 x10^3 ABCs), was not significantly different from mean ABCs for surgical controls (12.3 x10^3 ABCs) (*p=0.5*) (Table 6.1). The distribution of ABCs among cases and surgical controls was skewed towards the left (Figure 6.6).

Table 6.1: Flow cytometry results for cases and surgical controls

	Cases	Surgical Controls	*p-value*
Antibodies bound per cell (x10^3)			
Mean (SD)	13.5 (9.0)	11.9 (13.3)	0.5
Median	11.1	8.3	
Range	0.6 – 60.1	1.6 – 58.2	

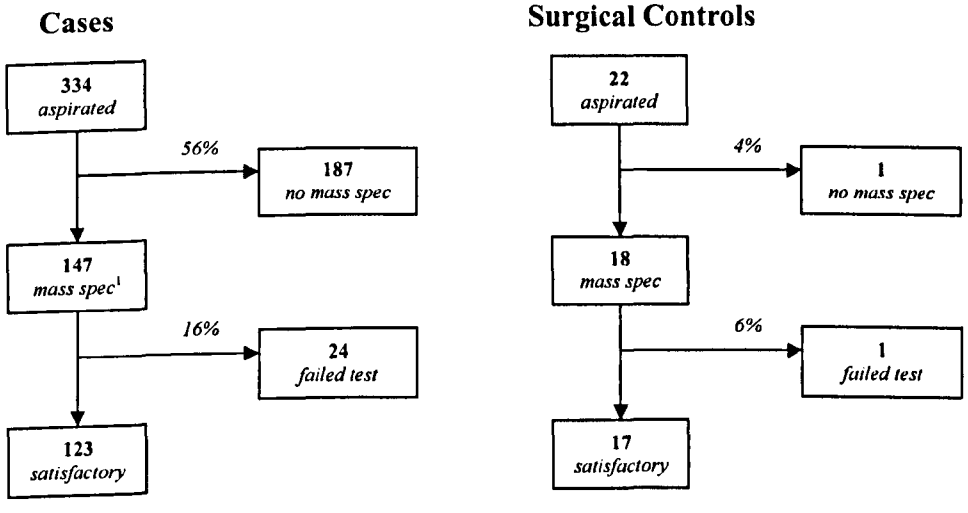
Figure 6.6: Distribution of antibodies bound per cell for cases and surgical controls



Mass spectrometry

Collection of bone marrow samples for iron determination using mass spectrometry started 12 months after the commencement of recruitment of cases, due to a delay in obtaining local ethical approval. A total of 187 (56%) of cases did not have samples for mass spectrometry collected and a further 24 (16%) had insufficient amount of marrow, producing failed test results (Figure 6.7).

Figure 6.7: Flow chart showing the proportion of cases and surgical controls that were processed for mass spectrometry



[1] mass spectrometry analysis

The mean weight of dry bone marrow for cases, at the end of the phase of preparing bone marrow for mass spectrometry, was significantly lower than the mean weight for surgical controls (63.5mg vs. 147.7mg, $p < 0.0001$). This may have been a direct result of collecting larger volumes of bone marrow in surgical

controls as compared to cases. The mean bone marrow weight for cases was 318mg before drying while for surgical controls was 553mg (difference, *p =0.03*).

The distribution of elemental iron concentration for both cases and surgical controls was slightly skewed to the left. The mean concentration for cases was 2.72 µg/mg marrow, and was not statistically different from surgical controls (Mean concentration 2.45 µg/mg, *p =0.7*) (Table 6.2).

Figure 6.8: Distribution of elemental iron in the bone marrow for cases and surgical controls

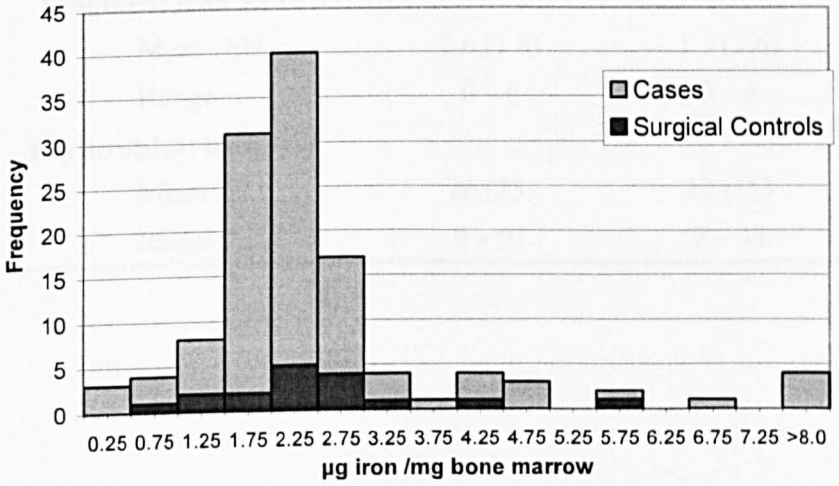


Table 6.2: Mass spectrometry results for cases and surgical controls

	Cases	Surgical Controls	*p-value*
Elemental iron (µg iron/mg marrow)			
Mean (SD)	2.72 (2.77)	2.45 (1.21)	0.7
Median	2.12	2.26	
Range	0.14 – 21.78	0.94 – 5.95	

Iron microscopy

Bone marrow iron smears were graded for fragment iron and erythroblast iron and the results are summarised in Table 6.3. These results have also been presented in Chapter 5. Out of a total of 381 cases, 334 (88%) had bone marrow aspirations performed, with 303 (91%) marrow smears prepared.

Table 6.3: Fragment and erythroblast iron results for cases and surgical controls

	Cases	Surgical Controls	*p-value*
Fragment iron score			
Mean (SD)	2.6 (1.8)	1.3 (1.6)	0.004
Range	0 – 6	0 – 4	
Erythroblast iron (%)			
Mean (SD)	26 (23)	13 (15)	0.006
Range	0 – 91	0 – 44	

Comparison of bone marrow iron determination methods

Fragment Iron correlated significantly with erythroblast iron (r = 0.38, *p<0.001*) but not with ABCs or elemental iron (Table 6.4). Erythroblast iron was negatively correlated with ABC's but this was not significant (r = -0.10, *p =0.2*). Flow cytometry and mass spectrometry showed no significant correlations in their iron values (r = 0.09, *p =0.4*).

Table 6.4: Correlations[1] between bone marrow iron determination methods (microscopy, flow cytometry, mass spectrophotometry) among cases

	Microscopy		Flow cytometry	Mass spectrometry
	Iron fragment score	Erythroblast iron	Antibodies bound per cell	Elemental iron concentration
Iron fragment score	r = 1	r = 0.38 (p<0.001) N= 196	r = 0.04 (p=0.6) N=195	r = 0.14 (p=0.2) N=91
Erythroblast iron	–	r = 1	r = -0.10 (p=0.2) N=181	r = 0.00 (p=0.9) N=85
Antibodies bound per cell	–	–	r = 1	r = 0.09 (p=0.4) N=98
Elemental iron concentration	–	–	–	r = 1

[1] Spearman correlation coefficients

6.5 DISCUSSION

Recent progress in flow cytometry and the development of new monoclonal antibodies have made it possible to study the number of transferrin receptors on different cells. In this study, the expression of transferrin receptors on erythroblasts was measured by the use of LDS, for selection of nucleated cells, CD235a, for defining erythroblasts in bone marrow, and CD71, for detection of the transferrin receptor. Cells which were reacting with all three monoclonal antibodies were the cells of interest, erythroblasts, which are active in haemoglobin synthesis.

There have been very few studies measuring the expression of transferrin receptors by using flow cytometry. Kuiper-Kramer *et al* (1998) reported a higher expression of transferrin receptors in iron deficient patients than in a group of healthy volunteers. Transferrin receptor expression was measured using the mean fluorescent intensity of a population of erythroblasts in relation to standard beads with known numbers of molecules of fluorochromes. This method may be used to compare expression among different groups of individuals but does not give the absolute number of receptors on the cell.

The use of QuantriBRITE™ beads offers a better opportunity to express the intensity of monoclonal antibody-antigen binding. In this study the mean antibodies bound per cell in cases was 13.5 $x10^3$. Due to lack of published research on the use of QuantriBRITE™ beads to measure mTfR expression, there are no reference values. There is a critical need for the development of reference values for children with normal iron status. Surgical controls in the

present study, were recruited from a population of healthy children, however, the recruitment criteria did not enable the exclusion of children with iron deficiency. Nevertheless, the results suggest that there was similar expression of transferrin receptors on erythroblasts.

There are some factors that may affect the accuracy of mTfR measurements. Calibration of a flow cytometer with beads gives estimates of the number of molecules of PE bound per cell. The more desirable unit, antibodies bound per cell (ABC), requires knowledge of the PE:monoclonal antibody ratio of the conjugate, i.e. the number of CD71-PE molecules that bind to a transferrin receptor. While 1:1 ratio is often assumed, this may not always be the case (Iyer *et al,* 1997). An additional source of inaccuracy may be instrument variability. It has been reported that within assay variability on a single FACSCalibur™ flow cytometer was ±2% and variability across instruments was ±5% (Iyer *et al,* 1997). As only one instrument was used in the present study, this error may have been minimised.

Published studies on chemical analysis of total bone marrow iron date back to the early 1960s when bone marrow microscopy methods were being developed. Most of these earlier studies used radio-isotope measurements. Gale *et al* (1963) in a study among patients undergoing thoracotomy due to various conditions, reported iron concentration in marrow to range from 0.01 to 7.94µg/mg. Johansson *et al* (1970), using radio-isotope iron ^{59}Fe, estimated the average iron concentration in biopsy bone marrow as 2.0µg/mg. However, they did not report their findings for healthy volunteers separately from patients and

patient diagnoses were not stated. Findings of mean concentration of 2.1µg/mg among cases and 2.3µg/mg among surgical controls of elemental iron in the present hospital-based study, were comparable to earlier studies, but reference levels from a healthy population with normal iron status are unavailable.

Use of mass spectrometry for the determination of iron in cortical bone and liver has been extensively reported but its use in bone marrow has been limited (D'Haese *et al,* 1985). This may be due to the fact that bone marrow, an admixture of serum and cells, poses more of a challenge than a homogenous tissue or fluid. Previous studies have expressed bone marrow iron in terms of weight of iron 'per dry or wet weight of marrow', or 'per weight of protein' (Trubowitz *et al,* 1970; van Dieijen-Visser *et al,* 1991). Iron expressed 'per wet weight of marrow' has been shown to correlate well with iron expressed 'per weight of protein', however one may argue that the haemo-dilution of bone marrow samples especially in aspirates can vary (Trubowitz *et al,* 1970). Iron content in blood, consisting of serum iron and haem iron cannot be completely excluded during marrow preparation for mass spectrometry.

There was lack of correlation between any of the bone marrow iron determination methods. This may be due to several reasons. Firstly, it might be as a result of poor diagnostic efficiency of either of the tests. Microscopy has already been reported to have a high rate of false negative results (Barron *et al,* 2001). The sensitivity and specificity of flow cytometry and mass spectrometry in determining iron deficiency is not known. If normal reference limits were available, it may have been possible to determine the diagnostic efficiency of all

three tests without using a 'gold standard' test, by using Bayesian or Latent Class statistical analysis (Moayyedi *et al,* 2004).

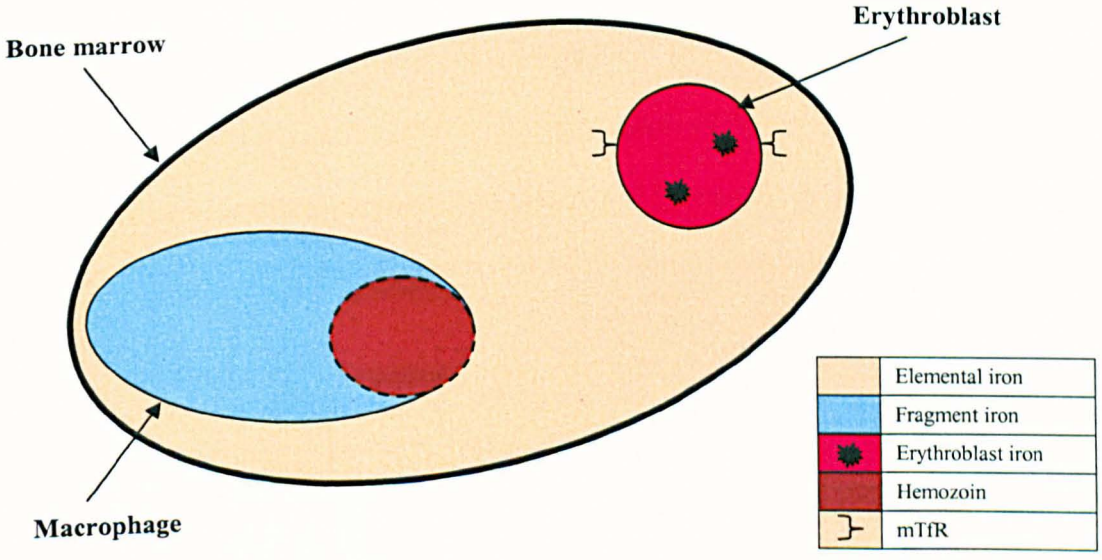
A second reason for non-correlation of the three methods may be that the relation between them is not linear. Van Djieijen-Visser *et al* (1991) reported that ferritin concentration increased with an increasing total liver iron content until a saturation of ferritin appeared to be reached at about 3g ferritin per kg protein. Although scatter plots were used in the present study to identify any relationship between tests, a non-linear relationship may still exist.

Bothwell (1980) noted that haem iron in the form of haemoglobin, myoglobin and haem enzymes may influence total iron measurements. Ignoring the contribution of haem iron may not significantly affect the accuracy of the methods when iron overload is present, however problems may arise when the iron concentration in the particular tissue is low. In the present study where a large proportion of children are assumed to be iron deficient, haem iron was not determined separately. This may have affected the interpretation of the mass spectrometry iron measurements hence a cause of non-correlation between the different bone marrow iron tests.

Another important reason that may explain the non-correlation findings between bone marrow iron tests is that each test is determining iron in different 'compartments' of the bone marrow. A more detailed and comprehensive understanding of each test and iron distribution in the bone marrow is necessary. Figure 6.9 gives a schematic illustration of the different iron 'compartments' measured by the bone marrow tests.

understanding of each test and iron distribution in the bone marrow is necessary. Figure 6.9 gives a schematic illustration of the different iron 'compartments' measured by the bone marrow tests.

Figure 6.9: Schematic representation of bone marrow iron measured by flow cytometry, mass spectrometry and microscopy



The size and importance of iron sequestered in hemozoin remains unknown. It has been shown that when an erythrocyte is parasitised by *P. falciparum*, 61% of erythrocyte iron is the trophozoite, of which 81% is in the form of hemozoin (Egan, Combrinck *et al* 2002). In the present study, 94% of cases had hemozoin visible on bone marrow iron smear (Figure 5.7). If this pool or iron is appreciably large, it may account for the non-correlation of iron measured by mass spectrometry, flow cytometry and microscopy.

Mass spectrometry iron results of the present study were comparable to previous studies, although there were no reference limits for non-iron deficient population. Mass spectrometry, despite its limitations, may be a reasonable option to microscopy to measure bone marrow iron.

There was no correlation of bone marrow iron measurements using flow cytometry, mass spectrometry, or microscopy. Iron sequestered in hemozoin may be an important confounder in determination of bone marrow iron content . There is need for more research into the role of hemozoin in iron metabolism.

CHAPTER 7

INFLUENCE OF INFLAMMATION ON SOLUBLE TRANSFERRIN RECEPTOR AND OTHER BIOCHEMICAL IRON MARKERS

7.1 INTRODUCTION

Biochemical iron markers are commonly used to determine iron status in the absence of bone marrow iron assessment as blood collection is less invasive than collecting marrow. Serum ferritin has been shown to be a good indicator of body iron stores with low levels reflecting depleted iron stores. However, in the presence of infectious or inflammatory conditions, ferritin is elevated (Trey and Kushner 1995). Some studies have further investigated the influence of malaria on markers of iron status (Das *et al,* 1997; Beesley *et al,* 2000). Das *et al* (1997), using albumin and caeruloplasmin as inflammatory markers, found that ferritin and other iron markers (serum transferrin, transferrin saturation) were altered during both asymptomatic and symptomatic malaria .

One way of controlling for a raised serum ferritin concentration resulting from infection would be to use the concentration of another acute phase protein to identify individuals whose measurements of both indicators are above an agreed threshold. This approach is not considered feasible in many parts of sub-Saharan Africa where people are commonly infected with malaria, as it would lead to the exclusion of the majority of individuals screened, hence other approaches are needed (WHO/CDC 2004). An alternative approach is to

determine the magnitude of the change in ferritin levels during inflammation and to correct for this by standardisation. Coenen, van Dieijen-Visser *et al* (1991) constructed nomograms of ferritin levels for different levels of CRP, erythrocyte sedimentation rate (ESR) and fibrinogen, but these were poor predictors of bone marrow iron status (Coenen *et al,* 1991).

Increased bone marrow iron stores have been associated with shorter survival times in HIV infected individuals (Gordeuk *et al,* 2001). Due to the depressed immune system, HIV infected children are prone to bacterial and other opportunistic infections (Lalloo and Amod 2005). However, it remains unclear the degree to which HIV infection influences biochemical markers of iron status.

sTfR has been extensively reported to be elevated in iron deficiency (Huebers *et al,* 1990; Cook *et al,* 1993). The rationale is that transferrin receptors, which mediate cellular uptake of iron, are expressed in proportion to the cell requirement for iron and to the erythroid progenitor mass. Membrane-bound transferrin receptor is subjected to proteolysis that generates a soluble form of the receptor (sTfR), which circulates in plasma. Since 80% of iron utilization is normally in the erythron, the concentration of sTfR in serum is proportional to the erythroid progenitor mass (Beguin 2003). Hence, erythropoietic activity of the bone marrow is an important determinant of sTfR levels. Decreased sTfR levels occur in situations characterized by erythroid hypoplasia, while haemolytic anaemia is associated with increased sTfR levels (Huebers *et al,* 1990). Performance of sTfR as an indicator for iron status has not

been investigated in severe anaemia where iron deficiency, malaria (through haemolysis) and other inflammatory conditions (through impaired erythropoiesis) may be the main aetiological factors.

7.2 OBJECTIVES

This study was carried out among severely anaemic children in Malawi to;

1. Verify whether biochemical iron markers are altered by inflammation in Malawian severely anaemic children
2. Determine whether biochemical iron markers are altered by malaria
3. Determine the main factors that influence sTfR levels among children with severe anaemia living in a malaria endemic area

7.3 METHODS

Plasma samples were used to determine ferritin using electrochemiluminescence immunoassay (Modular Analytics E170, Roche Diagnostics, Switzerland, detection limit 0.5µg/l) and serum iron using colorimetric assay (Modular P800, Roche Diagnostics, Switzerland, detection limit 0.9µmol/l). Immunoturbidimetric assay (Modular P800, Roche Diagnostics, Switzerland) was used to determine serum transferrin (detection limit 0.1g/l), haptoglobulin (detection limit 0.20g/l) and CRP (detection limit 1.0mg/l). TIBC was calculated from serum transferrin, and transferrin saturation from serum iron and TIBC. sTfR was measured using an enzyme immunoassay (Ramco Laboratories, TX, USA, detection limit 1.0µg/l). Approximately 100µl of EDTA

blood was used to measure red cell indices. These indices including MCV, absolute reticulocyte count, red cell count, hematocrit, was measured using a coulter counter (Beckman Coulter, Durban, South Africa). Reticulocyte count, the percentage of reticulocytes of all red blood cells, was calculated as a percentage (absolute reticulocyte count ÷ red cell count x 100%). Reticulocyte Index (RI) which corrects reticulocyte count for the degree of anaemia was calculated according to the formulae:

$$RI = \text{Reticulocyte count (\%)} \times \frac{\text{Patient Haematocrit}}{0.33}$$

A cut-off limit for inflammation was CRP ≥8mg/l (McGuire *et al,* 1996; Verhoef *et al,* 2001). Using individual biochemical iron markers, ferritin <30µg/l (WHO 2001), serum iron <3.6µmol/l, serum transferrin >3.6g/l, TIBC >72µmol/l (laboratory reference values), transferrin saturation <16% (WHO 2001), sTfR >8.3mg/l (test kit reference value) and MCV <67fl (1-2 year old children), <73fl (2-5 year old children) (WHO 2001) were inidividually taken as indicative of iron stores deficiency.

All tests except sTfR, MCV and RI were performed at the University of Amsterdam laboratories, Netherlands. sTfR, MCV and RI was done at the Wellcome Trust laboratories, Blantyre.

7.4 RESULTS

The results of biochemical determinations are shown in Table 7.1. Due to skewed distributions of CRP and ferritin, the median is a more informative measure of central tendency than the mean and showed decreasing levels of

CRP and ferritin from cases to surgical controls. There is was no significant variation of sTfR among study groups.

Table 7.1: Biochemical markers of iron status and inflammation by study group

	Cases	Hospital controls	Community Controls	Surgical Controls
CRP mg/l (N)	346	334	321	23
Mean (SD)	111.4 (84.8)	57.5 (66.5)[1]	22.2 (38.3)[2,4]	6.5 (9.8)[3,5,6]
Median	97.7	33.9	7.8	2.3
Range	1.0 – 450.0	1.0 – 341.2	1.0 – 303.1	1.0 – 37.6
Ferritin µg/l (N)	220	267	266	21
Mean (SD)	729.2 (1528.1)	161.7 (445.7)[1]	80.9 (111.8)[2,4]	37.9 (34.9)[3]
Median	340.0	60.0	50.5	32.0
Range	2 – 15015	0 – 6341	3 – 1259	6 – 154
Serum iron µmol/l (N)	278	319	304	22
Mean (SD)	16.0 (15.7)	6.5 (5.9)[1]	9.3 (6.8)[2,4]	9.6 (5.1)[3,5]
Median	10.1	4.8	7.9	7.9
Range	0 – 81.3	0 – 52.0	1.2 – 66.3	4.2 – 22.9
Serum transferrin g/l (N)	315	325	312	22
Mean (SD)	2.2 (0.7)	2.6 (0.6)[1]	2.6 (0.6)[2]	2.7 (0.6)[3]
Median	2.1	2.7	2.7	2.5
Range	0.3 – 4.3	0.6 – 4.4	0.4 – 4.7	1.9 – 4.3
TIBC µmol/l (N)	315	325	312	22
Mean (SD)	41.5 (12.7)	50.5 (11.3)[1]	50.2 (11.1)[2]	51.0 (11.5)[3]
Median	40.7	51.1	50.5	47.3
Range	7.8 – 79.0	13.8 – 82.1	10.6 – 86.2	36.8 – 80.5
Transferrin Saturation % (N)	278	314	299	22
Mean (SD)	41.4 (39.7)	14.2 (15.4)[1]	19.3 (14.9)[2,4]	20.2 (11.8)[3]
Median	26.8	8.6	16.0	19.2
Range	0 – 161	0 – 120	3 – 123	6 – 55
sTfR mg/l (N)	354	276	263	21
Mean (SD)	17.4 (15.8)	15.2 (8.7)[1]	15.0 (8.1)[2]	14.7 (11.4)
Median	13.3	13.2	13.5	12.5
Range	1.4 – 126.0	1.0 – 78.1	1.3 – 78.0	4.5 – 60.2

[1] $p<0.05$ between Cases and Hospital controls
[2] $p<0.05$ between Cases and Community controls
[3] $p<0.05$ between Cases and Surgical controls
[4] $p<0.05$ between Hospital and Community controls
[5] $p<0.05$ between Hospital and Surgical controls
[6] $p<0.05$ between Community and Surgical controls

Figure 7.1: Distribution of CRP (mg/l) by study group

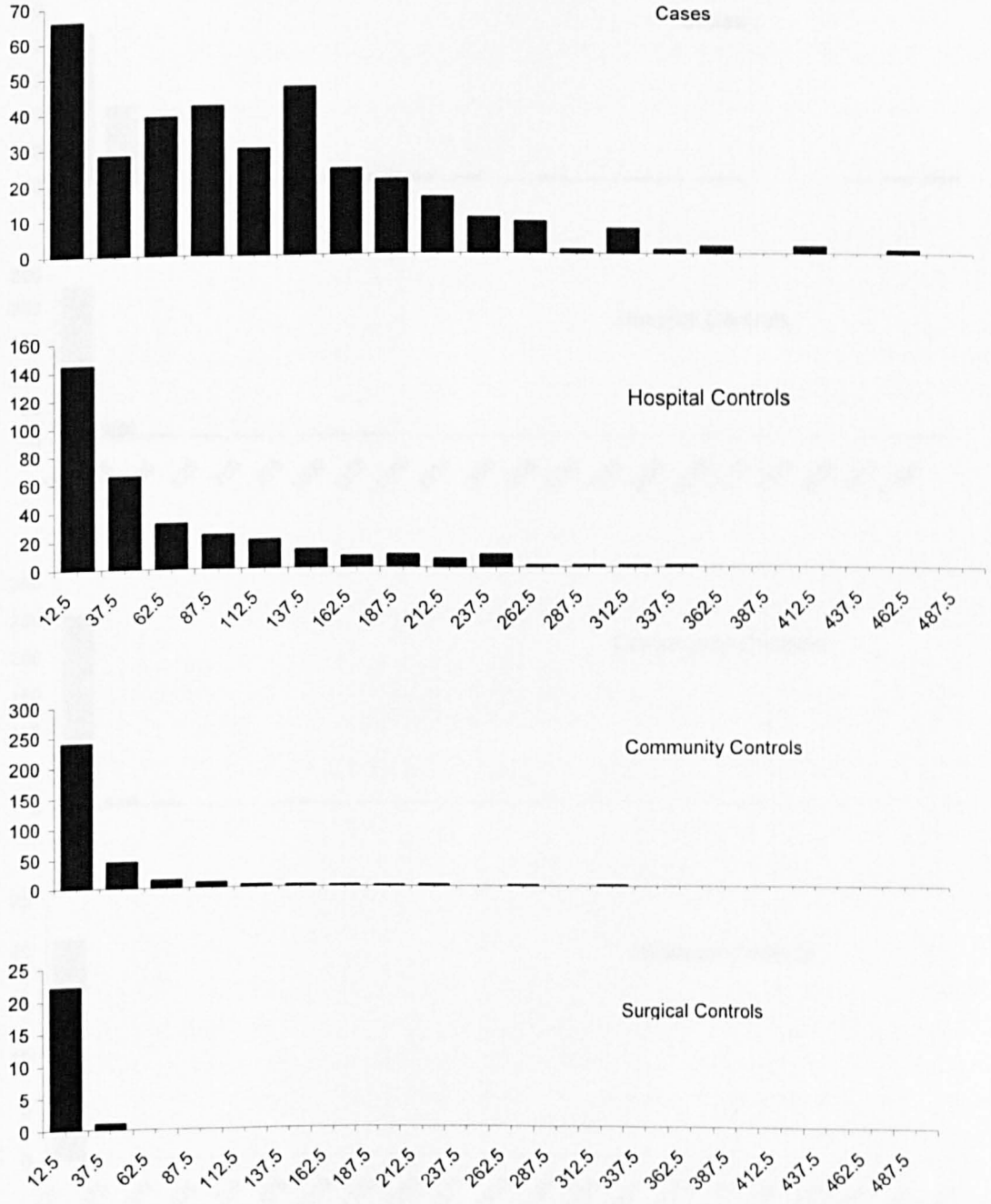


Figure 7.2: Distribution of ferritin (µg/l) by study group

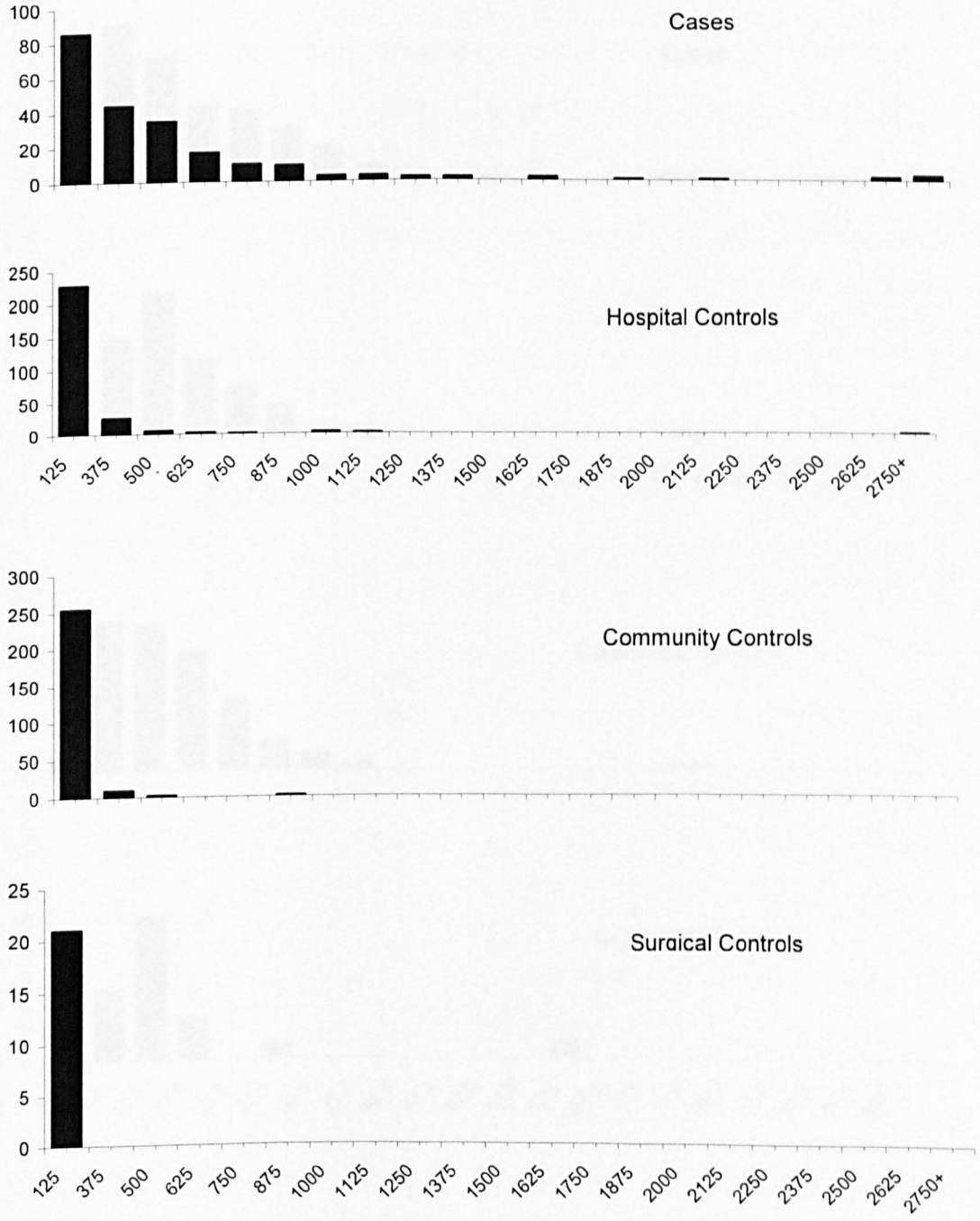
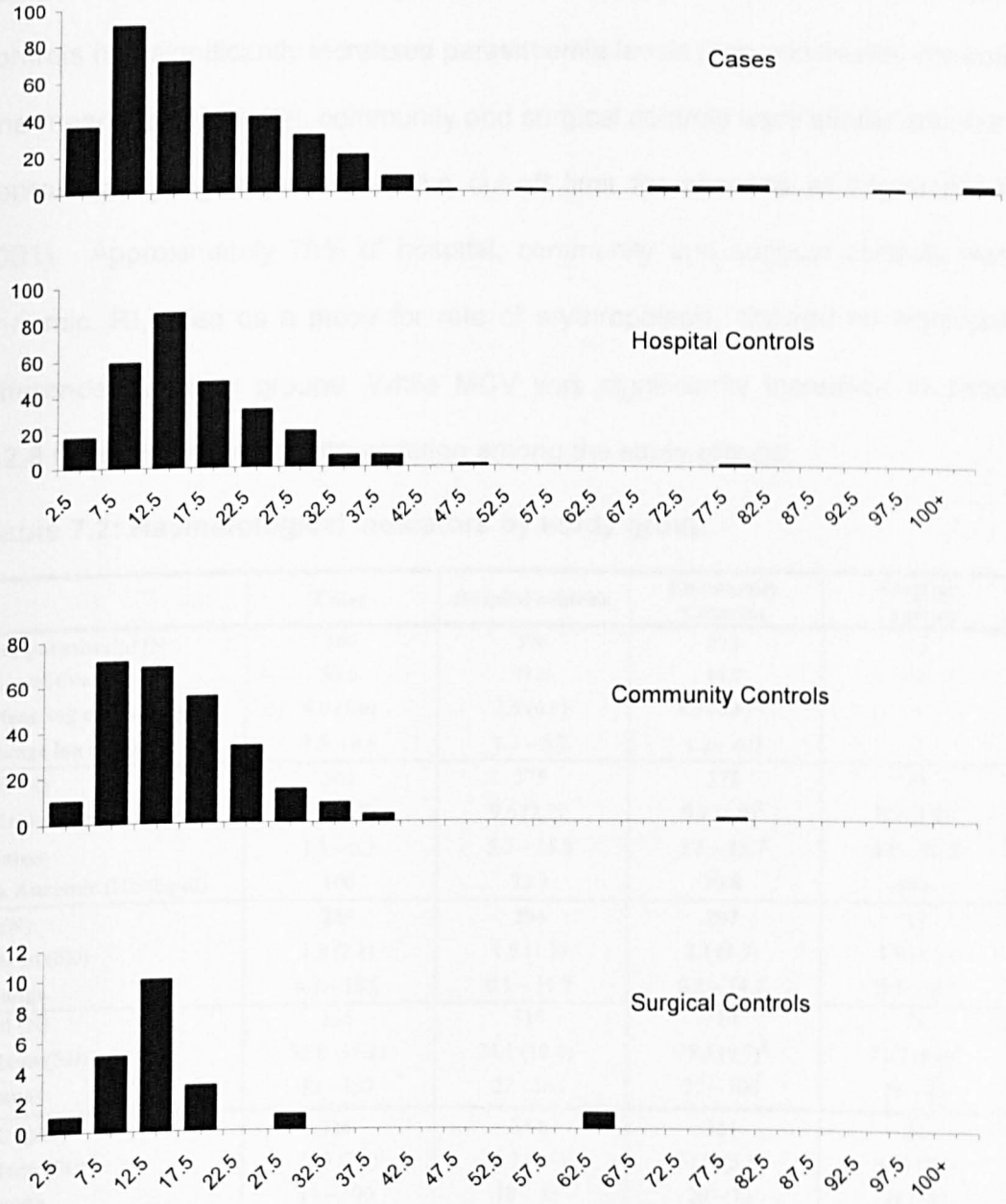


Figure 7.3: Distribution of sTfR (µg/ml) by study group



Malaria parasitaemia was more prevalent among cases (59.5%) as compared to hospital and surgical controls (Table 7.2). Cases and hospital controls had significantly increased parasitaemia levels than community controls. The mean Hb of hospital, community and surgical controls were similar and were approximately 1g/dl lower than the cut-off limit for anaemia of 11g/dl (WHO 2001). Approximately 70% of hospital, community and surgical controls were anaemic. RI, used as a proxy for rate of erythropoiesis, showed no significant difference by study groups. While MCV was significantly increased in cases (82.8 fl), MCHC showed little variation among the study groups.

Table 7.2: Haematological indicators by study group

	Cases	Hospital controls	Community Controls	Surgical Controls
Malaria parasites/µl (N)	380	376	374	23
% positive	59.5	41.0	44.7	0
Mean log count (SD)	4.0 (1.0)	3.9 (0.9)	3.5 (0.8)[2,4]	–
Range log count	1.5 – 6.4	1.7 – 5.7	1.2 – 6.0	–
Hb g/dl (N)	381	375	373	23
Mean (SD)	3.6 (0.8)	9.6 (2.2)[1]	9.9 (1.9)[2]	9.9 (1.8)[3]
Range	1.3 – 6.3	5.2 – 15.8	5.5 – 15.7	5.9 – 12.5
% Anaemic (Hb<5g/dl)	100	73.9	70.8	69.6
RI % (N)	289	296	297	17
Mean (SD)	1.9 (2.1)	1.9 (1.7)	2.1 (1.7)	1.9 (1.3)
Range	0.1 – 18.6	0.1 – 11.7	0.2 – 14.7	0.1 – 4.7
MCV fl (N)	316	315	324	21
Mean (SD)	82.8 (15.2)	74.1 (10.0)[1]	75.3 (9.7)[2]	71.2 (6.6)[3]
Range	52 – 137	27 - 104	27 – 100	59 – 83
MCHC (g/dl)	316	314	324	21
Mean (SD)	32.9 (7.8)	31.7 (4.3)[1]	31.9 (5.3)	33.3 (3.5)
Range	17 – 100	18 – 56	20 – 73	28 – 40

[1] $p<0.05$ between Cases and Hospital controls
[2] $p<0.05$ between Cases and Community controls
[3] $p<0.05$ between Cases and Surgical controls
[4] $p<0.05$ between Hospital and Community controls
[5] $p<0.05$ between Hospital and Surgical controls
[6] $p<0.05$ between Community and Surgical controls

To investigate the effect of inflammation on biochemical markers, CRP was used as a marker of inflammation to distinguish two groups of cases based

on the cut-off of ≥8mg/l. The mean of individual iron markers for each of these groups were compared using Students T-test. A similar analysis was done to investigate the effect of malaria parasitaemia on iron markers (Table 7.3).

Among the group of children with inflammation (CRP ≥8mg/l), there was a substantial increase in mean ferritin level which was consistent with an acute phase response (Table 7.4). Mean serum iron level was higher in children with inflammation. This was also reflected in an increased transferrin saturation concentration (Transferrin saturation = serum iron + TIBC). Further analysis (not presented) showed that the increased serum iron was not associated with increased bone marrow iron stores (using the conventional iron grading method).

Table 7.3: Differences in mean iron measures between cases with and without inflammation and malaria parasitaemia

Iron markers	Inflammation[1]			Malaria parasitaemia		
	Absent	Present	*p-value*	Absent	Present	*p-value*
Ferritin (µg/l)	147.4	817.5	<0.001	769.9	694.0	0.7
Serum iron (µmol/l)	10.4	16.7	0.008	17.1	15.2	0.3
Serum transferrin (µmol/l)	2.8	2.1	<0.001	2.1	2.2	0.9
TIBC (g/l)	52.6	40.3	<0.001	41.4	41.6	0.9
Transf Sat[2] (%)	20.6	43.9	<0.001	43.1	40.0	0.5
sTfR (mg/l)	42.3	14.4	<0.001	20.0	15.7	0.02
MCV (fl)	77.4	83.6	0.5	82.6	83.0	0.8
MCHC (g/dl)	30.8	33.0	0.2	32.5	33.1	0.5

[1] *defined as CRP ≥8mg/l*
[2] *transferrin Saturation*

Mean sTfR levels was significantly lower in the group with than in the group without inflammation. Levels were also significantly lower in those with than without malaria parasitaemia. sTfR was the only iron marker that differed significantly with malaria parasitaemia.

Table 7.4: Summary of expected and observed differences in biochemical iron measures between cases without and with inflammation and malaria parasitaemia

Iron markers	Expected differences: Inflammation	Observed differences: Inflammation	Observed differences: Malaria
Ferritin	↑	↑***	No difference
Serum iron	↓	↑**	No difference
Serum transferrin	↓	↓***	No difference
TIBC	↓	↓***	No difference
Transferrin Saturation	↓	↑***	No difference
sTfR	No difference	↓***	↓*
MCV	No difference	No difference	No difference
MCHC	No difference	No difference	No difference

* $p<0.05$
** $p<0.01$
*** $p<0.001$

Linear regression analysis was undertaken to identify predictors of sTfR levels among children with severe anaemia. This analysis could not be done in surgical controls due to the small number recruited. Variables included in the regression model were; a) iron stores status and erythroblast iron status using marrow smear microscopy (proxies of *iron status*); b) haemoglobin, RI (proxies for *erythropoiesis*); c) CRP, malaria parasitaemia, HIV status (proxies for *infection/inflammation*); and d) haptoglobulin (proxy for *haemolysis*). Variables

were fitted in the model simultaneously. Backward and forward fitting models gave very similar results.

The model predicted 50% of the variation in sTfR (R^2=0.497, F-Statistic=14.2, *p <0.001*). The standardized β coefficients and un-standardized β coefficient p-values were used to assess the contribution of each variable to the model (Table 7.5). The important predictors of sTfR that were identified in decreasing order were; CRP (<8mg/l=0 or >8mg/l=1); bone marrow iron stores deficiency (Absent=0 or Present=1); HIV status (Positive=0 or Negative=1), bone marrow erythroblast iron deficiency (Absent=0 or Present=1), and RI level. Presence or absence of malaria parasitaemia and level of haemoglobin or haptoglobin did not influence sTfR levels.

Table 7.5: Log sTfR linear regression model: β coefficients for predictor variables in severely anaemic children

Variable	Un-standardized β coefficient	SE	*p-value*	Standardized β coefficient
Constant	1.10	0.12		
Iron stores deficiency	0.28	0.05	<0.001	0.42
CRP	-0.39	0.08	<0.001	-0.36
Functional iron deficiency	0.17	0.05	0.001	0.26
HIV status	-0.22	0.06	0.001	-0.23
RI	0.02	0.01	0.03	0.16
Haemoglobin	0.02	0.03	0.4	0.06
Malaria parasitaemia	0.04	0.05	0.5	0.05
Log_{10} haptoglobin	-0.04	0.07	0.6	-0.04

7.5 DISCUSSION

Most children presenting to hospital in Malawi with severe anaemia have an infectious or inflammatory pathology (Boele van Hensbroek–personal communication). Inflammation markers such as CRP are expected to be high as observed in Table 6.1. Several acute phase proteins could be used as markers of inflammation including ESR, α-1-antichymotrypsin (ACT), α-1 acid glycoprotein (AGP), serum amyloid A, fibrinogen, haptoglobulin and more recently cytokines. The choice of the indicator depends on the sensitivity and specificity of the specific measurement, the decision on time of sampling and whether single or repeated measurements are required (Thompson *et al,* 1992). CRP, which responds rapidly to inflammation and raised concentrations in blood subside quickly, is widely used (Gabay and Kushner 1999). AGP, which rises and subsides more slowly than CRP or ACT, may be a better indicator of the presence of chronic or sub-clinical infection and may better reflect the changes in ferritin concentrations during infection (WHO/CDC 2004). Measurement of cytokines in plasma is difficult, partly because of their short plasma half-lives and the presence of blocking factors (Gabay and Kushner 1999).

During the acute phase response to inflammation, IL-1 mediates inhibition of synthesis of transferrin by the liver, causing a reduction of serum iron concentration (Tsuji *et al,* 1991). In the present study, the converse occurred, as mean serum iron concentration was in children with inflammation was higher than in those without inflammation.

Serum iron levels have larger diurnal variation than ferritin, TIBC and transferrin saturation, but there have been conflicting conclusions on its pattern (Costongs *et al,* 1985; Dale *et al,* 2002). In a study of healthy human subjects, the individual percentage change in an afternoon compared to a morning serum sample for iron concentration ranged from -54% to +107% (Dale *et al,* 2002). Additionally, in the same subjects, serum iron determined on two consecutive days showed that the between-day variation was greater than the within-day variation. Daily fluctuations of serum iron compounded by the effect of inflammation and analytical precision may partly explain the results of increased serum iron levels in the children with inflammation, found in the present study.

Although international reference values for sTfR are lacking (WHO/CDC 2004), the mean sTfR level among the community controls (15.0 mg/l) which reflects the general population reference, was moderately higher than values obtained from a population of healthy children living in a developed country which ranged from 1.5 to 3.3mg/l for children aged between 6 months and 4 years (Suominen *et al,* 1999; Suominen *et al,* 2001). In community-based surveys in sub-Saharan Africa, the mean sTfR levels among children under the age of five were 8.3mg/l in Kenya, 10.6mg/l in Cote d'Ivoire and 14.3mg/l in Tanzania (Asobayire *et al,* 2001; Menendez *et al,* 2001; Verhoef *et al,* 2001). It has been postulated that asymptomatic *P. falciparum* infection causes a slight but significant increase in erythropoiesis resulting in a moderate elevation of sTfR levels in the community (Menendez *et al,* 2001; Verhoef *et al,* 2001). In the

present study, 45% of community controls had asymptomatic malaria parasitaemia.

Univariate analysis showed that the mean sTfR level was lower in children with inflammation and malaria parasitaemia, and multiple regression analysis demonstrated that sTfR levels in severely anaemic children were significantly influenced by inflammation, HIV infection, iron status, and erythropoiesis.

The level of sTfR concentration in peripheral blood depends on both the number of erythroid precursors in the bone marrow, erythropoiesis, and the number of transferrin receptors per cell, a function of cellular iron need (Beguin 2003). There has been extensive evidence that iron status influences sTfR (Huebers *et al,* 1990; Cook *et al,* 1993). In the present study iron stores deficiency was a better predictor of sTfR than erythroblast iron deficiency. Skikne *et al* (1990) have described the relationship between iron status and sTfR. When normal volunteers underwent graded phlebotomy, ferritin decreased progressively while sTfR showed little change during the depletion of iron storage. However, sTfR increased significantly after the stores were depleted and iron-deficient erythropoiesis commenced (Skikne *et al,* 1990).

sTfR levels significantly depend on marrow erythropoietic activity, causing variations of up to 8-fold above, or 20-fold below normal values (Beguin 2003). RI has been used as a marker of erythropoiesis, but has limitations. A high RI has been observed in normal individuals and patients with hypoproliferative anaemia (Beguin 2003). Additionally, RI cannot differentiate between patients with hypoproliferative marrow (associated with low sTfR), ineffective

erythropoiesis (associated with high sTfR) or both. Nevertheless, if used in conjunction with sTfR, haematocrit, and EPO, it can help to identify different patterns of erythropoiesis (Beguin 2003).

Many studies have shown that sTfR is not increased by inflammation (Cook *et al,* 1993; Asobayire *et al,* 2001), where as others have demonstrated a decrease in sTfR as found in the present study (van den Broek *et al,* 1998; Menendez *et al,* 2001; Verhoef *et al,* 2001). These lowered levels have been attributed to blunted EPO production and suppression of erythropoiesis by cytokines such as TNF, or IL-1 (Hochberg *et al,* 1988). It is unclear whether HIV infection may cause lower sTfR due to a similar inflammatory mechanism. Although, one study among pregnant women did not find any association between HIV infection and sTfR, HIV infection was associated with increased iron stores and impaired development of bone marrow stem cells resulting in reduced numbers in all cell lines (Semba *et al,* 2000; Gordeuk *et al,* 2001). Early in infection, there is increased cytokine production, consequent to immune activation. In more advanced stages of the disease, the decline in immune function and imbalance of cytokine production can contribute to dysregulated erythropoiesis and blood cytopenias (Davis and Zauli 1995). Both cytokine suppression of erythropoiesis and increased iron stores, as observed in HIV infection, have been associated with lower sTfR levels.

The question arises whether in severely anaemic children sTfR is a marker of iron status or erythropoiesis. Studies to date have shown that sTfR is a useful marker of erythropoiesis when iron stores are adequate (Beguin 2003).

Although sTfR has proved a reliable marker of iron status, the interpretation of individual values may be complex in patients in whom both changes in erythropoietic activity and iron status occur simultaneously. Interpretation is further complicated if there is also inflammation. The relationship between iron status and sTfR will thus depend on the severity of inflammation, the status of iron stores, the degree of anaemia, the adequacy of EPO production and the effect of cytokines on the marrow activity (Beguin 2003).

In this study, malaria was not a significant predictor of sTfR. This may be due to two reasons. Firstly malaria may affect sTfR through the same mechanism as inflammation, therefore its influence on sTfR is not independent of CRP responses. Secondly, many children without malaria parasitaemia detected microscopically may nevertheless have had low grade malaria infections. Diagnosis of malaria by microscopy, is associated with a substantial number of false negatives (sensitivity ranges 71%–76%, specificity 72%–95% based on polymerase chain reaction diagnosis), due to human error or parasitaemia falling below detectable levels (Ohrt *et al,* 2002). The ubiquitous effect of malaria in the cases is further shown by the high prevalence of malaria pigment (95.8%) in bone marrows.

Active malaria is associated with increased haemolysis which leads to increased erythropoiesis (Beguin 2003) (which could increase sTfR), as well as inflammation (which could decrease sTfR). Previous studies have shown that the predominant effect is the suppression of erythropoiesis by inflammation resulting in decreased sTfR (Beesley *et al,* 2000; Menendez *et al,* 2001) while in

asymptomatic malaria, there are elevated sTfR levels in response to the milder degrees of anaemia (Verhoef *et al,* 2001). As a consequence, there may be limited use for sTfR on its own as a marker of iron status in children living in malaria endemic areas.

7.6 CONCLUSION

The commonly used iron markers, ferritin, serum iron, TIBC, transferrin saturation have been shown to be altered by inflammation in severely anaemic children. Further detailed analyses of the effect of inflammation on sTfR have shown that sTfR is significantly influenced by four main factors, namely, bone marrow iron status, erythropoiesis, inflammation and HIV infection. The possible mechanisms for each factor are discussed. CRP was a significant predictor of sTfR levels while malaria was not. These findings indicate that sTfR may not be an accurate marker of iron status in severely anaemic children.

CHAPTER 8

ASSESSMENT OF IRON STATUS USING BIOCHEMICAL IRON MARKERS - WHICH MARKERS ARE OPTIMAL?

8.1 INTRODUCTION

Estimates of prevalence of iron deficiency in children in sub-Saharan Africa range from 33 to 63% (Stoltzfus *et al,* 1997; Faber and Benade 1999; Asobayire *et al,* 2001; Nyakeriga *et al,* 2004). Comparison of criteria that have been used to define iron deficiency vary considerably between studies and are often not standardised. This variation arises from the use of different combinations of biochemical iron markers and the use of different cut-off values for the same iron markers in defining iron deficiency (Faber and Benade 1999; Asobayire *et al,* 2001; Nyakeriga *et al,* 2004).

The examination of stained aspirates of bone marrow for haemosiderin has been considered the 'gold standard' as a method for evaluation of iron status (Bain 2001). This technique is invasive and not suitable for screening. There are currently available several relatively non-invasive biochemical markers for the detection of iron deficiency and assessment of its severity. These conventional laboratory tests of iron status, which include ferritin, serum iron, serum transferrin, TIBC, and MCV, are widely used in clinical practice. However, these iron markers are considerably altered by inflammation, which complicates their clinical interpretation. It is still unclear the extent to which an adjustment of the recommended cut-off values for these iron markers would improve their

diagnostic efficiency for detecting or excluding iron deficiency. To date there are no studies which have validated these iron markers against bone marrow iron content in children living in malaria endemic areas.

There is a substantial clinical need e.g. targeting children to receive iron therapy or prophylaxis, for non-invasive and sensitive tests for distinguishing the deficiency of iron stores from functional iron deficiency associated with anaemia of inflammation. Recently, sTfR has been used as a promising new tool for the diagnosis of deficiency of iron stores (Beguin 2003). Transferrin receptors, which mediate cellular uptake of iron are expressed in proportion to the cell's requirement for iron. Membrane-bound transferrin receptor is subjected to proteolysis that generates a soluble form of the receptor (sTfR), which circulates in plasma and can be readily measured (Beguin *et al,* 1988). The performance of sTfR in predicting bone marrow iron content has not been evaluated among children living in malaria endemic areas.

8.2 OBJECTIVES

Among severely anaemic children living in a malaria endemic area:

1. To determine and compare the ability of different biochemical measures to predict bone marrow iron status
2. To investigate how to improve the performance of biochemical iron measures in predicting bone marrow iron status in a malaria endemic area

8.3 METHODS

Only those children with severe anaemia (Hb<5g/dl, cases, Chapter 3) were used for this analysis. Biochemical iron markers were determined as previously described (Chapter 6). Commonly-accepted cut-off levels for detection of deficiency of iron stores and used in this analysis were as follows: ferritin <30µg/l (WHO 2001); serum iron <3.6µmol/l; serum transferrin >3.6g/l; TIBC >72µmol/l (laboratory reference values); transferrin saturation <16% (WHO 2001); sTfR >8.3mg/l (test kit reference value); MCV <67fl (<2 years old) and <73fl (2-5 years old); MCHC <32g/l (WHO 2001). Transferrin-Ferritin (TfR-F) Index was defined as [sTfR + Log ferritin] (Punnonen *et al,* 1997).

A TfR-F Index value of >5.6 (using sTfR of >8.3mg/l and ferritin of <30µg/l) was defined as deficiency of iron stores. The 'gold standard' of bone marrow deficiency of iron stores was defined as deficient marrow iron stores (fragment iron <2) with normal or deficient cellular iron. Functional iron deficiency was defined as normal marrow iron stores (fragment iron ≥2) but with deficient cellular iron (erythroblast iron <30%) (Chapter 5).

Statistical analysis for sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy for individual iron markers was calculated using SPSS for Windows© version 11.0. Receiver operating characteristics (ROC) curves were described and corresponding areas under the curve (AUC^{ROC}) were compared. New cut-offs providing maximal sensitivity and specificity were determined from ROC curves.

8.4 RESULTS

A total of 381 children with severe anaemia, Hb <5g/dl, were analysed and the mean levels of their biochemical iron measures are shown in Table 8.1.

Table 8.1: Biochemical iron measures among severely anaemic children

Iron measure	Mean ± SD	Proportion iron deficient (%)[1]
Ferritin, µg/l	729.2 ± 1528.1	12.7
sTfR, µg/ml	17.4 ± 15.8	73.2
TfR-F Index	12.9 ± 28.1	46.4
Serum iron, µmol/l	16.0 ± 15.7	19.8
Serum transferrin, g/l	2.2 ± 0.7	97.5
Transferrin Saturation, %	41.4 ± 39.7	37.4
MCV, fl	82.8 ± 15.2	15.3
MCHC, g/dl	32.9 ± 7.8	43.3

[1] *using cut-off values as defined in the Section 8.3*

Detection of deficiency of iron stores

Biochemical iron markers having either a sensitivity or specificity <20% were arbitrary classified as poor predictors of iron stores deficiency. These included serum transferrin, TIBC and MCV which had a sensitivity of 0%, 0% and 8.6% respectively (Table 8.2). Ferritin, serum iron and transferrin saturation had lower sensitivity than specificity, i.e. they were performing poorly in identifying the presence of the deficiency and well in correctly identifying the absence of it. Conversely, sTfR had a high sensitivity of 90% and low specificity of 37%. MCHC, the measure of the amount of haemoglobin per red cell, had comparable values for sensitivity and specificity. The accuracy of the iron marker, defined as the percentage of children who were correctly identified as being positive or

negative for deficiency of iron stores, was highest for ferritin, 79% and lowest for sTfR, 49%.

Table 8.2: Sensitivity and specificity of biochemical iron markers to identify children with deficiency of iron stores using commonly-accepted cut-off values

		True		False		Sensitivity	Specificity	Accuracy
		positives	negatives	positives	negatives			
Ferritin		5	76	3	19	21	96	79
sTfR		35	52	87	4	90	37	49
TfR-F Index		16	55	18	7	70	75	74
Serum iron		7	93	13	22	24	88	74
Serum transferrin		0	127	0	33	0	100	79
TIBC		0	127	0	33	0	100	79
Transferrin Saturation		10	80	26	19	35	76	67
MCHC		22	81	40	13	63	67	66
MCV	<2yrs	1	71	8	22	4	90	71
	≥2yrs	2	36	7	10	17	84	69

ROC curves for the identification of deficiency of iron stores for ferritin, sTfR, TfR-F Index, and other iron markers were constructed (Figure 8.1). The area under the curve (AUC^{ROC}) for each marker represents their performance in the identification of deficiency of iron stores (Table 8.3). Ferritin, sTfR, TfR-F Index and MCHC had significantly higher AUC^{ROC} than 0.5, where 0.5 signifies a completely ineffective test. Serum iron and transferrin saturation were excluded from further analysis because they had AUC^{ROC} which were not significantly different from 0.5 (0.62 and 0.60 respectively).

Figure 8.1: ROC curves of sTfR, ferritin and TfR-F Index in the identification of deficiency of iron stores

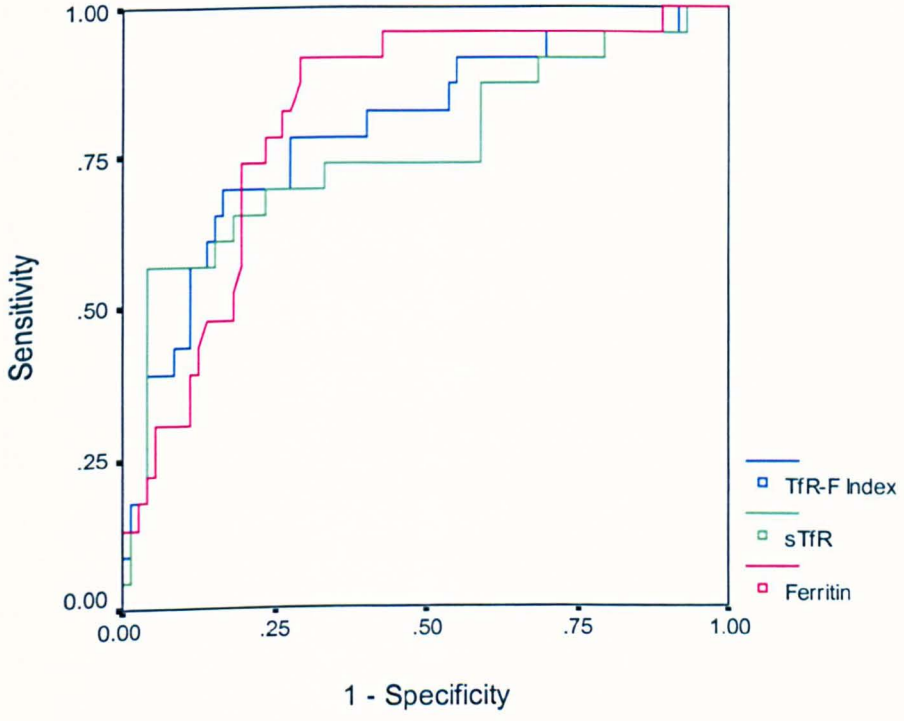


Table 8.3: AUC^{ROC} values for biochemical iron markers to identify children with deficiency of iron stores based on new cut-off values

		AUC^{ROC}	Standard Error	*p-value*
Ferritin		0.82	0.05	*<0.001*
sTfR		0.80	0.04	*<0.001*
TfR-F Index		0.79	0.06	*<0.001*
MCHC		0.68	0.06	*0.001*
Serum iron		0.58	0.06	*0.2*
Transferrin Saturation		0.57	0.06	*0.3*
Serum transferrin		0.52	0.06	*0.8*
TIBC		0.52	0.06	*0.8*
MCV	<2years	0.65	0.06	*0.03*
	≥2years	0.61	0.1	*0.2*

For the remaining biochemical iron markers, ferritin, sTfR, TfR-F Index and MCHC, new cut-off levels which provided maximal sensitivity and specificity were determined from the ROC curves. The resultant sensitivity and specificity, and the percentage change from the original cut-off are shown in Table 8.4. The ability for sTfR or ferritin to predict deficiency of iron stores was comparable and above 70% using the derived cut-offs of 273µg/l and 15.2µg/ml respectively. Although, the sensitivity and specificity for TfR-F Index or MCHC were lower than those for ferritin and sTfR (sensitivity 74%, 67% and specificity 73%, 64% respectively), they required smaller changes from their original to new cut-off levels in order to predict deficiency of iron stores.

Table 8.4: Ability of ferritin, sTfR, TfR-F Index and MCHC to identify children with deficiency of iron stores based on new cut-off values

	Original cut-off	New cut-off	% change in cut-off	Sensitivity[1]	Specificity[1]	Accuracy[1]
Ferritin	30 µg/l	273 µg/l	810	*75*	*76*	76
sTfR	8.3 µg/ml	15.2 µg/ml	83	*77*	*76*	76
TfR-F Index	5.6	5.3	-5	*74*	*73*	73
MCHC	32.0 g/l	32.1 g/l	0.3	*67*	*64*	65

[1] *Sensitivity, specificity or accuracy of new cut-off*

Detection of functional iron deficiency

Using commonly-accepted cut-offs (Section 8.3), the ability of different iron markers to correctly identify functional iron deficiency was assessed (Table 8.5). Ferritin, serum iron, serum transferrin, TIBC and MCV had either a sensitivity or a specificity less than 20%. For the remaining iron markers, sTfR had highest sensitivity (63%), and transferrin saturation had highest specificity (75%). Both sensitivity and specificity for sTfR, TfR-F Index, transferrin saturation and MCHC values were mostly lower than values estimated for predicting deficiency of iron stores (Table 8.2). This was reflected in lower accuracy values.

Table 8.5: Sensitivity and specificity of biochemical iron markers to identify children with functional iron deficiency using commonly-accepted cut-off values

		Sensitivity	Specificity	Accuracy
Ferritin		*97*	*11*	43
sTfR		*37*	*72*	58
TfR-F Index		*75*	*42*	54
Serum iron		*85*	*15*	43
Serum transferrin		*100*	*0*	41
TIBC		*100*	*0*	41
Transferrin Saturation		*71*	*25*	43
MCHC		*65*	*43*	52
MCV	<2years	*87*	*5*	42
	≥2years	*71*	*11*	29

ROC curves for all iron markers were constructed and the AUC^{ROC} for each determined (Table 8.6). AUC^{ROC} values for transferrin saturation, serum iron, serum transferrin, TIBC and MCHC were non-significantly different from 0.5.

Table 8.6: AUC^{ROC} values for biochemical iron markers to identify children with functional iron deficiency based on new cut-off values

		AUC^{ROC}	Standard Error	*p-value*
sTfR		0.62	0.04	*0.007*
Ferritin		0.62	0.06	*0.04*
TfR-F Index		0.64	0.06	*0.02*
MCV	**<2yrs**	0.61	0.06	*0.06*
	≥2yrs	0.64	0.08	*0.1*
Transferrin Saturation		0.59	0.05	*0.08*
Serum iron		0.59	0.05	*0.07*
Serum transferrin		0.54	0.05	*0.4*
TIBC		0.54	0.05	*0.4*
MCHC		0.56	0.05	*0.2*

Using the ROC curves for ferritin, sTfR, TfR-F Index and MCV, the optimal cut-off values providing maximal sensitivity and specificity for the identification of functional iron deficiency were determined (Table 8.7). TfR-F Index levels less than 3.7, gave a sensitivity of 64%, specificity of 62% and accuracy of 63% for predicting functional iron deficiency.

Table 8.7: Sensitivity and specificity of ferritin, sTfR, and TfR-F Index to identify children with functional iron deficiency based on new cut-off values

	Original cut-off	New cut-off	% change in cut-off	Sensitivity[1]	Specificity[1]	Accuracy[1]
Ferritin	30 µg/l	488 µg/l	1526	*55*	*57*	56
sTfR	8.3 µg/ml	11.5 µg/ml	39	*58*	*57*	57
TfR-F Index	5.6	3.7	34	*64*	*62*	63

[1] *Sensitivity, specificity or accuracy of new cut-off*

8.5 DISCUSSION

In the present study, the diagnostic efficiency of sTfR and a variety of more conventional laboratory tests for the identification of deficiency of iron stores or functional deficiency was evaluated. The results suggested that serum transferrin, TIBC and transferrin saturation were of limited value in diagnosis of deficiency of iron stores as their corresponding AUC^{ROC} values did not provide acceptable sensitivity and specificity estimates (Table 8.3). This may relate to the interference of soluble cytokines produced during the acute phase response, as these iron markers have previously been shown to vary with inflammation (Chapter 6).

The bone marrow iron smear was used as the 'gold standard' for the diagnosis of deficiency of iron stores. It has generally been considered the most reliable diagnostic test (Bain 2001), but has the limitations of being more invasive than peripheral blood iron markers and absence of iron stores result may be incorrectly determined in more than 30% of the cases (Barron *et al,* 2001).

Ferritin has been widely used to detect iron deficiency and has been shown to be a good indicator of the level of iron stores in individuals with non-inflammatory conditions (WHO 2001). Conversely, sTfR is considered to reflect the degree of tissue iron supply and there is evidence that it is a good indicator of iron status when the iron stores are depleted (Skikne *et al,* 1990). The reciprocal relationship between sTfR and ferritin describes a perfect log-linear relationship over a wide range of normal and depleted iron stores states. Punnonen *et al* (1997) evaluated various possibilities of combinations of sTfR and ferritin parameters. Their analysis indicated that the calculation of the sTfR/ferritin ratio did not improve diagnostic efficiency compared to sTfR alone. However, the calculation of the sTfR/log ferritin ratio (TfR-F Index) which utilises logarithmically transformed ferritin values, considerably improved diagnostic efficiency for the identification of deficiency of iron stores.

In the present study, serum ferritin had a high specificity and sTfR a high sensitivity for the detection of deficiency of iron stores, which is consistent with other studies (Means *et al,* 1999). These measurements are incorporated in the use of TfR-F Index. There is little benefit of changing the current conventional cut-off of TfR-F Index to improve its diagnostic efficiency (Table 7.4). This is in

contrast with ferritin and sTfR which required a change of 810% and 80% respectively to achieve maximal sensitivity and specificity (Tables 7.2 and 7.4).

During a study in which healthy volunteers were serially phlebotomised to induce iron deficiency, MCHC was found to be a sensitive early indicator of iron deficient erythropoiesis (Patton *et al,* 1991). A decrease in MCHC preceded changes in MCV which remained preserved until later during the development of iron deficiency. MCHC changes were less sensitive but more specific for iron deficiency when measured using impedance- based instruments (Bain 1995). In the present study, MCHC had a relatively good diagnostic efficiency. Although MCHC was measured using a coulter machine, which is an impedance-based instrument, it is an iron marker that is relatively cheaper to measure and more widely available in resource-poor settings than either ferritin and sTfR.

Results from the present study suggest that it is necessary to change the cut-off limit for ferritin from 30 to 273 µg/l in order to improve its diagnostic efficiency for detecting deficiency of iron stores. This proposed increase is consistent with other studies (Punnonen *et al,* 1997). This requirement probably reflects the effect of the acute phase response on ferritin levels. WHO reference levels for ferritin take this effect into account by increasing the cut-off limit for the detection of iron deficiency from 12 µg/l to 30 µg/l in areas of high prevalence of infection (WHO 2001). Witte *et al* (1988) developed a nomogram describing the relationship between ferritin and CRP or erythrocyte sedimentation rate in order to detect or exclude iron deficiency in patients with anaemia of inflammation in order to minimise bone marrow examination. Unfortunately, when this

nomogram, which corrected for the acute phase component of changes in ferritin, was applied in later studies, it performed poorly (Coenen *et al,* 1991).

The sensitivity of sTfR for detecting deficiency of iron stores was greatly improved by increasing the cut-off value to 14.9 µg/ml as determined from its ROC curve. Studies in sub-Saharan Africa, have shown that sTfR levels in children are generally higher than in children from developed countries which may be due to a slight increase in erythropoiesis from the effects of asymptomatic malaria (Verhoef *et al,* 2001). As a consequence, higher sTfR cut-offs are required to correctly classify children with deficiency of iron stores. However, establishing a cut-off value for sTfR may be problematic as there is currently a lack of an international reference with certified concentration of sTfR to standardise assays (WHO/CDC 2004). This hinders comparison of research findings using different sTfR assays. Nevertheless, work has been done to develop reference limits for sTfR levels, but these have been based on children living in non-malaria endemic areas (Suominen *et al,* 2001).

In the present study, the diagnostic efficiency of a variety of iron markers for the detection or exclusion of functional iron deficiency were investigated. Functional iron deficiency was defined as the presence of iron stores with an absence of cellular iron in the bone marrow. This classification may have limitations (Chapter 5). Nevertheless, after determining new cut-offs for the detection or exclusion of functional iron deficiency, the diagnostic efficiency of ferritin, sTfR and TfR-F Index were not noticeably improved. It may be necessary to combine an iron marker with a marker of inflammation, such as CRP, in order

to improve diagnostic efficiency. Ritchie *et al* (2004) have described a diagnostic protocol for iron deficiency anaemia in which a series of iron markers and CRP were used to distinguish children with iron deficiency anaemia from those with anaemia of inflammation. Their protocol was study specific and did not use bone marrow iron content as a 'gold standard', but instead used red cell indices and morphology.

Results from the present study suggested that ferritin, sTfR and TfR-F Index were the optimum markers for detecting deficiency of iron stores provided new cut-off values were used, particularly for ferritin and sTfR. TfR-F Index proved to be a better marker of deficiency of iron stores as it combined the inadequacies of ferritin and sTfR used alone. It also required little change from its original cut-off value to optimally detect or exclude children with deficiency of iron stores.

The results have been based on children presenting to hospital with severe anaemia. This was not a population based study, but a subgroup of severely anaemic children and therefore this may limit generalizability of these findings. However, the children that were recruited, represented the population of children who present the greatest diagnostic challenges to clinicians. An improved knowledge of diagnostic criteria for iron status assessment that avoids the need to do a bone marrow aspiration, should be of value for determining therapeutic practice.

8.6 CONCLUSION

There are a variety of laboratory tests that can be used to detect or exclude deficiency of iron stores which have differing diagnostic efficiency. Using conventional cut-off limits, ferritin was observed to have high sensitivity but poor specificity while sTfR had a poor sensitivity but high specificity. The TfR-F Index incorporates the high sensitivity of sTfR, a proxy for cellular iron need, and the high specificity of ferritin, a proxy for level of iron stores. After ROC curve analyses, ferritin, sTfR and TfR-F Index, all had reasonable sensitivity and specificity. TfR-F Index required least change in its cut-off to achieve the optimal diagnostic efficiency, although this could not be regarded as ideal. TfR-F Index, ferritin and sTfR were less useful for detecting or excluding functional iron deficiency. In this population of severely anaemic children the MCHC would be a potentially valuable screening test for detecting deficiency of iron stores in hospitals in resource-poor settings where provision of ferritin, sTfR or TfR-F Index assessment is too expensive.

CHAPTER 9

IRON DEFICIENCY AND RISK OF INFECTION

9.1 INTRODUCTION

A major unresolved concern that may influence iron deficiency control strategies is the interaction between iron status, iron supplementation and susceptibility to infection. There are two main postulates: firstly that iron deficiency aids immunity (the 'nutritional immunity' hypothesis), and secondly that it hinders host defence against infection. Conflicting results may arise from mis-interpretation of the effect of iron supplementation on infection, which is not simply the reverse of the effect of iron deficiency on infection (Oppenheimer 2001).

Iron deficiency aiding immunity: The idea that iron deficiency is protective against infection is based on the finding that the growth of a variety of bacteria and fungi are inhibited *in vitro* by transferrin and lactoferrin (Oppenheimer 2001). Most of the iron in the body is found intracellularly, in ferritin, haemosiderin or haem, and iron which is extracellular in body fluids is attached to high affinity iron-binding proteins, namely transferrin and lactoferrin. There is usually a reserve of free binding sites on circulating transferrin capable of binding iron, which is possibly an important safeguard against presence of free or loosely bound iron (Griffith and Williams 1999). It may be considered that by the further lowering of the saturation of iron in transferrin, as may occur during iron deficiency, that this will deprive pathogens from acquiring iron, and hence enhance immunity.

Iron deficiency impairing immunity: In vitro evidence shows that iron deficiency depresses certain aspects of cell-mediated immunity, including lymphocyte, neutrophil and macrophage function, while humoral immunity is relatively unaffected. Iron deficiency has been associated with: reduced neutrophil function resulting in impaired bacteriocidal activity; depression of T-lymphocyte numbers with thymic atrophy; defective T-lymphocyte-induced proliferative responses; impaired natural killer activity; impaired interleukin-2 production by lymphocytes; reduced production of macrophage migration factor; and reversible impairment of delayed cutaneous hypersensitivity (Oppenheimer 2001).

A major problem that limits the investigation of iron deficiency and its relation to infection is related to the ethics of studying control groups. To show causality there is need to follow-up iron deficient subjects for prolonged periods in order to measure the incidence of infection compared to non-iron deficient subjects. This situation is unethical as it is necessary to withhold iron treatment. As a result there may be inappropriate extrapolation of research findings using only iron intervention studies to examine the effect of iron deficiency on infection.

There have been a few observational studies in humans with iron deficiency. Weijmer *et al* (1990) reported that patients with furunculosis had significantly lower serum iron than controls and iron therapy alone was associated with resolution of furunculosis in all but one of 16 patients. In another study, oral lesions of chronic muco-cutaneous canididiasis regressed in iron deficient patients following iron therapy (Oppenheimer 2001). However, there was no control group and it was not clear whether they recieved iron alone or in combination with anti-fungals. Infections after post-operative surgery were

reported to be significantly more common in patients with low pre-operative serum ferritin compared with patients with normal ferritin (Harju 1988). These studies were undertaken in non-malaria endemic areas and support the theory that iron deficiency is associated with impaired immunity and therefore increased probability of infection.

Masawe *et al* (1974) in Tanzania reported fewer bacterial infections in patients admitted with iron deficiency anaemia compared to controls. Possibly the most quoted study indicating that iron deficiency may be protective are the observations made among Somali nomads entering a feeding camp (Murray *et al,* 1978). Iron deficiency was associated with no infection in 26 nomads versus 19 in 64 iron replete individuals.

It is unclear whether there is an association between iron deficiency and malaria or HIV infection risk. Conflicting results have been reported on whether iron deficiency is protective against (Murray *et al,* 1978; Nyakeriga *et al,* 2004), or a risk factor for malaria (Oppenheimer 2001). Of the few studies reporting on iron deficiency and HIV infection, most have suggested that 'high' iron status may adversely influence the outcome of HIV infection. Most of this work has been based on *in vitro* studies (Savarino *et al,* 1999; Gordeuk *et al,* 2001; Pugliese *et al,* 2002).

A major limitation to most of these studies is that they determined iron status using iron markers which are known to vary considerably during infection (Chapter 6). For example serum iron which was used in almost all the studies mentioned (Masawe *et al,* 1974; Murray *et al,* 1978; Harju 1988; Weijmer *et al,*

1990; Oppenheimer 2001), shows daily variations (Dale *et al,* 2002). In view of this there is need to re-assess the value of iron status markers allowing for the influence of concomitant infection.

9.2 OBJECTIVES

1. To determine the prevalence of iron deficiency in cases, and hospital, community, or surgical controls
2. To describe the common micro-organisms causing bacteraemia in cases and hospital controls
3. To determine the association between iron deficiency with bacteraemia, malaria or HIV infection

9.3 METHODS

Microbiological investigations

A bone marrow aspirate was collected from cases and surgical controls and a venous blood sample from hospital controls at the time of recruitment. If there was a failure to collect a marrow aspirate or there was insufficient sample, then a blood sample was collected for microbiological culture. A blood sample for culture was obtained from community controls on recruitment if they presented with fever that was not attributed to malaria parasitaemia.

Bone marrow and blood cultures were tested on a BacT/Alert machine (bioMérieux INDUSTRY, MO, USA) at the Wellcome Trust laboratories, Blantyre, Malawi. One millilitre of marrow or blood was collected into a 20ml BacT/Alert

MTB bottle which was immediately placed in an incubator at 37°C. The BacT/ Alert machine uses a colorimetric sensor and reflected light to determine the amount of CO_2 that is dissolved in the culture medium. If micro-organisms were present in the sample, CO_2 was produced as they metabolised the substrates in the culture medium. When CO_2 was produced, the colour of the sensor in the bottom of each culture bottle changed from green to yellow, which was detected by a photo-detector and transmitted to a computer. If after 7 days at these optimal conditions the CO_2 level did not change significantly, the sample was considered to be negative. Positive samples were plated out on an agar plate to obtain single colonies, and isolates were identified using a BSAC standardized disc susceptibility testing method (Andrews 2001).

Parasitological investigations

Malaria slides were double read by two separate individuals and if there was a discrepancy between the two readers, the slide was read independently by a third reader at the Wellcome Trust laboratories in Blantyre and the study clinic laboratory in Chikwawa. The number of WBCs per microlitre of blood, from the full blood count reading was used to express the number of malaria parasites per microlitre of blood. The proportion of malaria pigmented WBCs and the number of gametocytes seen per 200 WBCs were recorded. Children with malaria parasitaemia were not admitted for elective surgical procedures, hence all surgical controls were negative for malaria parasites.

Biochemical investigations

Ferritin was determined using an electro-chemiluminescence immunoassay (Modular Analytics E170, Roche Diagnostics, Switzerland, detection limit 0.5µg/l) at University of Amsterdam laboratories, Amsterdam, and sTfR using an enzyme immunoassay (Ramco Laboratories, TX, USA, detection limit 1.0µg/l) at the Wellcome Trust laboratories, Blantyre. Transferrin-Ferritin (TfR-F) Index was calculated as [sTfR ÷ Log ferritin] and a cut-off limit of >5.3 was used to define iron deficiency (Chapter 8).

HIV testing was done according to WHO recommendations using two rapid tests (Determine, Abbott Diagnostics and Unigold, Trinity Biotech) at the Wellcome Trust laboratories in Blantyre and study clinic laboratory in Chikwawa. For children aged less than 18 months, they were either re-tested at 18 months of age or tested using HIV PCR.

Socio-economic and nutritional status assessment

During recruitment, socio-economic and demographic information was collected by questionnaire and this was followed by a physical examination which included measurement of height (centimetres) and weight (nearest 100 grams). Socio-economic data included parental education level, and occupation, and property ownership.

Statistical analysis

Weight-for-height (W/H), height-for-age (H/A) and weight-for-age (W/A) and their corresponding Z-scores were calculated using Epi Info version 3.2.2

(CDC, Atlanta, USA). Children with a Z-score less than -2 standard deviations of the reference population were defined as wasted (W/H), stunted (H/A), or underweight (W/A) (WHO 1995).

A chi-square test for linear trend was used to assess prevalence of iron deficiency in sub-group categories by age. To determine the association between iron deficiency and infection, logistic regression was used to determine the crude odds ratio (OR) and adjusted odds ratios (AOR). Effect modifiers were identified and important confounders were selected. Multicollinearity and singularity among variables was tested using correlation statistics. Singularity, occuring when one variable is actually a combination of other independent variables, is not recommended in regression analyses. Variables with high correlations ($r > 0.9$) were therefore removed from the analysis. Sample size (N) versus the number of independent variables to be included in the analysis was considered using the formula $N > 50 + 8m$ (where m = number of independent variables). The final variables were then entered into a model using SPSS for Windows© version 11.0 to generate AORs of the variable of interest. The Student T-test was used to compare means for parameters of interest between iron deficient and iron replete groups.

9.4 RESULTS

A total of 1161 children (381 cases, 377 hospital controls, 380 community controls and 23 surgical controls) were recruited and included in the analysis. Of all cases, hospital and surgical controls, 93% (n=729) had a blood or marrow

culture performed. Ninety-nine percent (n=1153) had a malaria parasite slide available and 89% (n=1033) had their HIV status determined.

The prevalence of iron deficiency, varied significantly between cases, hospital controls, community controls, and surgical controls ($p < 0.001$) (Figure 9.1). The ORs for iron deficiency was significantly less amongst cases compared to all control groups (Table 9.1).

Figure 9.1: Prevalence of iron deficiency by study group

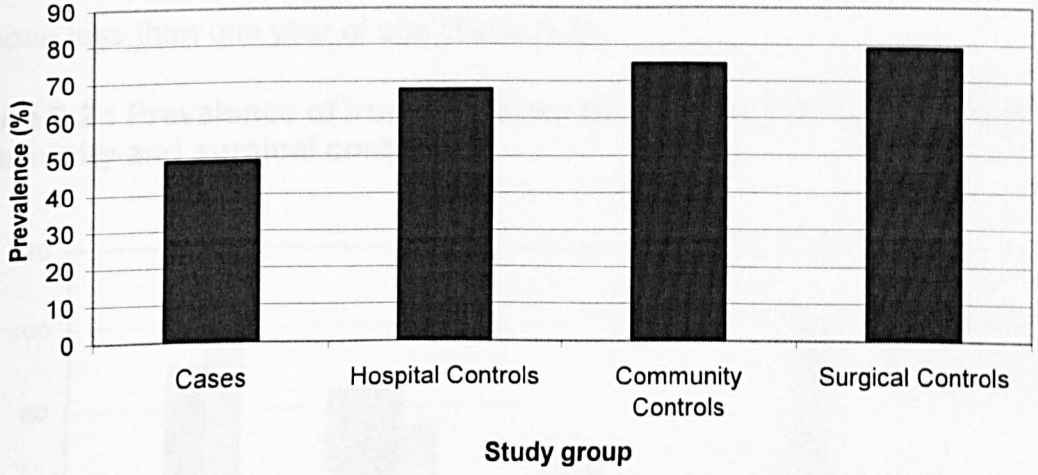


Table 9.1: Prevalence of iron deficiency[1] and odds ratio[2] by study group

	Cases	Hospital controls	Community Controls	Surgical Controls
Prevalence	49 %	68 %	75 %	79 %
OR (compared to cases)	1.0	2.2	3.1	3.9
95% Confidence Intervals	–	1.4 – 3.3	2.0 – 4.8	1.2 – 16.8

[1] TfR-F Index >5.3
[2] compared to cases

The prevalence of iron deficiency in cases, hospital, community and surgical controls differed by age group (Figure 9.2). There was a decreasing prevalence of iron deficiency with increasing age group in all the study groups except hospital controls. The prevalence estimate among surgical controls may be imprecise due to the small sample size (n=17). The prevalence in hospital controls increased from <1 year to 1-2 year age groups, and gradually decreasing in older age groups. This was reflected in the increased OR of 3.1 (95% CI 1.3 – 7.6, *p= 0.02*) among children in the 1-2 year age group compared to those less than one year of age (Table 9.2).

Figure 9.2 : Prevalence of iron deficiency by age group for cases, hospital, community and surgical controls

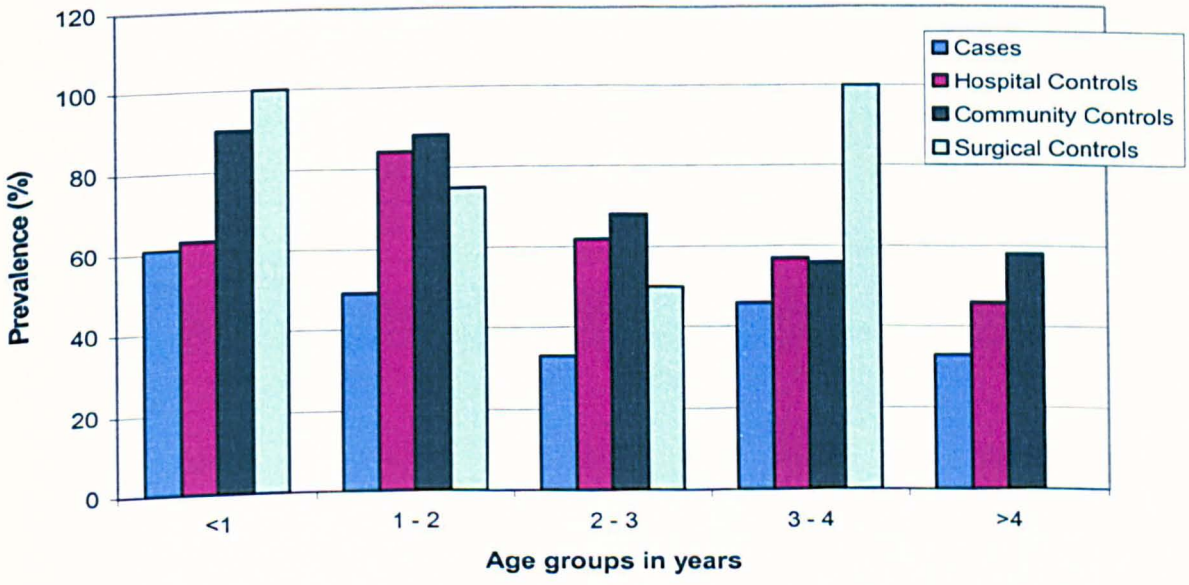


Table 9.2: Prevalence and odds ratio[1] for iron deficiency by age group

	Age group					χ^2 for linear trend	*p-value*
	<1yr	1-2yr	2-3yr	3-4yr	>4yr		
Cases							
Prevalence	61	49	33	46	33		
OR	1	0.62	0.32	0.56	0.32	4.75	*0.03*
95% CI	—	0.31 – 1.22	0.13 – 0.84	0.22 – 1.40	0.09 – 1.20		
Hospital controls							
Prevalence	63	84	62	57	46		
OR	1	3.12	0.95	0.79	0.49	3.43	*0.06*
95% CI	—	1.28 – 7.63	0.41 – 2.18	0.27 – 2.29	0.13 – 1.88		
Community controls							
Prevalence	90	88	68	56	58		
OR	1	0.81	0.23	0.13	0.15	17.98	*<0.001*
95% CI	—	0.19 – 3.38	0.06 – 0.84	0.03 – 0.52	0.03 – 0.66		
Surgical controls							
Prevalence	100	75	50	100	—	—	—
OR[2]	—	—	—	—	—		

[1] compared to age group <1year
[2] OR and χ^2 for trend not applicable as some cells had an expected count of less than 5

Of those specimens used for microbiological culture, 58% (n= 425) were blood samples and 42% (n= 304) were bone marrow aspirates. Cultures from bone marrow were twice as likely to be contaminated than blood samples (OR 2.0, $p < 0.001$). The rate of positive and contaminated cultures in each study group is shown in Table 9.3. The most common contaminants were *Staphylococcus epidermidis* and *Micrococcus* species.

Table 9.3: Results of blood and bone marrow culture

Bacteraemia	Cases	Hospital controls	Community Controls	Surgical Controls
Positive	**14** % (52)	**4** % (14)	**5** % (2)	**5** % (1)
Negative	**58** % (207)	**83** % (292)	**63** % (24)	**62** % (13)
Contaminated	**28** % (99)	**13**% (47)	**32** % (12)	**33** % (7)
Total	**100** % (358)	**100** % (353)	**100** % (38)	**100** % (21)

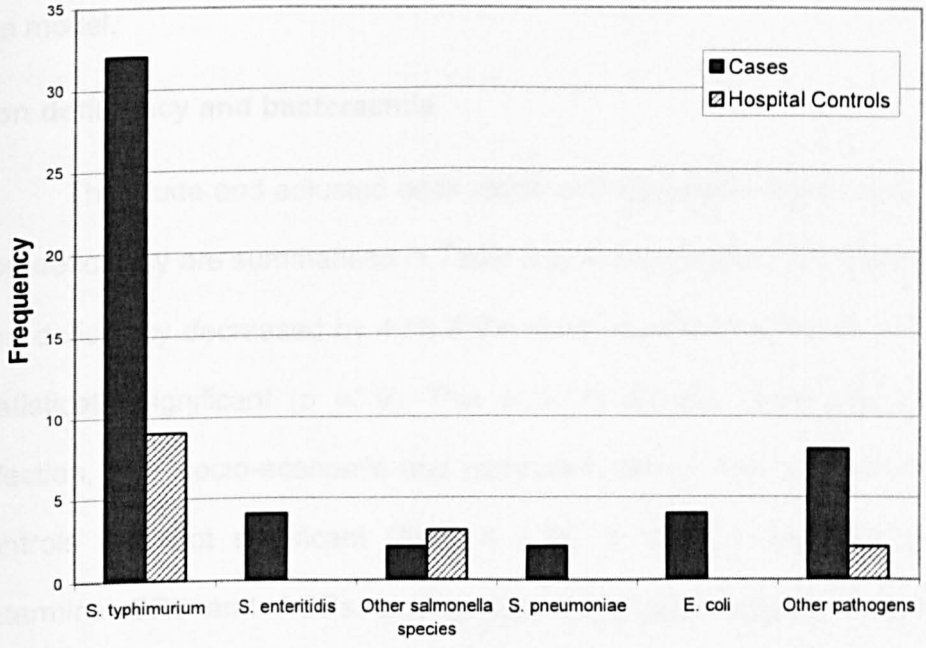
Excluding contaminated cultures, highest culture positivity was observed amongst cases (20%, n=52). Five percent (n=14) and 7% (n=1) of hospital and surgical controls respectively, were positive. In community controls, for whom blood for culture was not routinely collected on recruitment, a total of 8% (n=2) were positive.

Table 9.4 : Distribution of micro-organisms isolated from blood and bone marrow samples

Micro-organism	Cases	Hospital controls	Community Controls	Surgical Controls
S. typhimurium	61 % (32)	64 % (9)	100 % (2)	–
S. enteritidis	8 % (4)	–	–	–
Other *salmonella* species	4 % (2)	22 % (3)	–	–
S. pneumoniae	4 % (2)	–	–	–
E. coli	8 % (4)	–	–	–
Other pathogens	15 % (8)	14 % (2)	–	100 % (1)
Total	100 % (52)	100 % (14)	100 % (2)	100 % (1)

The most common organism isolated in cultures from cases, hospital and community controls was *Salmonella typhimurium* (Table 9.4 and Figure 9.3). *Salmonella* species accounted for bacteraemia in 73% (n=38) of cases and 86% (n=12) of hospital controls.

Figure 9.3 : Frequency of specific micro-organisms isolated from blood and bone marrow of cases and hospital controls



The odds of bacteraemia, malaria, or HIV infection, among iron deficient compared to non-iron deficient children was investigated using logistic regression. As child study group was an important effect modifier, analyses investigating the association between iron deficiency and infection were performed for each study group separately. This resulted in a decrease in sample size per analysis and restricted computation of some AORs.

Confounding variables included were the child's socio-economic and nutritional status. Due to the reduced sample size and high correlations among variables for socio-economic and nutritional status, the property number for the child's household alone was taken as an indicator for socio-economic status and the Z-score value for W/A alone was taken as the indicator for nutritional status

in the final logistic regression model. However, when Z-score value for H/A and W/H were used sequentially instead of W/A, there was no significant change in the model.

Iron deficiency and bacteraemia

The crude and adjusted odds ratios of bacteraemia being associated with iron deficiency are summarised in Table 9.5. Among cases, the adjusted odds of iron deficiency decreased by 40% if the child had bacteraemia but this was not statistically significant (*p =0.9*). This odds of disease were adjusted for HIV infection, age, socio-economic and nutritional status. The AOR among hospital controls was not significant (AOR = 0.14, *p =0.1*). It was not possible to determine ORs and AORs among community and surgical controls as the prevalence of bacteraemia in either the iron deficient or non-deficient group was zero.

Table 9.5 : Crude and adjusted odds ratio of bacteraemia as a risk factor for iron deficiency

	Iron deficient	Not Iron deficient	OR[1] (95% CI)	AOR[2] (95% CI)	*p-value*[3]
Cases (n=142)					
Bacteraemia prevalence	16 % (10)	17 % (13)	**0.96** (0.36 – 2.56)	**0.60** (0.20 – 1.79)	*0.9*
Hospital controls (n=168)					
Bacteraemia prevalence	1 % (1)	7 % (4)	**0.13** (0.01 – 1.38)	**0.14** (0.01 – 1.41)	*0.1*
Community controls (n=16)					
Bacteraemia prevalence	0 % (0)	20 % (1)	—	—	—
Surgical Controls (n=11)					
Bacteraemia prevalence	13 % (1)	0 % (0)	—	—	—

[1] Crude odds of a positive versus negative culture being iron deficient. Undeterminable for community and surgical controls
[2] Adjusted for age, HIV, socio-economic and nutritional status
[3] p-value for the adjusted odds ratio estimate

Iron deficiency and malaria

The odds of iron deficiency among children with malaria parasitaemia appears to be less than among those without malaria except for community controls (Table 9.6). These differences were not statistically significant. The prevalence of malaria parasitaemia among surgical controls was zero as it was an exclusion criteria for an elective operation.

Table 9.6 : Crude and adjusted odds ratio of malaria as a risk factor for iron deficiency

	Iron deficient	Not Iron deficient	OR[1] (95% CI)	AOR[2] (95% CI)	*p-value*[3]
Cases (n=207)					
Malaria prevalence	49 % (49)	60 % (64)	**0.62** (0.34 – 1.11)	**0.67** (0.37 – 1.20)	*0.4*
Hospital controls (n=207)					
Malaria prevalence	35 % (49)	37 % (25)	**0.90** (0.47 – 1.73)	**0.91** (0.48 – 1.73)	*0.8*
Community controls (n=207)					
Malaria prevalence	47 % (72)	40 % (21)	**1.34** (0.68 – 2.65)	**1.65** (0.81 – 3.36)	*0.2*
Surgical Controls (n=19)					
Malaria prevalence	0 % (0)	0 % (0)	—	—	—

[1] Crude odds of a positive versus negative malaria smear being iron deficient
[2] Adjusted for age, socio-economic and nutritional status
[3] p-value for the adjusted odds ratio estimate

Further analysis of mean values of malaria parameters showed that there were no significant differences between iron deficient and non-iron deficient children in terms of malaria parasite count and gametocytes/200WBCs in all study groups (Table 9.7). However, among cases, the mean number of white blood cells with malaria pigment was significantly reduced in iron deficient compared to non-iron deficient children (difference = 2.6%, *p= 0.01*).

Table 9.7: Mean concentration of malaria parameters in iron deficient and non-iron deficient children

		Mean (SD) concentration		*p-value*[1]
		Iron deficient	**Non-iron deficient**	
Log parasite count	Cases	3.8 (1.0)	4.1 (1.0)	*0.1*
	Hospital controls	3.8 (0.8)	3.9 (1.1)	*0.8*
	Community controls	3.5 (0.7)	3.7 (0.9)	*0.2*
WBCs with malaria pigment (%)	Cases	3.0 (5.6)	5.6 (7.9)	*0.01*
	Hospital controls	0.7 (1.8)	0.6 (1.4)	*0.7*
	Community controls	0.5 (1.1)	0.5 (1.8)	*0.9*
Gametocytes /200WBCs	Cases	0.7 (3.0)	0.6 (1.6)	*0.7*
	Hospital controls	0.5 (1.7)	0.4 (1.8)	*0.9*
	Community controls	0.8 (2.5)	1.7 (5.3)	*0.3*

[1] p-value of the difference between means

Iron deficiency and HIV infection

The association between iron deficiency and HIV infection is summarised in Table 9.8. The crude and adjusted odds ratios for all the study groups except surgical controls showed decreased odds of iron deficiency amongst HIV infected than non-HIV infected children, but this difference was not significant.

Table 9.8: Crude and adjusted odds ratio of HIV as a risk factor for iron deficiency

	Iron deficient	Not Iron deficient	OR[1] (95% CI)	AOR[2] (95% CI)	*p-value*[3]
Cases (n=194)					
HIV prevalence	8 % (8)	13 % (13)	**0.58** (0.21 – 1.60)	**0.67** (0.24 – 1.87)	*0.4*
Hospital controls (n=181)					
HIV prevalence	7 % (8)	14 % (8)	**0.43** (0.14 – 1.36)	**0.56** (0.27 – 1.04)	*0.3*
Community controls (n=189)					
HIV prevalence	2 % (3)	4 % (2)	**0.47** (0.05 – 5.84)	**0.64** (0.09 – 4.53)	*0.7*
Surgical Controls (n=7)					
HIV prevalence	0 % (0)	0 % (0)	—	—	—

[1] Crude odds of a positive versus negative HIV result being iron deficient
[2] Adjusted for age, socio-economic and nutritional status
[3] p-value for the adjusted odds ratio estimate

9.5 DISCUSSION

Trends in iron deficiency

Children with severe anaemia were less likely to be iron deficient (Table 8.1). Although this was not an individually matched case-control study, there was some degree of matching. All children were between 6 and 60 months, community controls were recruited from the same village as cases, and hospital controls were recruited within 48 hours after recruitment of a case and from the

same health facility. There were no striking differences in socio-economic status between study groups (Table 4.1). The main difference between the study groups was the degree of illness severity. Hospital controls, who were patients selected from the out-patient department rarely requiring hospital admission and were less severely ill than cases. Community controls were usually asymptomatic. Selection bias in community controls may have arisen from mother's with more 'sickly' children being more willing to participate in the study. The prevalence of iron deficiency was not statistically different between community and surgical controls (difference = 4%, $p = 0.8$).

CRP, a marker of inflammation, may be used as a proxy for infectious disease severity (Gabay and Kushner 1999). Results in Chapter 7 showed that there was a decreasing trend of CRP concentration among cases, hospital, community and surgical control which followed the trend of decreasing anaemia severity. When CRP was included in the logistic regression model, the observed trend for increasing of the odds of iron deficiency among the study groups was lost. This suggests that iron deficiency was associated with a decreasing odds of infection.

The prevalence of iron deficiency among the community controls was 75%. This estimate was higher than reported in other studies in sub-Saharan Africa (Stoltzfus *et al,* 1997; Faber and Benade 1999; Asobayire *et al,* 2001; Nyakeriga *et al,* 2004). However, other published studies have failed to control for the inflammatory effect on biochemical iron markers which has resulted in underestimation of the prevalence of iron deficiency. Asobayire *et al* (2001),

using a sTfR cut-off of >8.5mg/l, found a prevalence of iron deficiency of 65% in a population of children whom 46% had CRP levels greater than 10mg/l. Faber and Benade (1999) observed that 43% of children attending an under-five clinic in South Africa were iron deficient which was defined using a ferritin cut-off of <10µg/l. Stoltzfus *et al* (1997) in a study among Tanzanian schoolchildren, observed that 49% had erythroprotopophyrin levels >90µmol/mol haem. Results of the present study (Chapter 7) are in concordance with other studies (Witte *et al,* 1988; Hastka *et al,* 1993; Punnonen *et al,* 1997; Verhoef *et al,* 2001) which have demonstrated that in populations with high infection exposure and inflammatory response, cut-off values for ferritin, sTfR, and erythroprotopophyrin need to be adjusted in order to improve their diagnostic efficiency. One study that excluded children with malaria or increased CRP levels, reported that the prevalence of iron deficiency ranged from 33-38%, but this study included children aged up to 8 years, and is therefore not directly comparable to the present study population (Nyakeriga *et al,* 2004).

Figure 8.2 shows the prevalence of iron deficiency by age and illustrates that the most vulnerable ages for iron deficiency were the first and second year of life. The main source of iron in the newborn is acquired from the mother in utero. Due to the high requirements for iron to support the rapid increase in erythropoiesis during the first year of life, these iron stores quickly become depleted by 4 months of age, and even earlier in preterm and small-for-gestation babies. Infants become dependent on external iron sources to maintain an adequate iron nutritional status. Weaning foods in sub-Sahara African

communities have poor iron content and bioavailability which results in an increased incidence of iron deficiency by the first year of life (Gibson and Hotz 2001). This pattern is in agreement with the present study findings.

Blood and bone marrow culture

Twenty percent of children with severe anaemia had bacteraemia. A previous study in Malawi, found that 17% of blood cultures on children admitted to hospital grew a pathogen in blood, and anaemia was associated with a higher odds of having bacteraemia (OR 2.00, $p<0.0001$) (Walsh *et al,* 2000). Non-typhoidal salmonella (NTS), which included *S. typhimurium* and *S. enteritidis*, were the commonest infections isolated especially among children less than 5 years. NTS has been consistently shown to be the most common cause of bacteraemia in studies from sub-Saharan Africa, however, little is known about their source and mode of transmission in the African context (Graham *et al,* 2000). In the present study NTS was the predominant infection isolated from blood and bone marrow (Table 8.4).

The use of bone marrow culture in some children and blood culture in others to determine bacteraemia may be a source of bias, especially if the culture yield differs between the two methods. Bone marrow culture has been reported to be superior to blood culture for the detection of *Salmonella* species (Gasem *et al,* 1995). However, in a study of HIV infected individuals, bone marrow culture did not provide a significantly higher yield than blood culture (Ker *et al,* 2002). Failure to routinely collect a blood sample for culture among

community controls, limits generalizability of the study's findings to hospital-based populations of children.

No published reports on rates of contamination of microbiology specimens in sub-Saharan Africa were identified. Studies from laboratories in developed countries have reported 2.2% - 3.7% contamination, and this would be expected to be higher in resource-poor hospital settings (Bekeris *et al,* 2005). In the present study, approximately a third of microbiological cultures from cases, community and surgical controls, were contaminated, with bone marrow aspiration being associated with twice the odds of contamination than blood collection. Assuming that specimens were contaminated during collection, it is plausible for bone marrows to be associated with higher contamination risk. Marrow aspiration requires more physical manipulation to access marrow and much less time to transfer it from syringe to collecting tube, to avoid clotting, as compared to blood collection. Nevertheless, risk of contamination was high and may have led to underestimation of the positive cultures among cases and surgical controls who had predominantly bone marrow cultures.

9.5.1 Iron deficiency and infection

Studies of iron deficiency and its effect on infection are compromised without adequate control of confounding factors. This is because iron deficiency is part of a cluster of inter-related nutrient and social deprivations, ultimately resulting from poverty. A major problem incurred with observational study design is confounding, in particular from socio-economic and nutritional status which are important confounders in investigating the association between iron deficiency

and infection. The number of properties owned per household is part of the asset approach of measuring socio-economic status which has been used previously (Gwatkin *et al,* 2000). Using this approach, socio-economic status is defined in terms of assets or wealth as opposed to income or consumption. However, there is need to standardise weighting of different assets which vary according to different cultures and countries.

Nutritional status in children may be assessed using three anthropometric measures. Low height-for-age reflects long-term growth faltering (stunting), while low weight-for-height indicates short-term growth faltering (wasting), as weight is sensitive to recent growth disturbances. Low weight-for-age, which is the manifestation of both the above, was used in the present study to diagnose children who were underweight (WHO 1995).

Iron deficiency and bacteraemia

There was no significant association between iron deficiency and bacteraemia in the present study (Table 8.5). There have been few published, observational studies in humans investigating this association. In an often misquoted study, Masawe *et al* (1974) reported a decreased prevalence of bacteraemia in iron deficient individuals admitted to hospital (OR = 0.04, $p < 0.0001$). Unfortunately, they provided no details of whether patients were receiving iron therapy when assessed for bacteraemia, which would make it an iron trial rather than an observational study.

Studies on the effect of iron deficiency on infection have mostly been done *in vitro*, and indicated inconclusively that iron deficiency may be protective against bacteraemia. Under normal physiological conditions, the amount of free iron is far too small to support bacterial growth (Bullen *et al,* 1978). In addition, during infection the host reduces the total amount of iron bound to transferrin. This decrease, called hypoferraemia of infection, may be enforced by release of interleukin-1, a pro-inflammatory cytokine (Griffith and Williams 1999). Murray *et al* (1978) in their studies in Somalia collected serum from iron deficient and iron replete nomads, inoculated it with *S. typhimurium* before incubating at 36°C. After 18 hours, *S. typhimurium* did not grow in the iron-deficient sera, but grew in 6 out of the 8 iron replete sera.

However, other studies have demonstrated how pathogens causing bacteraemia have adapted to the severely iron restricted extracellular environment usually found *in vivo* and developed mechanisms to acquire iron necessary for their growth. These mechanisms and the expression of virulence, are extremely complex and diverse. For example, *Escherichia*, *Klebsiella*, *Salmonella* and *Shigella* species produce the iron chelators enterobactin and aerobactin, which have extremely high affinities for iron, while *Pseudomonas* species possess effective siderophores in pyochelin and pyoverdin (Griffith and Williams 1999). Although this study has shown that iron deficiency may not be associated with an increased or decreased risk of bacteraemia there is need for further observational studies to support this finding.

Iron deficiency and malaria

There have been conflicting results from observational studies on the effect of iron deficiency on malaria. Murray *et al* (1978) noted that nomads entering a feeding camp had no malaria parasitaemia if they were iron deficient, in contrast with 5 out of 64 in the iron replete nomads. Possibly the strongest observational study with evidence that iron deficiency may be protective against malaria, was reported by Nyakeriga and colleagues (2004). They followed a cohort of children aged between 8 months and 8 years for over two years and determined the incidence of malaria. At the end of the study, the children's iron status was determined using archived blood samples, thereby limiting observer bias. They found that the incidence of malaria among children who were iron deficient was significantly lower than among those who were iron replete (incidence ratio 0.70, $p<0.05$).

Recently, it has been reported that helminth infection may be associated with decreased risk of severe malaria (Shapiro *et al,* 2005). Researchers have suggested that by inducing elevated Th2 cytokine production and thus down-regulating the Th1 type immune response, helminth infections may alter susceptibility to malaria (Shapiro *et al,* 2005). It is possible that a protective effect of iron deficiency against malaria could explain these finding.

Evidence of iron deficiency being protective against malaria also comes from studies on the use of chelators in malaria infected individuals. The source of iron for intra-erythrocytic plasmodia is still uncertain but possibilities have included a small labile intracellular pool (Hershko and Peto 1988). Using iron

chelators and calcein as a fluorescent probe, it was demonstrated that this labile pool was significantly smaller in parasitised red blood cells as compared to normal cells (Loyevsky *et al,* 1999).

Other studies have been unable to support the hypothesis that iron deficiency is protective against malaria. In a study of Gambian children investigating whether iron status at the start of the malaria transmission season could predict malarial experience, no correlation between incidence of malaria during the transmission season and iron status was found (Oppenheimer 2001).

The present study demonstrated no association between iron deficiency and malaria parasitaemia. However, iron deficiency was associated with lower levels of malaria pigmented leukocytes in peripheral blood among cases. Hemozoin, the end product of haem digestion by the malaria parasite, is released together with merozoites when host red cells rupture. This is phagocytosed by scavenger neutrophils and monocytes (Egan *et al,* 2002). Previous clinical studies have consistently demonstrated that elevated proportions of pigmented leukocytes are associated with increased severity of *P. falciparum* infection, and with a worse clinical outcome (Lell *et al,* 2005). However, the proportion of pigmented neutrophils that are associated with increased severity and increased mortality vary widely (Nguyen *et al,* 1995). This study found that the proportion of pigmented leukocytes among iron deficient cases was 3.0% compared to 5.6% in iron replete cases. Another study observed that the proportion of pigmented leukocytes among survivors was 3.2% versus 7.7% among fatal cases (difference $p= 0.0001$) (Nguyen *et al,* 1995). Lell

et al (2005) have also demonstrated that pigmented monocytes, which have a longer half-life, were better markers for cerebral and severe malarial anaemia than neutrophils which have a half-life of approximately seven hours. Although in the present study, the type of leukocyte containing the malaria pigment was not recorded, the finding of a lower proportion of pigmented leukocytes in iron deficient cases suggests that iron deficiency may be associated with less severe malaria disease.

Iron deficiency and HIV infection

There has been little research on the association between iron deficiency and HIV infection and results to date are inconclusive. The present study found no association, which is in concordance with data for Ugandan children who had a prevalence of iron deficiency of 47% and 59% among HIV infected and non-infected children respectively (*p=0.3*) (Totin *et al,* 2002). Although, anaemia is often more prevalent among HIV infected individuals and is associated with a poor prognosis, its aetiology appears not be related to iron deficiency (Totin *et al,* 2002).

In a frequently quoted review by Gordeuk *et al* (2001), HIV infection has been associated with increased iron status. In one of the reviewed studies, HIV disease progression among thalassemic patients was faster among those receiving lower doses of desferrioxamine and with higher ferritin levels. In another retrospective study of bone marrow iron in HIV-positive patients, it was suggested that survival was shorter in those with high iron stores. In a study in

Malawi in which iron deficiency was determined using a bone marrow smear, it was found that HIV negative adults had a 10 times increased odds of iron deficiency (AOR 9.9, p =0.003) (Lewis *et al,* 2005). Pathogenesis studies have revealed that HIV alters metabolism of the iron by down-regulating CD71 surface expression, affecting the alternative iron pathway (citrate system) resulting in an increase in levels of intra-cellular iron as well as ferritin (Savarino *et al,* 1999; Pugliese *et al,* 2002).

One of the main limitations of the present study was the study design, as it is impossible to prove causality in observational studies. This is because the exposure and outcome under study (iron deficiency and infection), both occur at the time of assessment and their temporal relationship is not known.

9.6 CONCLUSION

The results from this study have demonstrated that there is an increased prevalence of iron deficiency among hospital, community and surgical controls as compared to cases. This may be interpreted as iron deficiency being associated with reduced illness in control children. Bacteraemia was more prevalent among cases than controls, and NTS infection was isolated in 73% of cases and 86% of hospital controls with positive cultures, but this was not associated with iron deficiency.

Although malaria parasitaemia was found not to be associated with iron deficiency, iron deficient children had a significantly lower proportion of malaria pigmented leukocytes than iron replete children. This suggests that iron

deficiency may be associated with less severe malaria disease. The problem of confounding and inability to establish causality are the main limitations of this study.

CHAPTER 10

SUMMARY AND CONCLUSIONS

This was a descriptive study with the broad objective of evaluating the assessment of iron deficiency and its association with infection in Malawian children living in a malaria endemic area. It was nested within a larger case-control study investigating aetiological factors for severe anaemia. A total of 1161 cases, hospital, community and surgical controls were recruited in Blantyre and Chikwawa districts in southern Malawi over a duration of 2 years.

10.1 Assessment of Iron Status using Bone Marrow Iron Methods

Bone marrow microscopy

Results from this study showed that the use of a detailed alternative bone marrow smear examination for iron, can be more informative than the conventional grading method. The alternative histological grading method combined assessment of iron stores in fragments and reticular cells, and cellular iron in erythroblasts. This enables classification of iron status into normal iron status, deficiency of iron stores and functional iron deficiency.

Definition of functional iron deficiency was noted to be an important limitation to the alternative grading method as it was based on bone marrow microscopy findings alone. Despite the limitations, there was reasonable agreement with biochemical iron markers in peripheral blood. Children classified as functionally iron deficient had increased CRP concentrations compared to

those with normal iron status or deficient iron stores. Functionally iron deficient and normal iron status children were found to not differ in mean ferritin and sTfR concentrations, which are indicators of iron stores. There is need for further research to evaluate the use of the alternative histological grading method for differentiating between deficiency of iron stores and functional iron deficiency, in children who are not severely anaemic, in adult populations, and in non-malaria endemic areas.

The use of nuclear fast red solution as a counter-stain was noted to not be optimal for the visualisation of cellular detail required for the assessment of erythroblast iron. There is need for further research on the use of better counter-staining reagents and methods e.g. silver stain, for improved erythroblast iron assessment.

Assessment of iron in bone marrow established that only 30% of children with severe anaemia presenting to hospital, had deficiency of iron stores. Malawi Ministry of Health guidelines recommend that therapeutic iron should be given to children at a dose of 6mg elemental iron/kg/day and prophylaxis iron at 1mg elemental iron/kg/day (Phillips *et al,* 1998). The guidelines do not specifically address the use of iron supplements as part of the management of children with severe anaemia. In practice in Malawian hospitals, most children with severe anaemia will be routinely prescribed iron. In areas where the prevalence of anaemia is above 40%, the World Health Organisation recommends iron supplementation of 2mg/kg/day in all children 6 – 60 months. A dose of 2mg/kg/day is recommended in children aged 6–23 months, to be taken until

they are 23 months old, and a same dose in those aged 23–60 months for a duration of 3months.

This 'blanket' treatment of children with iron, whether for prophylaxis or therapy, may have important implications not only in normal children (Sazawal *et al,* 2006) but even more in HIV infected children. Studies providing evidence that 'high' iron status was detrimentally associated with HIV infection, have been discussed in the Literature Review (Chapter 2). This study has found that iron deficiency was not associated with HIV infection, but this may not be the case during iron supplementation. To date, there have been no controlled trials of iron supplementation in HIV infection. The author plans to conduct a clinical trial of iron supplementation in HIV infected children to further investigate the relationship between iron and HIV infection.

As only a third of severely anaemic children had deficiency of iron stores, this emphasizes the importance of making a diagnosis of iron of deficiency before prescribing iron therapy. It is necessary to establish clear policy guidelines on diagnosis of iron deficiency and the role of iron therapy at both national and international level.

Bone marrow iron status using flow cytometry

Recent progress in flow cytometry has offered an alternative method to measure bone marrow iron content, compared to microscopy which has been the 'gold standard' test. Evidence was presented showing that bone marrow microscopy was occasionally associated false negative results and imprecision

when repeated samples were assessed. Determination of mTfR expression on erythroid progenitor cells has been considerably improved by the use of standardised beads, with known amounts of antigen for comparison. This has enabled the accurate estimation of the average number of transferrin receptors bound per cell in bone marrow samples of cases and surgical controls.

However, a major limitation in interpretation and comparison of these results, was the lack of reference limits for mTfR expression in iron replete children. Results of iron assessment using bone marrow microscopy and biochemical iron markers, presented in Chapters 5 and 7 showed that a large proportion of the limited group of children recruited as surgical controls were iron deficient, hence these were not an appropriate comparison group.

Flow cytometers are becoming more available in research laboratories and if they are to be used for mTfR quantitation as an alternative method to bone marrow microscopy, then it is imperative that studies be carried out to establish reference limits of mTfR expression among healthy, iron replete individuals. These reference limits will have to be age-related (Suominen *et al,* 2001). There is also need for further research on the number of CD71 molecules that bind to the transferrin receptor (i.e. PE:monoclonal antibody ratio).

Bone marrow iron status using mass spectrometry

Mass spectrometry offers a method of determining absolute concentrations of total elemental iron in the bone marrow. From published data, mass spectrometry has rarely previously been used to determine bone marrow

iron content. Studies have used radio-isotopes or other less precise chemical methods to estimate total elemental iron. Results from this study showed that the average concentration of elemental iron was in reasonable agreement with previous studies. Unfortunately, even from previous studies, as mixed patient populations and differing techniques of bone marrow preparation and measurement were used, reference limits for iron deficient individuals could not be determined.

In the discussion, it was noted that there was a discrepancy in the units used to express the amount of elemental iron in bone marrow. This varied from use of weight of iron per 'wet marrow', per 'dry marrow', or per 'protein'. This made comparison between studies complicated. Apart from the need to standardise the units used to report amounts of elemental iron in bone marrow, there is need for further research to establish cut-off values for defining iron deficiency. This may be done by determining the normal distribution of elemental iron concentration in a healthy iron replete group of individuals.

There was poor correlation between the three different methods used to assess bone marrow iron content, namely flow cytometry, mass spectrometry and microscopy. A reason suggested for this discrepancy, was that a significant amount of iron was sequestered in hemozoin in reticular endothelial cells such as macrophages. Studies have shown that a considerable amount of parasitised erythrocyte iron is contained in the parasite in the form of hemozoin. Additionally, this study established that a considerable proportion (94%) of children with severe anaemia had hemozoin visible on examination of their bone marrow

smear. It is unclear whether the quantity or duration it is sequestered and unavailable for erythropoiesis influences the correlation between the bone marrow iron assessment methods.

The author plans to determine the amount of elemental iron in hemozoin using mass spectrometry in a group of randomly selected bone marrow samples from the present study. The results will provide information on the importance of iron sequestered in hemozoin and will enable a meaningful comparison of the three methods used to assess bone marrow iron content. A finding of significant amounts of iron in hemozoin in bone marrow samples, may require the development and evaluation of a hemozoin grading scheme using bone marrow microscopy to help estimate the amount of hemozoin without performing an expensive mass spectrometric analysis. Such a grading scheme has been developed by the author but is not presented in this thesis.

10.2 Assessment of iron status using biochemical markers

Influence of inflammation and malaria

The influence of inflammation and malaria on sTfR and other commonly used biochemical iron markers, namely ferritin, serum iron, serum transferrin, TIBC, transferrin saturation, MCHC and MCV, was assessed among severely anaemic children. Previous studies had extensively reported that sTfR was not altered by inflammation, unlike the other commonly used biochemical iron markers. Results in this study showed that sTfR and most of the other biochemical iron markers were altered by inflammation. Malaria parasitaemia

was also found to only alter sTfR concentrations. This alteration of sTfR during inflammation, also observed in a few other studies, was further investigated by statistically modelling predictors of sTfR. Variables included in the regression model were proxies of iron status (iron in bone marrow fragments and erythroblasts), erythropoiesis (haemoglobin, RI), infection/inflammation (CRP, malaria parasitaemia, HIV status), and haemolysis (haptoglobulin). The important predictors of sTfR were found to be, in decreasing order; CRP; bone marrow iron stores deficiency; HIV status, bone marrow erythroblast iron deficiency, and RI level.

Previous studies showing that iron status and erythropoiesis were important predictors of sTfR were discussed. Possible mechanisms and supporting evidence of inflammation and HIV infection altering sTfR were presented. An important mechanism postulated was through production of inflammatory cytokines such as TNF or IL-1 that cause suppression of erythropoiesis.

It was found that the average sTfR concentrations in the study population were raised compared to concentrations in children living in developed countries. Studies carried out in malaria endemic areas suggested that this was a result of asymptomatic malaria parasitaemia. There is a need for further research to develop a standard reference for sTfR which could be used to standardise laboratory measurements.

CRP, the marker of inflammation used in this study, was one of several acute phase proteins that could be used to assess inflammation. The choice of

which indicator to use depended on the sensitivity and specificity of the specific measurement, and the decision on time of sampling and whether single or repeated measurements were required. It was noted that α-1 acid glycoprotein (AGP) which rises and clears more slowly than CRP or α-1-antichymotrypsin (ACT), may be a better indicator of the presence of chronic or sub-clinical infection. There is need for research to identify markers that can best reflect changes during specific phases of an infection.

Evaluation of optimal biochemical iron markers

Diagnostic efficiency of a variety of biochemical iron markers including sTfR for detecting or excluding iron stores deficiency using bone marrow microscopy, was assessed. Results showed that ferritin had a low sensitivity while sTfR had a low specificity. The iron marker, TfR-F Index, which combines sTfR and ferritin values, was found to have better diagnostic efficiency (sensitivity 74%, specificity 73%, accuracy 73%) than either iron marker used alone. MCHC, a red cell index, was also observed to show reasonable diagnostic efficiency (sensitivity 67%, specificity 64%, accuracy 65%).

After initial sensitivity and specificity determination of the markers, ROC curves for detecting iron stores deficiency were constructed, and the optimum cut-off values of the marker giving maximal sensitivity and specificity was determined. AUC^{ROC}, an indication of how efficient a diagnostic test performs in detecting or excluding iron deficiency, was significantly high for ferritin, sTfR, TfR-F Index and MCHC. Comparison of the original and new cut-off values

(derived from ROC curves) showed ferritin cut-off values increase substantially in order to improve the diagnostic efficiency. Conversely there was hardly any change required for TfR-F Index and MCHC.

Despite coulter machines not being available in many hospitals in resource-poor settings, MCHC, can potentially be utilised. For research purposes, the use of ferritin, sTfR and TfR-F Index may be recommended. For TfR-F Index it was not necessary to substantially change cut-off values to account for inflammation or malaria. By incorporating sTfR and ferritin, the TfR-F Index has been shown to have improved diagnostic efficiency than ferritin or sTfR using the currently accepted cut-off values. An ideal iron marker that has good diagnostic efficiency and that can be used in a health centre in resource-poor settings remains to be identified.

An important limitation to the study was that the diagnostic efficiencies of biochemical markers were tested against bone marrow iron content of children with severe anaemia. Although a full spectrum of bone marrow iron states was observed, and the effect of inflammation was taken into account, there may still be unknown confounders influencing biochemical iron markers.

This study showed poor diagnostic efficiency of most biochemical markers for detecting or excluding functional iron deficiency. There is a priority need for further research to identify new markers or combinations of current markers for diagnosis of functional iron deficiency.

10.3 Iron deficiency and risk of infection

The influence of iron deficiency on bacteria, malaria and HIV infection, was investigated. The possible pathogenesis underlying the two main postulates that iron deficiency aids immunity, or hinders host defence against infection were presented. It was noted that there were few observational studies investigating this association in humans. This may be due to the ethics of studying an iron deficient control group while withholding iron, or due to the problem of controlling for confounders in these studies. Iron deficiency is part of a cluster of inter-related nutrient and social deprivations, ultimately resulting from poverty.

It was observed that the risk of iron deficiency varied inversely with increasing age and children aged 1-2 years were the most vulnerable group. This may have been due to the poor endowment of iron in utero, followed by intake of weaning foods with low iron content and poor bioavailability. It is recommended that iron intervention strategies target the pregnant mother and the infant.

The prevalence of iron deficiency in children in the community, reflected by the prevalence among the community controls was observed to be higher than other community-based studies in the sub-Saharan region. Closer review of these studies showed that despite the use of varying biochemical iron markers with varying cut-off values, most failed to control for the inflammatory effect on biochemical markers which resulted in an underestimation of the prevalence of iron deficiency.

Results from blood and bone marrow bacterial cultures showed that bacteraemia was most prevalent among cases (20% excluding contaminated samples) and that non-typhoidal salmonella (NTS) was the commonest organism isolated (73% amongst cases and 86% amongst hospital controls). It was noted that due to the increased odds of contamination during bone marrow aspiration compared to blood collection, the observed prevalence of bacteraemia of 20% among cases may have been an underestimation.

Despite previous predominantly *in-vitro* studies showing an association between iron deficiency and bacteraemia, this association was not found in the present study. It was noted that there were very few observational studies in humans which emphasizes the need for further research. Additionally, in this study the infections studied were limited to bacteraemia, HIV infection and malaria. There is need to investigate the association between iron deficiency and other clinically important infections, e.g. pneumonia, diarrhoea, fungal infections.

Although there was no association between iron deficiency and malaria parasitaemia, which was contrary to some of the findings in other studies, it was observed that iron deficiency was associated with a lower proportion of malaria pigmented leucocytes. An increased proportion of pigmented leucocytes has been associated with more severe forms of malaria and a higher mortality. There is need to identify whether this difference between iron deficient and replete children was clinically significant and whether the proportion of pigmented leucocytes may be used as a prognostic factor for predicting outcome of clinical malaria in Malawi.

Literature evidence reviewed showed that 'high' iron status was detrimental in HIV infection, however this had been based mostly on *in vitro* studies and a few observational studies in humans. Recent population-based study suggested that iron supplementation may be deleterious in children living in areas of high malaria transmission (Sazawal *et al,* 2006). To date, a formal trial of iron supplementation has not been undertaken in HIV infected individuals. The present study showed no association between iron deficiency and HIV infection but this study did not take into account the stage of the disease. The use of CD4 counts and viral load as markers of HIV disease progression may improve understanding of the association between iron status and HIV infection.

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Appendix 3.1

Severe anaemia cases
Consent form

Your child has been admitted to the hospital because s/he is seriously ill and has severe anaemia (serious lack of blood). He/she may need a blood transfusion. The reason why your child has this serious lack of blood is not clear. It is also not known if your child will get the same problem in the future again. In the Malawi-Liverpool-Wellcome Trust programme in Blantyre and The Malawi College of Medicine, we are doing a research study to find out why children develop severe anaemia. We also want to try to find out if some children are more likely to develop severe anaemia than others.

As part of the normal hospital routine for children with severe anaemia, the blood of your child will be checked for anaemia and for malaria, and s/he will get a blood transfusion if necessary. S/he has to stay in hospital for a few days.

Study investigations:

1. As part of the study we would like to investigate the blood, the urine and stool of your child for possible causes of severe anaemia. The blood needed for the investigation will be taken from the IV line just at commencement of the blood transfusion, to make sure that it will not effect the health of your child.
2. As part of the study we would also like to investigate the bone marrow of your child. The bone marrow is the place in the body where the blood is made. Taking and studying a bone marrow sample is a way of investigating severe anaemia in hospitals. It may also help to give the right treatment (e.g. antibiotics) and to find out if a child has an increased risk to get this severe anaemia problem again. Before we do the investigation we will give your child medicine through either an injection in the buttock or application of some cream on the pelvic bone area to reduce the pain. The procedure is not dangerous and takes about a minute.
3. As part of the blood investigation we would like to test your child for HIV infection. HIV may be one of reasons why a child develops anaemia. A trained counsellor will explain more about the HIV virus and the HIV test.

You will also receive a special card, which gives you access to the special study clinic. We would like to ask you to bring your child to this clinic if the child falls ill. In addition, we will ask you to bring back your child to the clinic for regular health-checks after 1, 3, 6 and 12 months. At each of these visits we will ask about your child's health, the study staff will examine the child and will take a little bit of blood from the finger or the heel (¼ of a teaspoon). This will be used to see if the anaemia has improved or become worse. We will inform you of any test results, which will help you, understand why your child has anaemia.

The extra-care and regular health-checks will help to improve the health of your child and hopefully will help to detect illnesses at an early stage. If you do not want your child to go on with this study you can stop at anytime.

We hope the results of this study will help to improve the treatment and prevention for severe anaemia in this area. Thank you very much for your time.

Parent/Guardian *(or mark of consent)*	**Name**	**Signature**.....	***Date***.....
Nurse/doctor	**Name**	**Signature**.....	***Date***.....

Mgwirizano wolowera mu kafukufuku

Mwana wanu wagonekedwa ku chipatala chifukwa akudwala kwambiri ndipo ali ndi vuto loperewera magazi. Iye mwina azafunika kulandira magazi. Chifukwa chomwe mwana wanuyu akuperewera magazi sichikudziwika bwino. Sichodziwikanso ngati mwana wanuyu azapereweranso magazi mtsogolo. Mu bungwe la Malawi-Liverpool-Wellcome Trust ku Blantyre and Malawi College of Medicine, tikupanga kafukufuku ndi cholinga chopeza chifukwa chimene ana akumakhala ndi vuto loperewera magazi. Tikufunanso kufufuza chifukwa chomwe ana ena amakhala ndi vutoli kusiyana ndi ena.

Mwakhalidwe ku chipatala kwa ana onse okhala ndi vuto loperewera magazi, magazi a mwana wanu azalingidwa kuperewera kwake ndi ngati ali ndi malungo. Mwana wanu azalandira magazi ngati kuli kofunika kutero ndipo iye akhala kuchipatala masiku angapo.

Njira zofufuzira vutoli

1. Ana amene atakhale mukafukufuku adzayezedwa magazi, nkozo, ndi chimbuzi pofuna kuwunika nthenda zina zomwe zingabweretse kuperewera magazi. Magazi azatengedwa nthawi yomwe mwana wanu azayambe kulandira magazi.
2. Ana adzatengedwanso magazi amufupa. Mufupa ndi momwe magazi amapangidwa. Kutenga magazi amufupa ndi chinthu chomwe chimachitika ndipo ndi njira yimodzi yofufuzira zifukwa zomwe ana amakhalira ndi vuto loperewera magazi. Izi zitithandiza kudziwa chifukwa chake mwana wanu ali ndi vutoli. Ndiponso zitithandiza kuti timupase mankhwala woyenera ndiponso kufufuza ngati azakhale nayonso vutoli mtsogolo. Tisanatenge magazi amufupa timupatsa mankhwala woletsa ululu (pa thako). Kutenga magazi amufupaku sikowopsya ndipo sukutenga nthawi.
3. Tizafunanso kuyeza magazi a mwana wanu ngati ali ndi ka chilombo ka HIV. Kukhala ndi kachilombo ka HIV katha kukhala chifukwa chimodzi choperewerets a magazi mwa mwana wanu. Mlangizi adzakufotokoza zambiri za kachilomboka ndi kayezedwe kake.

Muzalandira chiphaso chokuyenerani kubwera ku chipatala chakafukufuku ka ana operewera magazi. Tikupempani kuti mubweretse mwana wanu nthawi zonse pamene adwala. Kuwonjezera apo, tikupempani kuti mumubweretse pakatha miyezi 1, 3, 6 ndi 12. Panthwa yimeneyi tizakufunsani za umoyo wa mwana wanu, ndiponso tizatenga magazi pang'ono pa chala kapena chidendeni. Izi zizathandiza kuwona ngati vuto loperewera magazi mwa mwana wanu lawonjezereka kapena kuchepa. Tizakuwuzani zosatira zili zonse zomwe zizathandize inu kumvetsa chifukwa chake mwana wanu ali ndi vutoli.

Kukupempani kubwera ndi mwana wanu ku chipatala kumuthandiza kukhala ndi umoyo wangwiro pakuti matenda ena azapezeka asanayambe kuwonekera. Mulinso ndi mwayi wosiya kafukufukuyu nthawi yina yiri yonse.

Tikhulupirira kuti zosatira za kafukufukuyu zithandiza kuchiza ndiponso kupewa vuto loperewera magazi mu dera lanu lino.

Zikomo kwambiri chifukwa cha nthawi yanu.

Kholo/oyan'ganira odwala **(kapena chidindo chovomereza)**	**Dzina:**.....	**Siginechala:**.....	***Tsiku*:**.....
Ogwira ntchito ya kafukufuku	**Dzina:**.....	**Siginechala:**.....	***Tsiku*:**.....

SEVERE ANAEMIA STUDY

Consent form for parent/guardian of Hospital controls

Background: In this part of Malawi anaemia (lack of blood) is very common. Anaemia affects the health, growth and development of children. Some children develop severe anaemia, which may be fatal. Through the Malawi-Liverpool-Wellcome Trust programme and The Malawi College of Medicine, we are doing a research study to find out why some children develop severe anaemia. We would also like to try to find out if some children are more likely to develop severe anaemia.

Routine management: As part of the normal hospital routine the blood of your child will be checked to find out why your child is ill. After this check he/she will receive treatment and it may be necessary to admit your child to the ward. In addition to the hospital routine we would like to investigate your child for anaemia.

Study investigations:

1. As part of the study we would like to investigate the blood, the urine and stool of your child for anaemia and possible causes of anaemia. The blood needed for the investigation will be taken at the same time as the blood investigation ordered by your doctor and will be not more than 5ml (table spoon).
2. As part of the blood investigation we would like to test your child for HIV infection. HIV may be one explanation why a child develops anaemia. A trained counsellor will explain more about the HIV virus and the HIV test.

Follow-up and study clinic:.
You will also receive a special card, which gives you access to the special study clinic. We would like to ask you to bring your child to this special clinic if the child falls ill. In addition, we will ask you to bring back your child to the clinic for regular health-checks after 1, 3, 6 and 12 months. At each of these visits we will ask about your child's health, the study staff will examine the child and will take a little bit of blood from the finger or the heel (¼ of a teaspoon). This will be used to check for anaemia and other diseases. Treatment at the clinic will be free of charge.

Benefits and Refusal: The extra-care and regular health-checks will help to improve the health of your child and hopefully will help to detect illnesses at an early stage. If you do not want your child to go on with this study you can stop at anytime.

We hope the results of this study will help to improve the treatment and prevention for severe anaemia in this area. Thank you very much for your time.

Parent/Guardian *(or mark of consent)*	Name.....	Signature	*Date*.....
Nurse/doctor	Name.....	Signature	*Date*.....

SEVERE ANAEMIA STUDY

MGWIRIZANO WOLOWERA MU KAFUKUFUKU (hospital control)

Tafuna tikupempheni ngati mungatilole kuti mwana wanu alowe mukaundula wa kafukufuku wofuna kudziwa chomwe chikuyambitsa vuto loperewera magazi m'matupi a wana oyambira miyezi isanu ndi umodzi kufikira zaka zisanu.
Mwana wanuyu adzakhala m'modzi mwa ana osiyanitsira ndi ana ena omwe akupezeka ndi vutoli kwambiri kuposa ena.

Kuperewera kwa magazi ndivuto lalikulu mdera lino. Ana opezeka ndi vutoli kwambiri amayenera kugonekedwa ku chipatala komanso kupatsidwa magazi. Akapanda kutero ena amatha kumwalira. Choncho a bungwe la Wellcome Trust lomwe limachita kafukufuku mogwirizana ndi sukulu ya za udotolo kuno ku Malawi, akupanga kafukufuku kuti apeze chifukwa chomwe ana ena amapezeka ndi vutoli kwambiri komanso kuti apeze njira zopewera kuperewera kwambiri kwa magazi m'matupi a wana.

Tili ndi chikhulupiliro kuti zotsatira za kafukufukuyu zidzathandiza kwambiri pogonjetsa vutoli.

NJIRA ZOFUFUZIRA VUTOLI.

Pa chipatala cha boma pa Chikwawa komanso pa chipatala chachikulu cha Queens ku Blantyre, patsegulidwa mbali ina yazipatalazi yomwe muzigwiridwa ntchito ya kafukufukuyu.

Ana amene akhale mukafukufukuyu adziyezedwa nkodzo, chimbudzi komanso magazi pofuna kuunika nthenda zina zomwe sizinaonekere. Malo otengera magazi ndipachala, pankono ndi pachitendene. Mwana aliyense adzayenera kulandira chithandizo molingana ndi nthenda yomwe yapezeka nthupi mwake.

Motsatira chithandizo choyambachi, kholo lidzayenera kubwera ndi mwanayo ku chipatala kudzayezedwanso pakatha mwezi umodzi, itatu, isanu ndi umodzi, pakatha chaka komanso pakatha chaka ndi theka. Komanso mwanayu adzakhala ndi mwayi olandila mankhwala kumbali ya ofesi ya kafukufukuyu pa nthenda ina iliyonse yomwe angadwale pa nthawi ina iliyonse.

Kholo lizadziwitsidwa za zotsatira zonse za zoyezayeza zomwe tikhulupilira kuti zidzakuthandizani kumvetsa bwino za matenda omwe mwana wanu angakhale nawo.

Zikomo kwambiri chifukwa cha nthawi yanu.

Kholo/oyan'ganira odwala. (kapena chidindo chovomereza)	**Dzina:**.....	**Siginechala:**.....	**Tsiko:**.....
Ogwira ntchito ya kafukufuku	**Dzina:**.....	**Siginechala:**.....	**Tsiko:**.....

Kholo likhoza kusayina kapena kungonena kuti lavomera kafukufukuyu ndipo mboni idzasainira.

SEVERE ANAEMIA STUDY

Consent form for parents/guardian of Community controls

In this part of Malawi severe anaemia (serious lack of blood) is a common problem, which may be fatal. Children with severe anaemia need to be admitted into hospital and get a blood transfusion. Through the Malawi-Liverpool-Wellcome Trust programme in conjunction with the Malawi College of Medicine, we are doing a research study to find out why only some children develop severe anaemia and if we can prevent these children from getting severe anaemia. A child from your village has been admitted at Chikwawa District Hospital with severe anaemia. We would like to ask you if you permit your child to join the study as a village control. This means that we will compare your child with the child with severe anaemia to find out in what respect the children are different.

A doctor or a nurse will check the health of your child on a regular basis at the under five clinic in your village or at the special study-clinic at CDH. We will also investigate the urine, stool and blood of your child for any unnoticed diseases that may be important in the development of severe anaemia (infectious diseases, malnutrition etc.). At a later stage a trained counsellor will ask you separate permission to include an HIV test in the infection screening. If necessary your child will receive treatment free of charge. You will also receive a special card, which gives you access to the special study clinic. Please bring your child to the study clinic at any time if it falls ill. At the clinic a nurse or doctor will then investigate the reason for the illness and check the blood for infections and anaemia.

Benefits and Refusal: The extra-care and regular health-check will help to improve the health of your child and hopefully will help to detect illnesses at an early stage. If you do not want your child to go on with this study you can stop at anytime.

We hope the results of this study will help to improve the treatment and prevention for severe anaemia in this area.

Thank you very much for your time.

Parent / Guardian *(or mark of consent)*	Name	Signature	*Date*.....
Nurse/doctor	Name	Signature	*Date*.....

SEVERE ANAEMIA STUDY

MGWIRIZANO WOLOWERA MU KAFUKUFUKU (community controls)

Tafuna tikupempheni ngati mungatilole kuti mwana wanu alowe mukaundula wa kafukufuku wofuna kudziwa chomwe chikuyambitsa vuto loperewera magazi m'matupi a wana oyambira miyezi isanu ndi umodzi kufikira zaka zisanu.
Mwana wanuyu adzakhala m'modzi mwa ana osiyanitsira ndi ana ena omwe akupezeka ndi vutoli kwambiri kuposa ena.

Kuperewera kwa magazi ndivuto lalikulu mdera lino. Ana opezeka ndi vutoli kwambiri amayenera kugonekedwa ku chipatala komanso kupatsidwa magazi. Akapanda kutero ena amatha kumwalira. Choncho a bungwe la Wellcome Trust lomwe limachita kafukufuku mogwirizana ndi sukulu ya za udotolo kuno ku Malawi, akupanga kafukufuku kuti apeze chifukwa chomwe ana ena amapezeka ndi vutoli kwambiri komanso kuti apeze njira zopewera kuperewera kwambiri kwa magazi m'matupi a wana.

Tili ndi chikhulupiliro kuti zotsatira za kafukufukuyu zidzathandiza kwambiri pogonjetsa vutoli.

NJIRA ZOFUFUZIRA VUTOLI.

Pa chipatala cha boma pa Chikwawa komanso pa chipatala chachikulu cha Queens ku Blantyre, patsegulidwa mbali ina yazipatalazi yomwe muzigwiridwa ntchito ya kafukufukuyu.

Ana amene akhale mukafukufukuyu adziyezedwa nkodzo, chimbudzi komanso magazi pofuna kuunika nthenda zina zomwe sizinaonekere. Malo otengera magazi ndipachala, pankono ndi pachitendene. Mwana aliyense adzayenera kulandira chithandizo molingana ndi nthenda yomwe yapezeka nthupi mwake.

Motsatira chithandizo choyambachi, kholo lidzayenera kubwera ndi mwanayo ku chipatala kudzayezedwanso pakatha mwezi umodzi, itatu, isanu ndi umodzi, pakatha chaka komanso pakatha chaka ndi theka. Komanso mwanayu adzakhala ndi mwayi olandila mankhwala kumbali ya ofesi ya kafukufukuyu pa nthenda ina iliyonse yomwe angadwale pa nthawi ina iliyonse.

Kholo lizadziwitsidwa za zotsatira zonse za zoyezayeza zomwe tikhulupilira kuti zidzakuthandizani kumvetsa bwino za matenda omwe mwana wanu angakhale nawo.

Zikomo kwambiri chifukwa cha nthawi yanu.

Kholo/oyan'ganira odwala. (kapena chidindo chovomereza)	**Dzina:**.....	**Siginechala:**.....	**Tsiko:**.....
Ogwira ntchito ya kafukufuku	**Dzina:**.....	**Siginechala:**.....	**Tsiko:**.....

Kholo likhoza kusayina kapena kungonena kuti lavomera kafukufukuyu ndipo mboni idzasainira.

Appendix 3.2

Severe Anaemia Study *Recruitment Form*

First name _____ **Study Number** _____

Surname _____

Study group ☐ **1** = Severe anaemia case, **2** = Hospital controls, **3** = Community controls

Consent ☐ **1** = Full consent, **2** = No consent, **3** = Only for BM, **4** = Only for HIV test

HIV counsel ☐ **1** = Yes HIV results, **2** = No HIV results, **8** = Not applicable, **9** = Missing

Study Centre ☐ **1** = QECH, **2** = CDH

Recruitment date ☐☐/☐☐/☐☐

Discharge date ☐☐/☐☐/☐☐

DEMOGRAPHIC DATA

Date of birth ☐☐/☐☐/☐☐

Age _____ *months* (only if DOB is not known)

Sex ☐ **1** = Male, **2** = Female

Ethnic origin ☐ **1** = Chewa, **2** = Yao, **3** = Sena, **4** = Tumbuka, **5** = Lomwe, **6** = Ngoni
7 = Tonga, **8** = Other *Specify*_____, **9** = Missing/Unknown

Religion ☐ **1** = Catholic, **2** = Prot-major, **3** = Prot-Adventist, **4** = Prot-Jehovah,
5 = Traditional, **6** = Muslim, **7** = others, **9** = Missing

Age mother ☐☐ **Estimated Age** (if unknown) ☐

Education **Mother** ☐ **1** = Non / minimal (illiterate), **2** = Primary
Father ☐ **3** = Secondary, **4** = Tertiary, **9** = Missing

Job **Mother** ☐ **1** = Unemployed, **2** = Self employed, **3** = Wage employed
Father ☐ **4** = White collar job, **9** = Missing

Health **Mother** ☐ **1** = healthy, **2** = died, **3** = sick, specify_____

Parents life ☐ **1** =Both alive, **2** =Mother died, **3** =Father died, **4** =Both died, **9** =Missing

Property no ☐ Record no of categories: Cattle, Land, Car/Motorbike, bicycle, TV, Radio/CD, House, **9**=missing

Number of siblings ALIVE ☐ **DIED** ☐ (*excluding patient & same mother and father*)

PREVIOUS MEDICAL HISTORY

Chronic illness ☐ **1** = No, **2** = Haematological, **3** = Cardiac, **4** = Nephrotic, **5** = Developmental **6** = Multiple, **7** = Other *Specify* _____ , **9** =Missing

	Y / N	
Previous Admissions	☐	*If yes Specify* _____
Previous Transfusion	☐	*If yes Specify* _____
Previous Ant malarial	☐	*In last 2 months, if yes Specify* _____
Previous Antibiotics	☐	*In last 2 months, if yes Specify* _____
Previous Haematinics	☐	*In last 2 months, if yes Specify* _____
Previous Multivitamins	☐	*In last 2 months, if yes Specify* _____

Weight loss or Failure to Thrive ☐ 1 = yes; 2 = no; 3 = less than three points on curve

CURRENT MEDICAL STATUS

Presenting problem 1 ☐ code and *Specify* _____

Presenting problem 2 ☐ code and *Specify* _____

Fever _____ *days*

Cough _____ *days*

Vomit _____ *days*

Diarrhoea _____ *days*

Last fluid intake _____ *hrs*

Diet details:

	Y / N
Bloody stool	☐
Bloody urine	☐
Jaundice history	☐
Respiratory distress	☐
Fitting	☐
Coma	☐

Meals with meat per <u>month</u>	☐	Concerning last month
Meals with fish per <u>month</u>	☐	More meals/day is possible
Poor appetite in last month	☐	**1** = Yes **2** = No
Breastfeeding	☐	**1** = Yes only BF, **2** = No **3** = Yes & additional feeds

History details:

CLINICAL EXAMINATION

Weight _____ *kg*

Length _____ *cm*

Temperature _____ *°C* (if axillary add 0.5)

Respiratory rate _____ */ min*

Pulse rate _____ *bpm*

Blood pressure _____ *mmhg*

O2 saturation _____ *%*

Respiratory rhythm ☐ **1** = Regular, **2** = Irregular, **9** = Missing

Respiratory amplitude ☐ **1** = Normal, **2** = Irregular, **3** = Deep, **9** = Missing

	Y / N		Y / N
Grunting	☐	**Cold periphery**	☐
Nasal flaring	☐	**Fitting on admission**	☐
Chest recessions	☐	**Neck stiffness**	☐
Anaemia	☐	**Skin changes**	☐
Jaundice	☐	**ENT/mouth abnormalities**	☐
Oedema	☐	**Chest signs**	☐
Xeropthalmia	☐	**Bleeding**	☐
Skeletal deformities	☐	**Koilonychia**	☐
Angular stomatitis	☐	**Glossitis**	☐

NB if any of these is confirmed please describe Abnormalities in <u>Clinical Examination</u>

General feeding condition ☐ **1** = good, **2** = fair, **3** = poor (describe in Clinical Examination)

Lymphadenopathy ☐ **1** = absent, **2** = regional, **3** = general

Pallor: **Conjunctival** ☐ **1** = absent, **2** = mild/moderate, **3** = severe **NB Assess before Hb!**

Palmar ☐ **1** = absent, **2** = mild/moderate, **3** = severe **NB Assess before Hb!**

Dehydration ☐ **1** = Mild, **2** = Moderate, **3** = Severe, **4** = No, **9** = Missing

Coma score ☐ **(0-5)**

Spleen _____ *cm* **Hb** _____ *g/dl*

Liver _____ *cm* **Malaria slide** _____ parasites/200 WBCs

Presenting diagnosis 1 ☐ code and *Specify* _____

Presenting diagnosis 2 ☐ code and *Specify* _____

Clinical examination details: