Aetiology, Pathogenesis & Consequences of Severe Anaemia in Malawian Children: HIV and other factors
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HIV and other factors

Job Calis

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HIV and other factors
**COLOFON**

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HIV and other factors

ACADEMISCH PROEFSCHRIFT

Ter verkrijging van de graad van doctor aan de Universiteit van Amsterdam
op gezag van de Rector Magnificus prof. dr. P.F. van der Heijden
ten overstaan van een door het college voor promoties ingestelde commissie,
in het openbaar te verdedigen in de Agnietenkapel
op donderdag 22 mei 2008, 12:00 uur

Door

Jacobus Clemens Johannes Calis

Geboren te Laren
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Faculteit der Geneeskunde
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ABBREVIATIONS

A&E    Accidents and emergency department
AIDS   Acquired immunodeficiency syndrome
AOR    Adjusted odds ratio
ART    Anti-retroviral therapy
ARV    Anti-retroviral (drugs)
AZT    Zidovudine
bp     Basepair
CC     Community control
CD     Cluster of differentiation
CI     Confidence interval
CMV    Cytomegalovirus
CRP    C-reactive protein
DNA    Deoxyribonucleic acid
EBV    Epstein-Barr virus
G6PD   Glucose 6 phosphate dehydrogenase (deficiency)
HAART  Highly active antiretroviral therapy
HC     Hospital control
HR     Hazard ratio
Hb     Haemoglobin concentration
HIV    Human immunodeficiency virus
IL     Interleukin
MCH    Mean cellular haematocrit
MCHC   Mean cellular haematocrit concentration
MCV    Mean cellular volume
NNRTI's Non-nucleoside reverse transcriptase inhibitors
NRC    Nucleated red cell
NRTI's Nucleoside and nucleotide analogs
NSI    Non-syncytium-inducing (see R5)
OR     Odds Ratio
PCR    Polymerase chain reaction
Pf     Plasmodium falciparum
PI's   Protease inhibitors
PMTCT  Prevention of mother to child transmission
R5     HIV strain that uses the CCR-5 co-receptor
RBC    Red blood cell
RCPF   Red cell production failure
RMSEA  Root mean square error of approximation
RNA    Ribonucleic acid
RR     Relative risk
RT     Reverse transcriptase enzyme
SevAna Severe anaemia, abbreviation used for the main study
SI     Syncytium-inducing (see X4)
sTfR   Soluble transferrin receptor
TNF    Tumor necrosis factor
WBC    White blood cell count
X4     HIV strain that uses the CXCR-4 co-receptor
Chapter

Introduction
SEVERE ANAEMIA

Natural history and definition
Anaemia is defined as a reduction of the red blood cell volume or haemoglobin concentration below the range of values occurring in healthy children. Newborn infants have a relative high haemoglobin level which decreases and reaches its lowest value at the age of 2-4 months, followed by a gradual rise with age until adulthood. In addition to age, haemoglobin levels can be influenced by racial differences. Black children have levels about 0.5 g/dl lower than those of white and Asian children of comparable age and socioeconomic status.

Although a reduction in the amount of circulating haemoglobin decrease the oxygen-carrying capacity, few clinical disturbances occur until the haemoglobin level falls below 7-8g/dl. Below this level clinical symptoms of anaemia occur including pallor, weakness, tachypnea and tachycardia which are all consequences of several physiological processes aimed at increasing oxygen transport to the (vital) organs.

Severe anaemia refers to a condition in which the haemoglobin level decreases to such an extent that the compensatory mechanisms fail and/or a severe outcome can be expected. Children aged 6-60 months are most vulnerable to this condition and in this population WHO has defined severe anaemia as:

- All children with a haemoglobin level < 6 g/dl
- Children with a haemoglobin level between 4-6 g/dl and dehydration, shock, impaired consciousness, deep and laboured breathing, heart failure or very high malaria parasitaemia.

For practical reasons the old definition of severe anaemia will be used (haemoglobin concentration <5g/dl).

Prevalence and mortality
Severe anaemia (haemoglobin concentration <5g/dl) is a major cause of paediatric morbidity and mortality in sub-Saharan Africa. Population based studies have reported a severe anaemia prevalence of 3-5% in African children under five year of age. In different geographical settings 12-29% of children admitted to hospital were severely anemic, with in-hospital mortality between 8-17%. In severely anaemic children following discharge from hospital an overall mortality of 30% has been reported.

Treatment and prevention
Severe anaemia is not a specific entity but results from many underlying pathogenetic and aetiological processes. The care for severely anaemic children may therefore exist of two
interventions: acute treatment of the anaemia (e.g. blood transfusion); and prevention or treatment of aetiologies and comorbidities.

The treatment of severe anaemia with blood transfusion, can be life-saving. However transfusions are expensive and logistically complex to deliver as only a minority of African children are transfused within six hours\(^4\,6\,7\). Transfusion increases the risk of transmission of blood born diseases including HIV and viral hepatitis\(^1\).

Since severe anaemia is a symptom rather than a disease, interventions should target prevention and treatment of the underlying causes\(^1\). Yet surprisingly little is known concerning the aetiology and pathogenesis of severe anaemia in African children. Published studies mostly relate to malarial anaemia\(^1\), or were limited by sample size\(^7\,14\) or to a restricted set of etiological factors investigated\(^4\,5\). This has contributed to the current opinion that malaria, folate and iron deficiency are the most common causes of severe anaemia, and World Health Organisation-advocated treatment guidelines specifically address these\(^1\).

**Pathogenesis**

Severe anaemia in African children may result from: a) an increased rate of destruction of red cells, due to either mechanical lysis or immune mediated destruction; b) acute or chronic blood loss, or c) impaired erythropoiesis with defective proliferation and/or maturation of red cell precursors.

The latter is probably the most important factor and may result from deficiencies of micronutrients (e.g. iron, folate), from chronic infections (e.g. malaria, HIV), or from an inappropriate host inflammatory response to intermittent infections\(^1\,15\,18\). In experimental models, a toxic or infective stimulus elicits pro-inflammatory cytokines such as interferon-gamma (IFN-\(\gamma\)) and tumour necrosis factor-\(\alpha\) (TNF), which modulate the production and effectiveness of erythropoietin, resulting in a suppression of erythroid progenitor cells\(^1\,20\). Studies in African children with severe (malarial) anaemia have demonstrated an inappropriate inflammatory response, with high serum levels of IFN-\(\gamma\) and TNF in the absence of normal counter-regulatory cytokine levels (IL-4 and IL-10)\(^1\,15\,17\,21\). In those children with persistent severe anaemia this cytokine imbalance persisted for weeks after effective antimalarial treatment\(^2\). An inappropriate (un-modulated) chronic cytokine response may not only explain why only some children develop severe anaemia, but also why there was a striking additional post-hospital mortality found in Kenyan children following a severe anaemia episode\(^2\). Very few studies have comprehensively studied the relative contribution of these mechanisms to the severe anaemia of African children.
Aetiology

Several aetiological factors may contribute to the development of severe anaemia which include nutritional factors, drugs, genetic disorders and infections.

Nutritional factors

There is a strong association between iron deficiency and mild to moderate anaemia in African children, but the attributable risk of iron deficiency for severe anaemia is uncertain. Only recently has a sufficiently powered study assessed the preventive value of iron supplementation against severe anaemia in African children. It showed a non-significant reduction in severe anaemia, but the study was stopped because iron supplementation was associated with an increased number of hospital admissions and deaths. The circumstantial evidence on iron status can be difficult to interpret as iron status mostly is assessed with proxy measures including serum ferritin, free iron, transferrin, and MCV which each can be altered by infection, malnutrition, or haemoglobinopathies, conditions that are common in African children. Examination of bone marrow iron stores is a more accurate measure that has rarely been used in studies of severe anaemia in African children. The most promising measure of body iron status could be a combination of sTfR and ferritin levels (measuring cellular iron needs and body iron stores), with an additional non-specific marker of inflammation (e.g. C-reactive protein). Again data on this in African children remains limited.

Vitamin B12 and Folate deficiency lead to diminished DNA synthesis and can cause megaloblastic/macrocytic anaemia. The main dietary source for folate is green vegetables and fruits, whilst vitamin B12 is derived from animal sources. Both deficiencies are thought to frequently occur in developing countries. Although these deficiencies are generally considered to be causes of mild and moderate anaemia, little information is available on their contribution to severe anaemia in African children. Folate supplementation in anemic children with malaria failed to raise haemoglobin concentrations in a study from The Gambia. Vitamin B12 deficiency has mostly been studied in adults and this data suggests it may play a more important role than currently expected.

Vitamin A supplementation has been associated with the prevention of infections such as malaria and in deficient populations should prevent anaemia. Supplementation trials in women improved the haemoglobin levels of newborns, although the evidence in older children is limited. In fact the same study that showed a protective effect against malaria in children did not observe a protective effect against anaemia. Other micronutrients that may influence haemoglobin concentration include zinc, riboflavin and vitamin E. The data on these in relation to the prevention of severe anaemia, especially in children, is limited.
Drugs
Several drugs, mostly prescribed as treatment of infections, can cause anaemia in African populations. These include; antibiotics (mainly anti-folate drugs and chloramphenicol), tuberculostatics; and to a lesser extent anti(retro)virals (especially AZT), and cytostatic agents. Some of the mechanisms are known to relate to genetic polymorphisms affecting the red cell.

Genetic disorders
Glucose-6-phosphate dehydrogenase deficiency (G6PD), alpha-Thalassaemia and haemoglobinopathies such as sickle cell trait and haemoglobin C commonly occur in African populations and have a protective role against the development of malaria. However homozygosity as in sickle cell disease may lead to severe anaemia. There is increasing evidence that genes regulating the immune response may also play a role in host susceptibility to severe anaemia, one possible pathway being through genetic regulation of cytokine production. Recently polymorphisms in the tumor necrosis factor gene have been associated with severe malaria anaemia (TNF-238 A allele) and some polymorphisms in the IL-10 gene are associated with decreased IL-10 production. Whether this and other polymorphic markers are of true functional importance (directly affecting cytokine production) or are just linked genetic markers requires further investigation.

Infections
Malaria, a major aetiological factor for severe anaemia, causes haemolysis and impaired erythropoiesis. The relative contribution of both mechanisms to malaria related severe anaemia are poorly understood. Haemolysis may be intravascular through red-cell destruction by schizont rupture, or extravascular, aggravated by increased clearance of parasitized and non parasitized red-cells by the spleen. Recent detailed studies have indicated that even with high parasitaemia, the extravascular component is the dominant mode of haemolysis in malaria. Impaired erythropoiesis in children with ‘malaria related severe anaemia’ appeared not to be related to hypoxic damage due to parasite sequestration. The evidence was more suggestive of a non-specific activation of the inflammatory cytokine network related to infections. In areas with high malaria endemicity, malaria is likely to be the major contributor to the (chronic) infectious component, but other pathogens (e.g. HIV and chronic bacterial infections) may well have been underestimated (or under-diagnosed) in the past. Antimalarial prophylaxis can prevent severe anaemia in holoendemic areas but has been associated with increased susceptibility to malaria and increased risk of severe anaemia after the intervention was stopped.

Both Hookworm and Schistosoma are well known to be associated with the development of anaemia. Hookworm (Necator americanus and Ancylostoma duodenale) can be very prevalent in rural populations and this prevalence increases with age. Blood loss (and
iron deficiency) appears to be the main causal mechanism and the magnitude is closely related to the burden of infection\textsuperscript{56,57}. Hookworm infections have been associated with severe anaemia\textsuperscript{58}. In Schistosomiasis both blood loss and cytokine responses have been identified as main mechanisms leading to mild and moderate anaemia\textsuperscript{59,60}.

HIV infection is associated with anaemia in African children\textsuperscript{36,61-63}, although its link to severe anaemia is less well described\textsuperscript{37}. The pathogenesis of anaemia in HIV-infected adults, although multi-factorial, relates primarily to a reduced production of erythrocytes\textsuperscript{64-67}. This reduction is influenced by several aetiological factors including infection and neoplasms, drugs such as zidovudine, a direct effect of HIV on erythropoiesis, a blunted response to erythropoietin and nutritional deficiencies\textsuperscript{64-67}. Compared to adults there is very little information available about the association between HIV infection and anaemia in children. This evidence on anaemia and HIV in children is reviewed in detail in Chapter 5.

HIV

Until thirty years ago, HIV was an unknown disease and few persons had been infected\textsuperscript{68}. By 2007 an estimated 33 million people are infected worldwide and another 25 million have died of AIDS\textsuperscript{69}. Approximately 7.5% (2.5 million) of all infected persons worldwide are younger than 15 years of age. In 2007 16.8% of all new infections occurred in children and 15.7% of HIV-related mortality occurred in this age group. These numbers are striking given the fact that maternal-infant transmission, which is the main cause of new infections in this age group, can largely be prevented with minimal interventions.
which could be made accessible and cost-effective\textsuperscript{48,70,71}. In Western & Central Europe and North America less than 2500 children were infected in 2007, in sub-Saharan Africa the estimate was 370 000 (Figure 1).

**Viral Characteristics**

HIV is a human retrovirus which can be subdivided into two types: HIV-1, the most common variant throughout the world, and HIV-2 a related but less pathogenic and relatively uncommon type which is concentrated in West Africa. HIV-1 can be further subdivided into several subtypes which possess varying pathogenicity. Clade B is the most common subtype in Western Europe and the Americas. In sub-Saharan Africa subtype A was most common in the early epidemic. Nowadays the more virulent subtype C has become dominant and accounts for 55% of HIV-1 infections worldwide\textsuperscript{72}. Several other subtypes and recombinants exist and others are being discovered throughout the world.

In addition to this genetic distinction, HIV can be sub-divided by the type of co-receptor the virus uses to infect human cells. To infect a cell, the virus binds to both the CD4 receptor and to a second receptor, the so-called co-receptor, both located on the cellular membrane. Most HIV-strains have affinity for the CCR5 co-receptor and are called R5 or non-syncytium-inducing (NSI) viruses. Several other strains using different co-receptors exist of which the strain binding to the CXCR4 receptor, the X4 or Syncytium Inducing (SI) strain, is the most common\textsuperscript{73,74}. Most infections, and probably all perinatal infections, are thought to be caused by the R5 strain\textsuperscript{75}. During the course of infection some viruses may evolve to express X4 affinity\textsuperscript{73}. This viral switch is associated with a more rapid decline in CD4+ T-cells and disease progression\textsuperscript{73}. The switch to X4 affinity is a consequence of changes to the viral genome of the envelope protein gp120\textsuperscript{76}. It is best studied and most prevalent in subtype B infected persons. In subtype C infected persons the X4 strain is relatively uncommon and little studied\textsuperscript{76}.

**Natural course of HIV infection**

HIV can be transmitted sexually, through parenteral-exposure to blood or vertically from mother to child. In contrast to other viral infections, the risk of serious illness and death will increases over time in infected individuals. In adults the initial infection is followed by a symptom free interval which on average lasts 8 to 10 years. During this period CD4+ or T-helper cells, primary targets of HIV, decline. Once T-helper cells, which have an immuno-regulatory function, reach a low-threshold, the infected person becomes immunodeficient and several opportunistic infections and neoplasms may occur. This condition, called Acquired Immunodeficiency Syndrome (AIDS), will eventually, if left untreated, lead to death.
Although in children the infection follows a similar pattern, some differences are present. Firstly children are mainly infected perinatally, either in-utero, during delivery or through breast milk. All children born to HIV-infected mothers will carry antibodies which can persist up to the age of 18 months and may complicate diagnosis. If no preventive measures are taken 12-30% of exposed children in western settings will become infected. In developing countries these numbers are even higher with 25-52% of HIV-exposed children becoming infected.

Secondly children are known to have higher viral loads and more rapid progression to AIDS than adults. In western countries 15-25% of infected children die before the age of one. The majority of children (60-80%) survive longer (median 6 yrs) and a small group survives beyond the age of 10 (5%). In developing countries up to 85% of those infected follow a rapid progression and die within months after birth.

Thirdly a different classification system is used in children. Immunological staging, based on CD4+ T-cells, generally shows higher cell counts in children, is (therefore) expressed in % of total lymphocytes rather than cells per liter and uses age dependent cut offs. Clinical staging is done using guidelines specifically adapted to children. These were developed as some HIV-related conditions are relatively uncommon in children (e.g. toxoplasmosis and Cryptococcal meningitis), whilst others may not necessarily be restricted to immunodeficient children (e.g. oral candidiasis and recurrent upper respiratory tract infections). Overall these differences complicate diagnosis, staging and therefore timely and accurate treatment in HIV-infected children, especially in resource poor settings.

**Anti-Retroviral Therapy**

HIV cannot be eliminated from infected persons, and antiretroviral therapies (ART) are aimed at diminishing HIV replication. Four classes of antiretroviral agents directed against three steps in the HIV life cycle are currently available.

- Reverse transcriptase enzyme (RT): nucleoside and nucleotide analogs (NRTI’s) and non-nucleoside reverse transcriptase inhibitors (NNRTI’s) inhibit the function of this enzyme.
- Fusion inhibitors attach themselves to the envelope glycoprotein gp41 which prevent fusion between the virion and the host membrane.
- Protease enzyme (Pro): protease inhibitors (PIs) inhibit the function of the viral protease.

During the reverse transcription of HIV to proviral DNA many mutations occur (1/2000 bp) and drug resistance may follow. Therefore highly active antiretroviral therapy (HAART) with combinations of antiretroviral agents is applied, and the chance that the virus will develop drug resistance is minimised. Drug intolerance and drug toxicity
are significant problems for all drugs used to treat HIV infection. Monitoring patients based on the dynamics of CD4+ cells and viral load gives a good indication of potential resistance and disease progression. However these tests are costly and this restricts good monitoring of patients started on ART in sub-Saharan Africa. Reliable and yet affordable monitoring may be one of the most important challenges for the future of ART in Africa.

The drugs used in ART can also be applied to prevent in pregnant HIV-infected mothers transmission to their children. The PACTG076 study first described that zidovudine monotherapy reduced transmission drastically. Later several more practical regimens have been successfully tested in developing countries. Although elective caesarean section and bottle milk still are advocated in western countries, probably the most practical strategy in African countries may be maternal reduction of viral load by HAART. Despite a few gifts of ART can prevent a new infection, only a minority of pregnant women infected with HIV living in Malawi received this treatment in 2006.

**METHODS**

**Study setting**

*Malawi* is situated in south-east Africa and is bordered on the north by Tanzania, the west by Zambia, and the north and east by Mozambique (Figure 2). 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, approximately three times the size of the Netherlands, 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. Malawi has three seasons: a dry hot season from September to November, a rainy season from December to April. May to August is marked by colder dry weather. Temperature and rainfall vary with proximity to Lake Malawi and altitude. Some demographic indicators are presented in Table 1.

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. The stable diet consists of maize and to a lesser extent rice and cassava. According to the Malawi Annual and Economic survey (2001) the economy depends on substantial inflows of economic assistance from the IMF, the World Bank, and individual donor nations. Agriculture comprises 55% of gross domestic product (GDP). The main exports are tobacco, tea, coffee, peanuts and wood products.
Malawi’s health service consists of 23 District Hospitals which are surrounded by health centres that provide basic services. Health care is free of charge in the governmental institutions, although mission hospitals charge a small amount for their services. Both systems are constrained by lack of financial and human resources. Since 1998 medical doctors were able to fully complete their training in Malawi at the College of Medicine.
in Blantyre. For nurses, medical assistants and clinical officers training facilities already existed in Blantyre and Lilongwe.

**Study sites**

*Blantyre* is the main commercial town of Malawi. Located in the southern region (Figure 3), it has a predominantly urban population of half a million people. At an altitude of 800m above sea level, malaria and anaemia are mainly seasonal. Its main hospital, the Queen Elizabeth Central Hospital, is not only a District and referral hospital, but also the main teaching hospital for Malawi’s only medical university: the College of Medicine. The hospital has recently renovated its Accidents and Emergency department (A&E) which caters for children presenting to hospital. During the rainy season 300 children are seen daily in this department of which 100 are admitted. All children who are admitted to hospital are routinely screened for malaria and anaemia. Reasons for admissions are presented in table 2. Individuals living in this area will endure approximately one infectious malaria bite per person per year (T Mzilahowa personal communication) and the overall HIV-prevalence in adults living in this urban area is 25%.[86] 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, two research assistants and a driver. Study patients were managed in this clinic during recruitment and follow-up visits.

*Chikwawa* district is, located 50km from Blantyre in the Shire valley (altitude 250m above sea level) and experiences malaria transmission year round (Figure 3.1). Individuals living

![Figure 3. Location of the study sites Blantyre and Chikwawa](image-url)
in this area will endure approximately 170 infectious malaria bites per person per year (T Mzilahowa personal communication) and the adult HIV-prevalence in this rural area is estimated at 13%\textsuperscript{86}. Schistosoma haematobium and hookworm infections are thought to be common among children living in this area\textsuperscript{88,89}. The Chikwawa District Hospital caters for a predominantly rural population of approximately 400,000 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. Several other studies on anaemia from our study group have been performed in this area.

**Study design and groups**

To improve our understanding of severe anaemia, we conducted a case-control study to assess causative factors in Malawian children with severe anaemia. Between July 2002 and July 2004 three groups of children, aged between 6 and 60 months were recruited: cases, hospital controls, and community controls. All children needed to be living within the pre-defined catchment area and should not have received a blood transfusion within four weeks prior to recruitment. Additional recruitment criteria per study group were:

**Cases:**
- Hb less than 5g/dl on admission
- Should not have trauma or malignancy as a recognised specific cause of severe anaemia

**Hospital Controls:**
- Hb greater or equal to 5g/dl on recruitment
- Should be presenting to hospital due to an illness

**Community controls:**
- Hb greater or equal to 5g/dl on recruitment
- Share same residency as a case
- Physically healthy child

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<th>Percentage</th>
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<td>Malaria</td>
<td>32%</td>
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<tr>
<td>Respiratory tract infection</td>
<td>18%</td>
</tr>
<tr>
<td>Abscess</td>
<td>12%</td>
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<tr>
<td>Malnutrition</td>
<td>8%</td>
</tr>
<tr>
<td>Gastro-enteritis</td>
<td>7%</td>
</tr>
<tr>
<td>Bacterial infection other</td>
<td>7%</td>
</tr>
<tr>
<td>Severe anaemia</td>
<td>4-12%</td>
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The aim of recruiting hospital controls was to control for factors that influence health-seeking behaviour in the population. Community controls were recruited to control for environmental factors that may influence development of severe anaemia.

Cases, hospital and community controls were recruited in a ratio of 1:1:1. Informed consent was requested from the guardians of the children if they fulfilled the inclusion criteria for the study. During recruitment, a questionnaire collecting demographic information was administered, followed by a medical history and full physical examination. Prior to administering a blood transfusion, venous blood was collected and a bone marrow aspirate was performed under local or general anaesthesia. Hospital controls were recruited the day following recruitment of a case. The first child at the front of the queue waiting to be seen by medical staff in A&E (Blantyre) and under-five clinic (Chikwawa) was selected. Community controls were recruited on discharge of a case. The case was escorted home by a member of the study team and the first child fulfilling the selection criteria at a distance of 100-1000 metres from the compound was selected. Informed consent, data and sample collection procedures were the same as in cases except for the bone marrow collection which was not performed in either control population.

Follow-up study
To determine outcome in children who experienced an episode of severe anaemia all children (also the control populations) were followed during a period of 18 months from recruitment. Follow-up consisted of scheduled visits to the study clinic (active follow-up) which took place on 1, 3, 6, 12 and 18 months from recruitment. If children did not report for their follow-up, families were visited by a study team member. In addition guardians were asked to present children in case of illness during the follow-up period (passive follow-up). At both visits a standardized history and physical examination was completed.

Main aims of severe anaemia study
To investigate in Malawian children with severe anaemia:

- 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.
- The contribution of the bone marrow inflammatory cytokine network to the pathogenesis of severe anaemia, and its relation to genetic determinants.
- The natural history of severe anaemia through longterm 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.
Additional aims of this PhD
To study in Malawian children:
- If HIV is a risk factor for developing severe anaemia.
- The pathogenesis of HIV-associated severe anaemia.
- The use of severe anaemia as a potential predictor of mortality in HIV-infected children.
- The association between HIV and anaemia in children in different geographical settings; and to summarize currently available data on pathogenesis, aetiology and treatment.
- The erythroid response and importance of bone marrow apoptosis in severely anaemic HIV infected children.
- Potential aetiological factors of HIV-associated severe anaemia.
- Viral differences among severely or non-severely anaemic HIV infected children

Chapter overview
2. Severe anaemia in Malawian children.
5. HIV-associated anaemia in children, a systematic review from a global perspective.
7. Severe anaemia is not associated with HIV-1 env gene characteristics in Malawian children.
8. Discussion and summary.
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Chapter 2

Severe anaemia in Malawian children


ABSTRACT

Background
Severe anaemia is a major cause of morbidity and mortality in African children. Current preventive and curative strategies focus on treatment of malaria and hookworm and supplementation with iron and folic acid. Yet the causes of the anaemia have been inadequately studied.

Methods
We conducted a case-control study of 381 preschool children with severe anaemia (haemoglobin concentration <5g/dL) and 757 children without severe anaemia in urban and rural settings in Malawi. Causal factors associated with severe anaemia in the past were studied. The data were examined using multivariate analysis and structural equation modelling.

Results
Bacteraemia (Adjusted Odds Ratio, AOR:5.3, 95%-Confidence Interval, 95%-CI:2.6-10.9), malaria (AOR:2.3, 95%-CI:1.6-3.3), hookworm (AOR:4.8, 95%-CI:2.0-11.8), HIV infection (AOR:2.0, 95%-CI:1.02-3.8), G6PD\textsuperscript{202/-376} (AOR:2.4, 95%-CI:1.3-4.4), and deficiency of vitamin A (AOR:2.8, 95%-CI:1.3-5.8) or vitamin B12 (AOR:2.2, 95%-CI:1.4-3.6) were associated with severe anaemia. Folate deficiency, sickle cell disease, and laboratory signs of an abnormal inflammatory response were uncommon. Iron deficiency was less prevalent in case-patients (AOR:0.37, 95%-CI:0.22-0.60) and was negatively associated with bacteraemia. Malaria was associated with severe anaemia in the urban area (seasonal transmission), but not in the rural (holoendemic) setting. Seventy-six percent of hookworm infections were found in children aged under two years.

Conclusions
In severely anemic children current recommendations promoting iron and folate-supplements and ignoring bacteraemia and vitamin B12 deficiency may not be applicable. Even in the presence of malaria parasites, additional or alternative causes of severe anaemia should be considered.
BACKGROUND

Severe anaemia (haemoglobin concentration <5g/dl) is a major cause of morbidity and mortality in sub-Saharan African children. In different settings, 12-29% of hospitalized children are severely anemic, and in these children the in-hospital case fatality rate is 8-17%. Little is known of the cause of severe anaemia in African children. Most studies have been confined to the anaemia of malaria, or other individual factors. As a result, treatment guidelines advocated by the World Health Organization deal specifically with malaria, folate deficiency, and iron deficiency, which are widely held to be the commonest causes of severe anaemia in African children. To improve our understanding of severe anaemia, we conducted a case-control study to assess causative factors in Malawian children with severe anaemia.

METHODS

Study sites
The study was conducted in Malawi at Chikwawa District Hospital in a rural area where malaria infections occur throughout the year (~170 infectious bites/person/year) and at Queen Elizabeth Central Hospital, a referral hospital in urban Blantyre, where malaria is seasonal (~1 infectious bites/person/year, T. Mzilahowa, personal communication). Predefined catchment areas were used; the urban area was confined to the city limits.

Study design
Between July 2002 and July 2004 a consecutive sample of children (382 cases) who presented at the outpatient department during working hours with a primary diagnosis of severe anaemia (defined as a haemoglobin concentration <5.0 g/dL) were recruited into a prospective case-control study. Additional inclusion criteria were: age 6-60 months and no transfusion within the previous month. For each case, a community control and a hospital control were enrolled. Community controls were recruited from apparently healthy residents living within 100-1000 meters of the case; hospital controls were recruited by selecting the first child presenting at the outpatient department on the same time of the working day following presentation of the case. Community controls and hospital controls were eligible for recruitment if their haemoglobin was ≥5.0 g/dL and they were aged 6-60 months, no other matching was applied. Written consent was obtained from a parent or guardian of children in all three study groups. The study was approved by the ethics committees of the College of Medicine, Malawi, and the Liverpool School of Tropical Medicine, UK.
Clinical assessment and management

On enrolment, a clinical research form, including a medical and dietary history, socio-demographic data and physical examination was completed, and samples of blood, urine, and stool were collected. In case-patients, if the clinical condition permitted, a bone marrow aspirate was obtained under local anaesthesia. Children requiring admission were treated in a study ward. All conditions were managed according to standard protocols.

Anthropometry

Nutritional Z-scores were calculated according to the WHO-growth reference curves using EPI info 2000. ‘Wasting’ (weight-for-height), ‘stunting’ (height-for-age) or ‘underweight’ (weight-for-age) applied to children with Z-scores <-2 and were considered ‘severe’ if <-3.

Laboratory methods

Laboratory tests (haematology, bacteriology and parasitology) were performed within 24 hours and aliquots were stored at -80°C for later analysis. Laboratory staff were blinded to the child’s study group.

Haematology:

Haemoglobin concentration was measured on site using a Hemocue system (Angelholm, Sweden). A complete blood count, including reticulocytes, was performed by Coulter counter (Coulter, Hialeah, Fla). In case-patients, bone marrow slides were stained (HaematoGnost Fe, Darmstadt, Germany) and graded for iron content; these results were used to validate peripheral blood markers for iron deficiency. The soluble transferrin receptor/log ferritin-index (TfR-F Index) best predicted bone marrow iron status irrespective of the presence of infection, and was used to define iron deficiency (TfR-F Index>5.6: sensitivity 70%, specificity 75%).

Chemistry:

Plasma levels of C-reactive protein, haptoglobin, transferrin, iron, ferritin, folate and vitamin B12 were analyzed on a Roche p800/e170 system (Roche, Switzerland). Inflammatory cytokine profiles were measured by Cytometric Bead Array on a FACS-Calibur flow-cytometer (Becton-Dickinson, South Africa). Serum vitamin A (retinol) and soluble transferrin receptor were measured using high performance liquid chromatography and enzyme linked immuno-sorbant assay (Ramco Laboratories, TX) respectively.

Parasitology:

Plasmodium falciparum asexual parasites were counted against 200 white blood cells and expressed per microliter blood. Malaria slides were read by two independent readers,
a third being used if results differed by >25%. ‘Malaria’ was defined by the presence of asexual *P. falciparum* parasites. ‘Recent or current malaria’ was defined by the presence of asexual *P. falciparum* parasites in erythrocytes or malaria pigment in monocytes or macrophages. ‘Hyperparasitaemia’ was defined as >100,000 parasites/μL. Stool samples were examined for helminths using the Kato-Katz method. For hookworm, ‘heavy infection’ was defined as >1000 ova/g feces. A polymerase chain reaction (PCR) was used to confirm microscopy and define subspecies (*Ancylostoma duodenalis* and *Necator americanus*). Urine specimens were examined for *Schistosoma haematobium*, using a semi-quantitative concentration method.

**Bacteriology:**
A bone marrow or venous blood sample (1-2ml) was inoculated into BACTEC Myco/F-Lytic culture vials and incubated in a BACTEC 9050 automated system during 56 days. Sub-culturing, susceptibility testing and isolate identification were in accordance with standard techniques. Cultures were checked for mycobacteria using Ziehl-Neelsen stained slides. Mixed growth or growth of micrococci, *Bacillus* species or coagulase-negative staphylococci were considered contaminants.

**Virology:**
Whole blood isolates were assessed for Epstein-Barr virus (EBV) and cytomegalovirus (CMV) infection by semi-quantitative PCR and for parvovirus by real-time PCR. Infections were considered clinically important if the number of viral copies exceeded 1000c/mL blood. HIV testing was performed using two rapid tests (Determine, Abbott-Laboratories, Japan; Unigold, Trinity-Biotech, Ireland). Discordant and reactive results in children less than 18 months were resolved by PCR.

**Genetics:**
DNA was extracted using a Nucleon extraction kit (Amersham biosciences, UK) and genotyped by primer extension mass-spectrometry using MassArray (Sequenom). Sickle cell disease (homozygous HbS) and single nucleotide polymorphisms in the promoter region of *IL10* (-1117, -3585, +4949) and *TNF* (-238, -308, -1031) were analyzed. The term G6PD-202/-376 is used to denote boys who were hemizygous and girls who were homozygous for both the G6PD202A and G6PD376G allele; which is strongly predictive of glucose-6-phosphate dehydrogenase deficiency. The Hardy-Weinberg equilibrium was applied (cut-off: P<0.001) and there was no significant evidence of population stratification. We chose the allele frequency, dominant model or haplotype that was most strongly associated with severe anaemia.

**Statistical Methods**
The prevalence of each factor was compared individually across the three groups using the Fisher exact and chi-square tests. The combined association of characteristics
Table 1. Characteristics of the study groups.

<table>
<thead>
<tr>
<th></th>
<th>Case-patients</th>
<th>Community Controls</th>
<th>Hospital Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total sample size</td>
<td>381</td>
<td>380</td>
<td>377</td>
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<tr>
<td>Aread:</td>
<td></td>
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<tr>
<td>Urban</td>
<td>205 (53.8%)</td>
<td>203 (53.4%)</td>
<td>201 (53.3%)</td>
</tr>
<tr>
<td>Rural</td>
<td>176 (46.2%)</td>
<td>177 (46.6%)</td>
<td>176 (46.7%)</td>
</tr>
<tr>
<td>Sex:</td>
<td></td>
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</tr>
<tr>
<td>Female</td>
<td>203 (53.3%)</td>
<td>191 (50.3%)</td>
<td>180 (47.7%)</td>
</tr>
<tr>
<td>Male</td>
<td>178 (46.7%)</td>
<td>189 (49.7%)</td>
<td>197 (52.3%)</td>
</tr>
<tr>
<td>Age in months(^a)</td>
<td>mean (s.d.)</td>
<td>20.4 (12.8)</td>
<td>25.3 (13.1)</td>
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<tr>
<td>Jaundice(^b)</td>
<td>19 / 379 (5.0%)</td>
<td>1 / 380 (0.3%)</td>
<td>0 / 376 (0.0%)</td>
</tr>
<tr>
<td>Splenomegaly (&gt;1 cm)(^b)</td>
<td>237 / 372 (63.7%)</td>
<td>108 / 363 (29.8%)</td>
<td>86 / 349 (24.6%)</td>
</tr>
<tr>
<td>Fever (&gt;37.5°C axillary)(^c)</td>
<td>189 / 376 (50.3%)</td>
<td>41 / 375 (10.9%)</td>
<td>172 / 374 (46.0%)</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)(^b)</td>
<td>mean (s.d.)</td>
<td>3.6 (0.8)</td>
<td>9.9 (1.9)</td>
</tr>
<tr>
<td>MCV (fL)(^b)</td>
<td>mean (s.d.)</td>
<td>82.9 (15.2)</td>
<td>75.5 (9.3)</td>
</tr>
<tr>
<td>Reticulocytes ((^10^9/L))(^a)</td>
<td>median (iqr.)</td>
<td>53.2 (30.2-91.7)</td>
<td>76.8 (46.4-114.7)</td>
</tr>
<tr>
<td>Admitted to hospital(^d)</td>
<td>381 (100%)</td>
<td>3 (0.8%)</td>
<td>17 (4.5%)</td>
</tr>
<tr>
<td>Died in hospital(^d)</td>
<td>24 (6.3%)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^a\) Differences between all three groups statistically significant (Tukey post hoc test or Kruskal-Wallis test with Tukey multiple comparisons p < 0.05), \(^b\) Community and hospital controls significantly different from case-patients (Tukey post hoc test, p < 0.05), \(^c\) Community controls significantly different from case-patients and hospital controls (Tukey post hoc test, p < 0.05), \(^d\) No statistical tests applied.

MCV: Mean cellular volume; s.d.: standard deviation; iqr: interquartile range; Splenomegaly: > 1cm palpable below the left costal margin in the mid axillary line

associated with the risk of severe anaemia (P≤0.10, unless uncommon) was examined using an unconditional multivariate logistic regression model correcting for potential confounding factors (age, sex, recent use of anti-malarials or haematinics, and previous transfusions). Missing observations were included in the analysis by creating ‘missing value’ categories. Alternative definitions for malaria, hookworm and nutritional deficiencies and status were tested. Attributable risk percentages were calculated using Adjusted Odds Ratios (AOR)\(^25\). The primary analysis compared all case-patients with both control groups combined. To explore the possibility that different patient characteristics were important in the two study locations, secondary analyses were performed stratified by location and with the community control and hospital control groups separated. More complex associations and alternative strategies for handling missing data (e.g. maximum likelihood imputation) were explored using structural equation modeling\(^26\). All reported P-values are two-sided. Data were analyzed using STATA 9 (Stata Corporation, TX), SPSS 12 and AMOS 6.0 (SPSS, IL).
RESULTS

Over a two year period, we enrolled 1141 children. Five protocol violations occurred: two hospital control children had severe anaemia and were re-designated as case-patients; one case, with a haemoglobin concentration of ≥5 g/dL, and two controls, aged less than six months, were excluded. Table 1 summarizes characteristics of the 1138 children included in the analysis. Haemoglobin levels were significantly different between the case-patients and the two controls groups, but similar between the control groups. Splenomegaly (>1cm palpable) or severe splenomegaly (≥8 cm) were more common in case-patients (P<0.001 and P=0.03, respectively). Severe splenomegaly, present in 11

* Cultures only performed in case-patients and hospital controls. ** Hookworm was not entered in the 'urban' model because the prevalence was <5%. CI: Confidence interval; Wasting was defined as a Z-score for weight-for-height <−2; Iron deficiency was defined as TfR/log(ferritin) ratio >5.610;11; Concentrations of vitamin B12 <200 ng/L and vitamin A <20 μg/dL were considered deficient; HIV: Human immunodeficiency virus; EBV: Epstein-Barr virus; The rs-classification for IL10 +4949: wis rs3024500.

The model was corrected for possible confounders: age, sex, recent anti-malarial treatment, recent hematinic treatment, previous transfusions and death of a parent. Owing to the high correlation between the three IL-10 polymorphisms, only one (most strongly associated to severe anemia) was included in the multivariate model. In the combined model interaction existed between malaria and site (p<0.001). The goodness-of-fit of the model was evaluated using the Hosmer and Lemeshow test (P=0.65).

Figure 1. Adjusted odds ratios and 95% confidence intervals for factors associated with severe anaemia by study group and recruitment site.
Table 2. Distribution of possible aetiological and confounding factors amongst study groups by recruitment site.

<table>
<thead>
<tr>
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<th>Both Sites</th>
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<tr>
<td></td>
<td>Cases n=381</td>
<td>CC+HC n=757</td>
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<td><strong>History</strong></td>
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<td>Mother did not attend secondary school</td>
<td>323 / 366 (88%)</td>
<td>554 / 753 (74%)***</td>
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<td>Death of a parent</td>
<td>25 / 284 (8.8%)</td>
<td>22 / 554 (4.0%)**</td>
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<tr>
<td>Recent Hematinics</td>
<td>85 / 376 (23%)</td>
<td>61 / 754 (8.1%)***</td>
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<tr>
<td>Recent Antimalarials</td>
<td>232 / 375 (62%)</td>
<td>346 / 755 (46%)***</td>
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<tr>
<td>History of Transfusion</td>
<td>57 / 378 (15%)</td>
<td>38 / 756 (5.0%)***</td>
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<td><strong>Malnutrition</strong></td>
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<td>Wasting</td>
<td>52 / 330 (16%)</td>
<td>43 / 695 (6.2%)***</td>
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<td>Iron deficiency</td>
<td>97 / 208 (47%)</td>
<td>288 / 415 (69%)***</td>
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<td>Vitamin B12 deficiency</td>
<td>95 / 310 (30%)</td>
<td>94 / 603 (16%)***</td>
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<td>Vitamin A deficiency</td>
<td>228 / 247 (92%)</td>
<td>172 / 262 (66%)***</td>
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<td><strong>Viral infections</strong></td>
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<td>HIV</td>
<td>45 / 357 (13%)</td>
<td>41 / 682 (6.0%)***</td>
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<td>Parvo B19</td>
<td>5 / 294 (1.7%)</td>
<td>2 / 609 (0.3%)*</td>
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<td>EBV</td>
<td>89 / 269 (33%)</td>
<td>102 / 566 (18%)***</td>
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<tr>
<td><strong>Bacterial infections</strong></td>
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<td>Bacteremia</td>
<td>54 / 359 (15%)</td>
<td>14 / 353 (4.0%)***</td>
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<td><strong>Parasitic Infections</strong></td>
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<td>Malaria parasites</td>
<td>226 / 380 (59%)</td>
<td>321 / 750 (43%)***</td>
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<tr>
<td>Hyperparasitemic malaria</td>
<td>45 / 380 (12%)</td>
<td>24 / 750 (3.2%)***</td>
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<tr>
<td>Recent or current malaria infection</td>
<td>243 / 334 (73%)</td>
<td>336 / 696 (48%)***</td>
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<td><strong>Genetic disorders</strong></td>
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<td>G6PD-202/-376</td>
<td>44 / 318 (14%)</td>
<td>54 / 601 (9.0%)*</td>
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<tr>
<td>Sickle Cell Disease</td>
<td>4 / 238 (1.7%)</td>
<td>4 / 404 (1.0%)</td>
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<tr>
<td>IL10 -1117 (C/C+C/T vs. T/T)</td>
<td>196 / 324 (60%)</td>
<td>332 / 607 (55%)†</td>
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<tr>
<td>IL10 -3585 (A/A vs. A/T+T/T)</td>
<td>22 / 308 (7.1%)</td>
<td>25 / 575 (4.3%)†</td>
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<tr>
<td>IL10 +4949 (G/G vs. G/A+A/A)</td>
<td>97 / 322 (30%)</td>
<td>134 / 606 (22%)**</td>
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<tr>
<td>Abnormal IL-10/TNF-α ratio</td>
<td>4 / 276 (1.4%)</td>
<td>n/a</td>
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</table>

All comparisons vs. cases. †: p ≤ 0.100 *: p ≤ 0.050 **: p ≤ 0.010 ***: p ≤ 0.001
CC: Community Controls; HC: Hospital Controls; Recent use of antimalarials or hematinics applied to a period of 4 weeks prior to recruitment. HIV: Human Immunodeficiency Virus; EBV: Epstein-Barr Virus; Wasting was defined as a Z-score for weight-for-height < -2. Iron deficiency was defined as TR/ log(ferritin) ratio >5.6. Concentrations of folate <3.0 μg/L, vitamin B12 <200 ng/L and vitamin A <20 μg/dL were considered deficient; Malaria was defined as the presence of *P. falciparum* parasites, Hyperparasitemia was defined as >100,000 *P. falciparum* parasites per microliter blood; An IL-10/TNF-α ratio <1 was defined as abnormal.
### Table 2.

**Distribution of possible aetiological and confounding factors amongst study groups by recruitment site.**

<table>
<thead>
<tr>
<th></th>
<th>Rural</th>
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<th>Urban</th>
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<tbody>
<tr>
<td></td>
<td>Cases</td>
<td>CC</td>
<td>HC</td>
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<tr>
<td></td>
<td>n=176</td>
<td>n=177</td>
<td>n=176</td>
</tr>
<tr>
<td>162 / 172 (94%)</td>
<td>154 / 176 (88%)*</td>
<td>143 / 176 (81%)**</td>
<td>161 / 194 (83%)</td>
</tr>
<tr>
<td>13 / 168 (7.7%)</td>
<td>5 / 163 (3.1%)*</td>
<td>3 / 164 (1.8%)*</td>
<td>12 / 116 (10%)</td>
</tr>
<tr>
<td>40 / 176 (23%)</td>
<td>7 / 177 (4.0%)**</td>
<td>13 / 176 (7.4%)**</td>
<td>45 / 200 (23%)</td>
</tr>
<tr>
<td>107 / 176 (61%)</td>
<td>79 / 177 (45%)*</td>
<td>90 / 176 (51%)+</td>
<td>125 / 199 (63%)</td>
</tr>
<tr>
<td>24 / 176 (14%)</td>
<td>9 / 177 (5.1%)*</td>
<td>13 / 176 (7.4%)+</td>
<td>33 / 202 (16%)</td>
</tr>
<tr>
<td>24 / 169 (14%)</td>
<td>9 / 174 (5.2%)*</td>
<td>19 / 175 (11%)</td>
<td>28 / 161 (17%)</td>
</tr>
<tr>
<td>71 / 101 (70%)</td>
<td>76 / 95 (80%)</td>
<td>63 / 92 (68%)</td>
<td>26 / 107 (24%)</td>
</tr>
<tr>
<td>46 / 142 (32%)</td>
<td>20 / 143 (14%)**</td>
<td>30 / 149 (20%)*</td>
<td>49 / 170 (29%)</td>
</tr>
<tr>
<td>113 / 126 (90%)</td>
<td>44 / 83 (53%)**</td>
<td>60 / 74 (81%)+</td>
<td>115 / 121 (95%)</td>
</tr>
<tr>
<td>7 / 176 (4.0%)</td>
<td>5 / 176 (2.8%)</td>
<td>9 / 172 (5.2%)</td>
<td>38 / 181 (21%)</td>
</tr>
<tr>
<td>2 / 143 (1.4%)</td>
<td>0 / 147 (0%)</td>
<td>1 / 146 (0.7%)</td>
<td>3 / 151 (2.0%)</td>
</tr>
<tr>
<td>43 / 128 (34%)</td>
<td>34 / 133 (26%)</td>
<td>24 / 127 (19%)**</td>
<td>46 / 141 (33%)</td>
</tr>
<tr>
<td>20 / 171 (12%)</td>
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<td>9 / 166 (5.4%)*</td>
<td>34 / 188 (18%)</td>
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<td>93 / 175 (53%)</td>
<td>93 / 176 (53%)</td>
<td>135 / 204 (66%)</td>
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<tr>
<td>17 / 176 (10%)</td>
<td>3 / 175 (1.7%)*</td>
<td>11 / 176 (6.3%)</td>
<td>28 / 204 (14%)</td>
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<tr>
<td>113 / 169 (67%)</td>
<td>98 / 171 (57%)+</td>
<td>98 / 175 (56%)*</td>
<td>130 / 165 (79%)</td>
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<tr>
<td>27 / 154 (18%)</td>
<td>4 / 160 (2.5%)*</td>
<td>8 / 156 (5.1%)*</td>
<td>2 / 142 (1.4%)</td>
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<td>4 / 160 (2.5%)</td>
<td>4 / 156 (2.6%)</td>
<td>0 / 142 (0%)</td>
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<tr>
<td>4 / 159 (2.5%)</td>
<td>6 / 168 (3.6%)</td>
<td>1 / 162 (0.6%)</td>
<td>0 / 148 (0%)</td>
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<tr>
<td>21 / 152 (14%)</td>
<td>11 / 141 (7.8%)+</td>
<td>9 / 145 (6.2%)*</td>
<td>23 / 166 (14%)</td>
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<tr>
<td>2 / 118 (1.7%)</td>
<td>2 / 101 (2.0%)</td>
<td>0 / 86 (0%)</td>
<td>2 / 120 (1.7%)</td>
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<td>98 / 155 (63%)</td>
<td>75 / 141 (53%)+</td>
<td>83 / 147 (56%)</td>
<td>98 / 169 (58%)</td>
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<tr>
<td>8 / 148 (5.4%)</td>
<td>6 / 134 (4.5%)</td>
<td>5 / 140 (3.6%)</td>
<td>14 / 160 (8.8%)</td>
</tr>
<tr>
<td>48 / 155 (31%)</td>
<td>31 / 140 (22%)+</td>
<td>34 / 147 (23%)</td>
<td>49 / 167 (29%)</td>
</tr>
<tr>
<td>1 / 122 (0.8%)</td>
<td>n/a</td>
<td>n/a</td>
<td>3 / 154 (1.9%)*</td>
</tr>
</tbody>
</table>

The rs-classification for the genetic markers were **IL10** -1117: rs1800896; **IL10** -3585: rs1800890; **IL10** +4949: rs3024500; **TNF** -238: rs361525; **TNF** -308: rs1800629; **TNF** -1031: rs1799964. Among variables not included in the table (because they did not meet the pre-set cut of significance) are: Parental unemployment, Number of household assets, Folate deficiency, Trichuriasis, Ascariasis, CMV infection, Hemoglobin C and TNF-α alleles/genotypes (-238, -308, -1031).
case-patients (3.0%), was not associated with thrombocytopenia or leukopenia. Jaundice was more common in cases (5%, Table 1) but was not associated with sickle cell disease, G6PD-202/-376 or splenomegaly (P=1.00, 0.70 and 0.30 respectively). Twenty-four case-patients (6.3%) died during admission, nine (38%) before receiving a transfusion. We obtained 1105 (97%) peripheral blood, 1024 (90%) stool, 1042 (92%) urine, and 348 (91% of case-patients) bone marrow samples. Table 2 lists the features we investigated in the three groups and indicates P values for differences. Factors significantly associated with severe anaemia were further explored in a multivariate and structural equation model (Figure 1 and 2).

Malaria

*P.falciparum* was identified in 226 (59%) case-patients and 321 (43%) controls and was the predominant malaria species overall (97.5%). *P. malariae* was found in 1.6% and a mixed infection in 0.9%, equally distributed between the study groups. The attributable risk of *P.falciparum* to severe anaemia was 34% overall and 47% in the urban setting.
In the rural setting, a significant association between malaria and severe anaemia was found only in the subgroup (10%) with hyperparasitaemia (AOR: 7.1, 95% CI: 1.4-34.6, case-patients vs. community controls).

**HIV**
HIV infection was found in 86 children (13% of case-patients and 6% of controls). The attributable risk of HIV to severe anaemia was 6.2% overall, and 15% in the urban setting. In severely anemic children, the presence of EBV (15/30 vs. 69/226, P=0.03) or bacteraemia (11/42 vs. 38/300, P=0.02) was more common among HIV-infected children than among uninfected children, while hyperparasitaemia (2/44 vs. 42/312, P=0.09) and vitamin B12 deficiency (5/39 vs. 85/254, P=0.009) were less common.

**Bacteraemia**
Fifty-four (15%) case-patients and 14 (4%) controls had bacteraemia. The attributable risk of bacteraemia to severe anaemia was 12.2%. The most common pathogen was non-typhoid salmonella, which was present in 38 (70%) of the case-patients and 11 controls (79%, P=0.54) with bacteraemia. None of the specimens grew mycobacteria. Fever was absent in 37% of children with bacteraemia. In both case-patients and controls bacteraemia was less common in children with malaria than in those without (21/208 vs. 32/150, P=0.003 and 3/146 vs. 11/207, P=0.12).

**Nutrition**
Fifty-two (16%) case-patients and 43 (6%) controls had wasting, the attributable risk to severe anaemia being 6.2%. Severely anemic children were commonly stunted or underweight (53% and 49%), but for both conditions the unadjusted and adjusted odds ratios were similar to those for wasting (data not shown). Severe wasting occurred in 3.7% of severely anemic children. Vitamin B12 deficiency was found in 95 (30%) case-patients and 94 (16%) controls and was severe (<136 ng/L [100 pmol/L]) in 11% of case-patients and 2.8% of controls (AOR: 4.3, 95%CI: 1.9-9.9). Macrocytosis was more common in vitamin B12 deficient children than in children with normal B12 levels (P=0.02), though the sensitivity for vitamin B12 deficiency was low (18%). Severely anemic children with vitamin B12 deficiency had a history of less meals with meat than those not deficient (1.9 vs. 2.7 per month, P=0.02). Folate deficiency was not found in any child enrolled in the study. Vitamin B12 and folate levels were inversely correlated to each other among severely anemic children (Pearson correlation coefficient: -0.22, P=0.01). Vitamin A deficiency was found in 92% of case-patients and 66% of controls, and was considered severe (<10 μg/dL) in 33% of all case-patients and 15% of controls (AOR: 1.6, 95%CI: 0.91-2.8). Deficiency was associated with malaria and bacteraemia in the structural equation model. Iron deficiency was found in 47% of case-patients and 69% of controls. Further exploration indicated this finding was not affected by the definition used (Table...
Table 3. Prevalence of iron deficiency in relation to the development of severe anemia using several peripheral blood markers.

<table>
<thead>
<tr>
<th>Prevalence of iron deficiency</th>
<th>Multivariate Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Case-patients</td>
</tr>
<tr>
<td><strong>Original definition</strong></td>
<td></td>
</tr>
<tr>
<td>TfR/Ferritin-Index</td>
<td>97 / 208 (47%)</td>
</tr>
<tr>
<td><strong>Alternative definitions</strong></td>
<td></td>
</tr>
<tr>
<td>CRP-containing Index</td>
<td>35 / 208 (17%)</td>
</tr>
<tr>
<td>Microcytosis</td>
<td>48 / 316 (15%)</td>
</tr>
<tr>
<td>Hypochromasia</td>
<td>137 / 314 (44%)</td>
</tr>
<tr>
<td>Microcytosis &amp; Hypochromasia</td>
<td>26 / 316 (8.2%)</td>
</tr>
</tbody>
</table>

The odds ratios were obtained by replacing the original variable for iron deficiency in the multivariate analysis by the alternative definition. Iron deficiency was defined as:

Transferrin Receptor (TfR)/Ferritin-Index>5.6 [TfR (mg/L) / Log ferritin (µg/L)]\(^{10,11}\).

CRP-containing index<0 \((0·34+0·0043 \times ferritin-(2·7 \times TfR)/ferritin+0·00696 \times CRP+0·05 \times TfR)\) (all expressed in mg/L)\(^{27}\).

Microcytosis: Mean Cellular Volume <67fL (<2 years old) and <73fL (2-5 years old) (WHO).

Hypochromasia: Mean Cellular Haematocrit Concentration <32g/L (WHO).

Others markers assessed but not presented since they predicted iron deficiency less well (sensitivity and/or specificity<40%) were ferritin, TfR, serum iron, serum transferrin, total iron binding capacity and transferrin saturation.

3) In the structural equation model iron deficiency was found to be negatively associated with bacteraemia (P=0.006).

**Hookworm**

Hookworm was the most common helminth infection. Thirty-one (76%) of the hookworm infestations occurred in children less than two years old. The attributable risk to severe anaemia in the rural site, where 95% of infections were seen, was 16%. In this site heavy infections occurred in 10% of all case-patients and 0.6% of controls (AOR: 9.4, 95%CI: 2.0-45). A PCR was performed on 36 of 41 positive samples (88%). *A.duodenale* was found in 81%, *N.americanus* in 8% and a mixed infection in 11%. Hookworm infestation was associated with iron deficiency (P=0.003, Figure 2).

**Genetics**

No association was found between severe anaemia and sickle cell disease or trait (P=0.40, 0.20). Jaundice was uncommon in severely anemic children with sickle cell disease or *G6PD\(^{202/-376}\) (0% and 2% respectively). Haptoglobin levels were commonly decreased (<0.30 g/L) in *G6PD\(^{202/-376}\) case-patients (78%). Boys accounted for 68% of children with *G6PD\(^{202/-376}\) but after stratification *G6PD\(^{202/-376}\) remained significantly associated with severe anaemia in both sexes (AOR-girls: 4.1, 95%CI: 1.2-13.3; boys: 2.2, 1.1-4.7).
DISCUSSION

In many African hospitals, severe anaemia is a leading cause of admissions and a major contributor to death. The cause of the anaemia has not been comprehensively investigated, but we found several important associations in this study.

Malaria is commonly considered to be a principal cause of severe anaemia in Africa\(^7\). In this study, \(P.falciparum\) parasitaemia was strongly associated with severe anaemia in the area with seasonal transmission but not in the holoendemic transmission area. However, the cumulative impact of malaria on the individual is difficult to assess in holoendemic settings where children are repetitively infected. Our findings therefore do not exclude malaria as a predisposing cause of severe anaemia in the rural area, but indicate that additional or alternative diagnoses should be considered in severely anemic children who are diagnosed with a malaria infection. In the structural equation model, malaria and bacteraemia were identified as variables modifying the association between vitamin A deficiency and severe anaemia. This is in line with earlier observations that vitamin A deficiency is associated with an increased susceptibility to infection\(^28\). A vitamin A supplementation trial showed a reduction in the incidence of malaria\(^29\), though this and another study failed to show that vitamin A supplementation reduced the incidence of severe anaemia\(^29;30\).

We found a negative association between iron deficiency and severe anaemia. The structural equation model partly explains this finding by indicating that iron deficiency was negatively associated with bacteraemia. This finding supports the hypothesis that iron deficiency protects against infection by creating an unfavourable environment for bacterial growth\(^31;32\). It is also in agreement with observations of increased morbidity and mortality in iron supplementation studies in areas where bacterial infections are common\(^32;33\). Although iron supplementation may play a role in preventing anaemia\(^33\), supplementation following severe malaria-anaemia had no haematological benefits and resulted in an increased morbidity in Tanzanian children\(^34\).

In rural children with severe anaemia, we found an increased prevalence and intensity of hookworm infections, \(A.duodenale\) being the predominant species. Three-quarters of the hookworm infested children were less than two years of age. This age group would have remained untreated according to the current guidelines\(^7\). Although usually hookworm is increasingly found in older children, younger children might be more vulnerable to severe haematological complications especially in the presence of heavy infections with \(A.duodenale\)^{35}.

Bacteraemia, most commonly due to non-typhoid salmonella, was strongly associated with severe anaemia. This association has been described previously\(^17;36;37\) but is not
reflected in current management guidelines for severe anaemia in children\(^7\). In the structural equation model, bacteraemia was also identified as a mediating variable of the effect of HIV on severe anaemia. Although bacteraemia might not necessarily be a cause of severe anaemia, its high prevalence may justify antibiotic treatment in the standard management of severe anaemia in settings where HIV is prevalent and blood culture facilities are not available.

Although folate supplementation is recommended by WHO, deficiency was not found in our study groups. We may have underestimated its prevalence, because folate deficiency can be masked by vitamin B12 deficiency\(^38\) and we measured plasma rather than erythrocyte folate concentrations. However, our findings concur with previous reports\(^39\) and observations that folate supplementation in anemic children with malaria failed to raise haemoglobin concentrations\(^40\). Unlike folate, vitamin B12 is not recommended in the management of severe anaemia. In our population vitamin B12 deficiency was found in 30% of case-patients and was associated with severe anaemia. This is in line with findings in adults in this region\(^41;42\) and may be explained by the lack of animal products in the diet of Malawian children.

\(G6PD^{-202/-376}\) was found in 14% of case-patients and was associated with severe anaemia, while sickle cell disease was uncommon in our setting. The roles of these mutations may be different in West and Central Africa. Possible associations between IL-10 and TNF-\(\alpha\) and severe malaria anaemia have been described\(^23;43\), but in our study an imbalance in circulating plasma levels of IL-10 and TNF-\(\alpha\) was uncommon.

We found that several independent yet overlapping conditions are associated with severe anaemia in Malawian children. Our findings indicate that even in the presence of malaria parasites, additional or alternative diagnoses should be considered. Current treatment recommendations promoting iron and folate-supplements and ignoring bacteraemia, vitamin B12 deficiency and, in young children, hookworm infections appear to be of limited applicability in our setting. Our findings, if confirmed in different settings, will contribute to the assessment of new therapeutic and preventive strategies for Africa.
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(38) Dierkes I, Domrose U, Ambrosch A et al. Supplementation with vitamin B12 decreases homocysteine and methylmalonic acid but also serum folate in patients with end-stage renal disease. Metabolism 1999; 48:631-635.


Severe anaemia in Malawian children
A descriptive profile

Michael Boele van Hensbroek, Job Calis, Kamija Phiri, Raymond Vet,
Francis Munthali, Henk vd Berg, Brian Faragher, Imelda Bates and
Malcolm Molyneux

Submitted
ABSTRACT

Background
Severe anaemia is a major cause of morbidity and mortality in African children. The aetiology is recognized to be multi-factorial, but interventions have often targeted only one or a few causal factors, with limited success.

Methods
We conducted a detailed descriptive study of severe anaemia in Malawian children. We assessed the prevalence of different pathophysiological mechanisms (red cell production failure [RCPF], haemolysis and blood loss), and compared the pattern of important aetiologies across the mechanism sub-groups. More complex associations were explored using Structural Equation Modelling.

Findings
In 381 severely anaemic children studied, RCPF, haemolysis and blood loss were found in 48.1%, 21.7% and 6.9% of children, respectively. RCPF was the most commonly identified mechanism, in the children with a single as well as those with multiple aetiologies, and in each of the major aetiological subgroups, with 38.7 - 59.7% of children fulfilling the RCPF definition. In the structural equation model, aetiologies, including infectious as well as nutritional, were directly or indirectly associated with RCPF, but not with haemolysis. This was also true for the malaria factor.

Interpretation
RCPF is the most common pathophysiological pathway leading to severe anaemia from a variety of often multiple aetiologies. Our approach to the description of this syndrome, from the point of view of both aetiologies and mechanisms, allows: a profile of paediatric severe anaemia to be developed for a particular population; explain the limited success of the single factor interventions applied in the past; and, most important, provide a basis for the design of a locally relevant package of measures that can be deployed in the prevention and treatment of severe anaemia in children.
BACKGROUND

Severe anaemia (haemoglobin concentration <5g/dl) is a common cause of morbidity and mortality in African children\textsuperscript{1-4}. Of all children admitted to hospital, 12-29\% receive a blood transfusion and in-hospital mortality in this group is commonly between 4 and 10\%\textsuperscript{3,5,6}.

The pathogenesis of anaemia is complex because several distinct mechanisms may lead to a reduced number of circulating red cells. In African children, mechanisms known to contribute to severe anaemia include haemolysis (intra or extra vascular), acute or chronic blood loss, and red cell production failure (RCPF)\textsuperscript{7}. Each of these mechanisms may be activated by a variety of aetiological factors and some single aetiologies may affect more than one mechanism\textsuperscript{4,8}. For example, malnutrition, HIV and malaria may each cause RCPF, while a malaria infection may cause both haemolysis and RCPF. A single aetiology may predominate in some patients, while in others multiple aetiologies and mechanisms may combine to result in severe anaemia\textsuperscript{9}.

Despite the size of the problem, severe anaemia has received little research attention and its pathophysiology is still poorly understood\textsuperscript{10}. Interventional studies of either prevention or treatment of severe anaemia have often evaluated only one factor at a time\textsuperscript{7,11}. The limited success of this approach is not surprising if, as recent studies suggest, in children most of the severe anaemia is the result of multiple aetiological factors\textsuperscript{10}. In order to adopt a rational approach to reducing the burden of severe anaemia it is important to be able to identify the important mechanisms and the specific factors that contribute to these mechanisms.

We have attempted to identify both the mechanisms and aetiologies associated with severe anaemia in 381 rural and urban Malawian children. With these data we have produced a profile of the syndrome in these populations, by which more effective preventive and treatment strategies may be developed.

PATIENTS, MATERIALS, AND METHODS

Population

The study was conducted in southern Malawi at Queen Elizabeth Central hospital in Blantyre (urban site) and Chikwawa District hospital (rural site). Between July 2002 and July 2004 consecutive children with a primary diagnosis of severe anaemia, defined as a blood haemoglobin concentration less than 5 g/dl, were recruited as cases into a prospective case-control study. Children were eligible for enrolment if they were aged
6-60 months and had not received a blood transfusion within the previous month. For each case, two controls were recruited: a hospital control (HC) attending the same outpatient department for a condition other than severe anaemia, and a community control (CC), recruited from among apparently healthy children residing within 100-1000 meters of the home of the respective case. HC and CC were aged between 6 and 60 months, and had a haemoglobin concentration of >5g/dl. Informed consent was obtained from a parent or guardian in all three study groups. The study was approved by the ethics committees of the College of Medicine, University of Malawi and the Liverpool School of Tropical Medicine, United Kingdom.

**Procedures**

This study formed part of large severe anaemia research programme. Admission procedure and management have been described in detail elsewhere. In summary, on enrolment a standardized study questionnaire and physical examination were completed, and samples of blood, urine and stool were collected. In cases only, if the clinical condition permitted, a fine needle bone marrow aspirate was obtained under anaesthesia from the posterior superior iliac spine. Children requiring admission were treated in a study ward and all conditions were managed according to standard protocols.

**Laboratory measurements**

Laboratory tests crucial to patient care were performed within 24 hours, and sample aliquots were stored at -80°C for later analysis. Whole blood haemoglobin and plasma haemoglobin concentrations were measured using the HemoCue systems® (Angelholm, Sweden). Plasma haemoglobin levels were assessed in all Cases and in a smaller, randomly selected, sub-group of 92 Control children. A full blood count, including absolute reticulocyte count, was performed by Coulter counter analyzer® (Beckman Coulter, Durban, South Africa). Bone marrow aspirates were used for microscopy and for the cytokine assays. Wedged spread films were air dried, fixed in methanol and stained with May-Grüwald-Giemsa and iron stain (HematoGnost Fe, Darmstadt, Germany). Bone marrow differential count was conducted by a laboratory technician with more than 10 years experience (RV). Iron stained bone marrow slides were graded according to Gale’s criteria for iron content in stroma, macrophages and red cell precursors.

C-reactive protein, erythropoietin, haptoglobin, transferrin, iron, ferritin, folate and vitamin B12 concentrations were analyzed in heparin plasma on a Roche p800 system (Roche, Switzerland). Inflammatory cytokine profiles were measured by Cytometric Bead Array on a FACS-Calibur flow-cytometer (Becton-Dickinson, South Africa), which was also used to measure the red cell precursor fractions in the bone marrow aspirate (details have been described elsewhere). Serum vitamin A (retinol) and soluble transferrin receptor (sTfR) were measured using high performance liquid chromatography and enzyme linked immunosorbant assay (Ramco Laboratories, TX).
respectively\(^1\). *Plasmodium falciparum* asexual parasites were counted against 200 white blood cells. Stool samples were examined for helminths using the Kato-Katz method\(^1\) and polymerase chain reaction (PCR)\(^2\). Urine specimens were examined for *Schistosoma haematobium* using a semi-quantitative concentration method\(^3\).

A bone marrow or venous blood sample (1-2ml) was inoculated into BACTEC Myco/F-Lytic culture vials and incubated in a BACTEC 9050 automated system for 56 days\(^4\). Cultures were checked for mycobacteria using Ziehl-Neelsen stained slides. Mixed growth or growth of micrococci, Bacillus species or coagulase-negative staphylococci were considered contaminants. HIV testing was performed using two rapid tests (Determine, Abbott-Laboratories, Japan; Unigold, Trinity-Biotech, Ireland); discordant and reactive results in children less than 18 months being resolved by PCR.

**Definitions used**

**Severe anaemia:** The criteria used to define the severe anaemia mechanisms are given in table 1.

**Malaria infection:** was defined as the presence of asexual *Plasmodium falciparum* parasites on a blood film; Parasitaemia in excess of 10,000 asexual parasites/\(\mu\text{L}\) was referred to as ‘\(\text{Pf}>10^4/\mu\text{L}\)\(^5\).

**Important aetiological factors:** in the study population were previously defined by multivariate analysis and included: malaria, HIV, bacteraemia, hookworm infection and iron, vitamin B12 and vitamin A deficiency\(^6\).

**Dyserythropoiesis:** was defined by the following nuclear features in bone marrow smears: (a) multi-nuclearity; (b) karyohexis; (c) intercellular chromatin bridging; and (d) incomplete mitoses. Dyserythropoiesis was expressed quantitatively as the percentage of RBC precursors fulfilling at least one of these criteria\(^7\).

**Nucleated red cells:** (NRC) were defined as all mononuclear bone marrow cells positively stained with LDS-751 (a DNA dye) and expressing CD235a (Glycoprotein A) on flow-cytometric analysis.

**Iron deficiency:** In our study population, an stTfR/log ferritin-index of more than 5.6 has been shown to be the best predictor of bone marrow iron status irrespective of the presence of infection, and was used to define iron deficiency (sensitivity 70%, specificity 75%)\(^8\).
**Statistical Methods**

This is primarily a study of the diversity of mechanisms of severe anaemia, the identification of which requires an assessment of bone marrow appearances. Since bone marrow could only be examined in Cases, the principal statistical comparisons are of categories within the group of severe anaemia cases. The prevalence of each of the three anaemia mechanisms was assessed in the Cases group, and important aetiological factors were compared across the various mechanism sub-groups. More complex associations between factors and with the main mechanisms were explored using Structural Equation Modelling\(^7\). Reported p-values are always two-sided. Data were analyzed using STATA 9 (Stata Corporation, TX), SPSS 14 and AMOS 6.0 (SPSS, IL).

**RESULTS**

Three hundred and eighty one children with severe anaemia (cases), 205 (53.8%) from Blantyre and 176 (46.2%) from Chikwawa, were enrolled over a two year period (2002-04). Of these, 235 (62%) had a complete dataset for all the mechanism-defining variables (table 1) and were included in the main analysis. A further 54 children, with one or more data points missing, were included in the subgroup analyses. Of the cases with a complete dataset, 55.0%, 9.8% and 1.4% fulfilled the definitions for one, two and all three anaemia mechanisms, respectively (figure 1).

<table>
<thead>
<tr>
<th>Mechanism</th>
<th>Percent prevalence among severe anaemia cases (n) #</th>
<th>Definitions used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red Cell Production Failure (RCPF)</td>
<td>48.1 (113/235)</td>
<td>Whole blood haemoglobin &lt;5.0 g/dL and Reticuloytes &lt;50,000/uL †</td>
</tr>
<tr>
<td>Hemolysis</td>
<td>21.7 (51/235)</td>
<td>Plasma Haemoglobin &gt;0.15 g/dl (IV) and/or Unconjugated bilirubin &gt;16.9 mmol/L (EV)</td>
</tr>
<tr>
<td>Blood loss</td>
<td>7.2 (17/235)</td>
<td>UT: Urine dip-stick &gt;1+ for erythrocytes and/or GI: Hookworm load &gt;1000 eggs/gram stool *</td>
</tr>
<tr>
<td>Non defined</td>
<td>34.5 (81/235)</td>
<td>Not fulfilling any of the above criteria</td>
</tr>
</tbody>
</table>

IV = intravascular, EV = extra vascular, GI = Gastro-Intestinal, UT = Urine Tract.

# Includes patients with a complete dataset only.

* Gastro-intestinal (GI) blood loss was measured indirectly, using a ‘high hookworm load’ as a substitute marker.

† If reticulocyte cut off <150,000/uL is used (3% of erythrocytes in a non anaemic child): RCPF = 86.4% (203/235) with 23.0% (54/235) overlap with haemolysis and blood loss.

Control population (HC & CC combined): 1.8% (10/566) and 1.1% (8/705) fulfilled the definition for haemolysis and blood loss, respectively.
Mechanisms
Red cell production failure (RCPF) was identified in 113 (48.1%) of whom 24 (10.2%) were overlapping with the other mechanisms (figure 1). When exploring the RCPF group in more detail, it was found that in these children 11.4 % (inter quartile range 4.3-36.9) of the mononuclear fraction was defined as red cell precursors (nucleated red cells [NRC]). Dyserythropoietic features in the NRC were found in 61.3% of the children with RCPF with a mean of 3.6 ±2.8 % of cells affected (table 2).

**Table 2:** Laboratory markers associated with severe anaemia mechanisms

<table>
<thead>
<tr>
<th>Markers</th>
<th>RCPF % (n)</th>
<th>Haemolysis % (n)</th>
<th>Blood loss % (n)</th>
<th>Non defined % (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRP: Elevated</td>
<td>85.3 (93/109)</td>
<td>89.4 (42/47)</td>
<td>64.7 (11/17)</td>
<td>93.8 (75/80)</td>
</tr>
<tr>
<td>Erythropoietin: Not elevated</td>
<td>13.8 (8/58)</td>
<td>7.7 (2/26)</td>
<td>0.0 (0/6)</td>
<td>10.6 (5/47)</td>
</tr>
<tr>
<td>Dyserythropoiesis</td>
<td>61.3 (49/80)</td>
<td>62.9 (22/35)</td>
<td>69.2 (9/13)</td>
<td>55.9 (33/59)</td>
</tr>
<tr>
<td>Haptoglobin: Low</td>
<td>68.6 (72/105)</td>
<td>86.4 (38/44)</td>
<td>52.9 (9/17)</td>
<td>78.9 (60/76)</td>
</tr>
<tr>
<td>NRC fraction</td>
<td>11.4 (4.3-36.9)</td>
<td>16.5 (4.9-41.9)</td>
<td>26.1 (16.1-34.2)</td>
<td>17.0 (7.7-38.9)</td>
</tr>
</tbody>
</table>

CRP: C-reactive protein (elevated if blood levels >10 mmol/L), Erythropoetin: normal in non-anaemic children if ≤1200 U/L. Dyserythropoiesis: Defined by the presence in >2% of red cell precursors with dyserythropoietic features.

Haptoglobin: Low if <0.2mMol/L.

NRC fraction: Percentage of nucleated red cell (red cell precursors) of the mononuclear bone marrow cells.
Hemolysis was found in 51 out of 235 cases (21.7%) with 17 children (7.2%) also having criteria for RCPF and/or blood loss. Of all cases 19.3% had elevated plasma haemoglobin levels, indicative of intra-vascular hemolysis, and 5.0% had a raised unconjugated bilirubin level. In the haemolysis group 24.4% (IQR 16.2-40.9) of the mononuclear fraction were NRCs and dyserythropoiesis was found in 62.9% of children.

Direct or indirect evidence of blood loss was found in 16 cases (6.9%): this was through the urinary tract in 4 (1.4%) and the gastro-intestinal tract in 12 (5.4%).

**Aetiologies**

Among all previously identified important aetiologies of severe anaemia, RCPF was the most common associated mechanism, with 38.7 - 59.7% of cases, in the various aetiological subgroups, fulfilling the RCPF definition (table 3).

Infections, associated with anaemia, were common in the severe anaemia cases of whom 59.4% (n=164) had one infection, an additional 38 (13.8%) had two and 9 (3.3%) three infections. A single (isolated) infection was relatively uncommon (22.6%) among the HIV infected cases, but common among the malaria, hookworm and bacteraemia infected children, in whom 60.0, 52.2 and 37.9% were single infection, respectively (table 3). In the sub-groups of children with a single infection as aetiology, RCPF was the most important...
mechanism. Only in the six children with bacteraemia was haemolysis more commonly found.

In the children with a positive malaria slide, RCPF and haemolysis were found in 42.1% and 17.9%. In the sub-group with a malaria parasite count >10⁴/uL, the equivalent proportions were 36.6% and 17.1%.

**Structural Equation Model**
In order to further explore the association between the aetiological factors and anaemia mechanisms and to correct for interaction between factors a structural equation model was developed\(^\text{10}\). In the model, with a goodness of fit (RMSEA) of 0.053 (0.038-0.068), iron deficiency, vitamin B12 deficiency and malaria were directly and significantly associated with RCPF (standard regression coefficient of +0.14, -0.13, +0.15, respectively). Associations between RCPF and HIV, hookworm and vitamin A were indirect, through the previous 3 aetiologies and no significant associations were found with haemolysis (figure 2).
DISCUSSION

Severe anaemia in children in Africa has usually been investigated in the context of a condition that may contribute to it, such as malaria, malnutrition or helminth infection. We have studied the syndrome in its own right, by enrolling consecutive Malawian children presenting to hospital with severe anaemia, irrespective of its possible aetiology. This has allowed us to build up a picture of contributory causes in both an urban and a rural population in southern Malawi.

To describe such causes, we have aimed to identify, for each child, both the mechanistic pathway leading to their anaemia (failure of red cell production, reduced red cell lifespan, or loss of red cells through haemorrhage) and the aetiologies that may have led to the operation of the mechanisms found. A description of severe anaemia from these two different perspectives provides a profile of the syndrome, and may contribute to the development of more effective treatment and preventive strategies.

Our results confirm that in Malawian children, although severe anaemia is associated with many potential aetiological factors, one mechanistic pathway – failure of red cell

Figure 2. Structural Equation Model:

Exploratory model describing the relationship between the important aetiologies and the mechanisms: Red Cell Production Failure and haemolysis. Reduced model presented, in which the significant (→) and theoretical important non-significant (---->) relationships are indicated (other non-significant arrows are taken out for clarity). Size of the associations is indicated by numbers (standardized regression coefficients; range: -1.0/+1.0). The overall model fit was valid (RMSEA: 0.053 (0.038-0.068))
production (RCPF) – predominates as the final pathway leading to anaemia. This was true when using stringent criteria for RCPF (reticulocytes <50,000/uL and haemoglobin <5 g/dl) and became even more apparent when the cut-off for reticulocyte count was put at <150,000/uL (3% of erythrocytes in a non-anaemic child)\(^1\), when 86% of all severely anaemic children would fall into the RCPF category. The importance of RCPF may have been expected, since the study was conducted in an area with a high infection pressure, reflected in a high prevalence of single or mixed infections, and in the fact that the majority of cases had a raised plasma CRP concentration. In these circumstances, RCPF may be the result of pro-inflammatory cytokine activation giving rise to apoptosis of red cell precursors, dyserythropoiesis and down-regulation of erythropoietin production, with additional blunting of the effect of erythropoietin on the bone marrow. This interpretation – that severe anaemia is largely mediated by inflammatory mechanisms – is in part supported by the results listed in Table 2. However, inflammatory responses may not fully explain the RCPF, since nearly a quarter (23.6%) of children with a RCPF were not found to have an infectious aetiology, while nutritional deficiencies, which are on their own able to affect red cell production, were found in over 40% of RCPF cases.

Although RCPF was the dominant mechanism of anaemia in our patients, irrespective of associated aetiologies (summarised in table 3), the majority of cases had multiple aetiologies (nutritional deficiencies, single infections and, in one fifth of cases, multiple infections). This may explain why interventions directed against single aetiologies, either to treat or to prevent severe anaemia, are often limited in their success\(^1\). It may also be, next to antimalarial treatment failure, an additional explanation of the high post-discharge mortality rate in Kenyan children following a severe malaria anaemia episode treated with blood transfusion and antimalarial drugs only\(^3\).

The Structural Equation Model (Figure 2) underlines both the importance of RCPF and the complexity of the syndrome, and indicates the apparently minor role of haemolysis in the patients we studied. There were no significant associations between haemolysis and any of the aetiologies, including malaria. This was the case whether malaria was defined as any parasitaemia or a parasite density >10\(^4\)/l.

A limitation of a study of this kind is that data can be collected at one time point only, in a disease process that may have lasted for weeks with markers fluctuating from day to day. We have tried to limit this effect by using a large sample size. Another limitation is the difficulty of quantifying haemolysis and gastro-intestinal blood loss. For haemolysis, the kinetics of free haemoglobin in plasma are not well known, and 1g/dl of free haemoglobin is sufficient to saturate circulating haptoglobin, limiting its value as quantitative marker\(^2\). For gastro-intestinal blood loss we assumed that hookworm is the only likely cause and that blood loss is likely to accompany a heavy egg-load in the stool\(^8\).
These limitations are unlikely to affect the main findings of our study and their implications for the control of severe anaemia in Malawi and similar settings. The syndromic approach that we have adopted allows a profile of paediatric severe anaemia to be developed, based a description of both aetiologies and mechanisms for a particular population. This provides a basis for the design of a locally relevant package of measures that can be deployed in the prevention and treatment of severe anaemia.
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Chapter 4

Long term outcome of severe anaemia in Malawian children

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Submitted
ABSTRACT

Background
Severe anaemia is a common, frequently fatal, syndrome in African children admitted to hospital, but its long term outcome is unknown. Early reports that survivors may be at risk of additional late morbidity and mortality may have significant implications for child survival in Africa. We assessed the short and long term outcome of severe anaemia in Malawian children and identified potential risk factors.

Methods and Findings
We conducted a prospective case-control study of children presenting to hospital with severe anaemia (haemoglobin ≤5g/dl) and their (hospital and community) controls. All children were aged between 6 and 60 months and were followed up for 18 months to assess the incidence of (in-hospital and post-discharge) mortality and further severe anaemia.

A total of 377 cases, 377 hospital controls and 380 community controls were recruited. The in-hospital mortality was 6.4% among the cases. Mortality in the 18-month follow-up period among cases was 12.6%, significantly greater than in hospital controls (2.9%) or community controls (1.4%)(p<0.001). HIV was the most important risk factor for mortality (HR 10.5, 95% CI 4.0-27.2). The incidence of severe anaemia during the follow-up period among the cases was 80 per 100 person-years (95% CI 57-113), significantly higher than the 5 per 100 person-years (95% CI 2-11) in the combined controls (p<0.001).

Conclusions
Severe anaemia carries a high ‘hidden’ morbidity and mortality occurring in the months after initial diagnosis and treatment. Because severe anaemia is very common, this is likely to contribute importantly to overall under-five mortality. If not adequately addressed, severe anaemia may be an obstacle to achievement of the fourth millennium development goal on child survival. Strategies to diagnose and properly treat HIV-infected children early may reduce the high post-discharge mortality in severe anaemia.
INTRODUCTION

Of children admitted to hospital in Africa, 12-29% have severe anaemia requiring a blood transfusion\textsuperscript{1,2,3}. In-hospital mortality in this group is commonly between 4% and 10\%\textsuperscript{1,2,3,4}.

Investigators in Kenya reported unexpectedly high post-discharge mortality and recurrent severe anaemia rates in children within two months of a severe anaemia episode\textsuperscript{3}. There has been no subsequent attempt to confirm or investigate these findings. An excess risk of death in this large group could contribute an important, and potentially correctable, component to the high mortality rate among young children in Africa.

In this study our aims were, firstly, to document mortality and the incidence of recurrent severe anaemia during a period of 18 months after admission for severe anaemia, and secondly, to identify risk factors for post-discharge mortality, in order to develop more effective management and preventive strategies.

METHODS

This study was carried out as part of a follow-up to a large case-control study investigating the aetiological factors for severe anaemia in children\textsuperscript{5}. It was conducted in southern Malawi at Queen Elizabeth Central hospital in Blantyre and Chikwawa District hospital. In Blantyre (urban site) malaria is seasonal, while in Chikwawa (rural site) malaria transmission is intense throughout the year.

Children were recruited from July 2002 for 2 years and followed up until February 2006 from the hospital’s Paediatric Accident and Emergency Unit (Blantyre) or Under-fives Clinic (Chikwawa). Each of these is a facility available to the public without charge, functioning in daylight hours for six days each week. Severely anaemic children (cases) were recruited if they had a blood haemoglobin concentration ([Hb]) of less than 5.0 g/dl, were aged 6-60 months and had not received a blood transfusion during the preceding four weeks. For each case a Hospital Control (HC) was recruited on presentation to the same out-patient department for a condition other than severe anaemia, and when the index case was discharged from hospital a Community Control (CC) was recruited from among apparently healthy children residing within 100-1000 metres of the home of the case. HC and CC had to be aged between 6 and 60 months, and to have a blood [Hb] of 5g/dl or more. Informed consent was obtained from the guardians of the children and the study was approved by the ethics committees of the University of Malawi and the Liverpool School of Tropical Medicine, UK.
Procedures

The process of recruitment included a detailed medical history and physical examination. Samples of venous blood, urine and stool were collected. Children requiring hospitalisation (all cases and a minority of HCs) were managed in a research ward and according to standard protocols.

All three study groups (cases, HC and CC) were actively followed up at 1, 3, 6, 12 and 18 months. Guardians were additionally asked to return with the child to a study clinic whenever the child was sick. During each follow-up visit, a standard clinical form was completed and, if necessary, the child was treated by the attending clinician. At the time of this study, anti-retroviral therapy (ART) was not provided to children in Malawi. Deaths and severe anaemia episodes were recorded, and if they occurred outside the study clinics, they were investigated as completely as possible using the patient’s ‘Health Passport’ book, hospital records and home interviews with parents or guardians. Deaths occurring during the initial hospitalisation period were recorded as in-hospital mortality. Deaths occurring after discharge but during the study follow-up period were recorded as post-discharge mortality.

All laboratory assays were done blinded to patient study group. Haemoglobin was measured on site using the HemoCue B-Haemoglobin analyser (HemoCue, Ängelholm, Sweden) and subsequently by Coulter counter analyser (Beckman Coulter, Durban, South Africa). Ferritin was measured using the electrochemiluminescence immunoassay (Modular Analytics E170, Roche Diagnostics, Switzerland). Soluble transferrin receptor (sTfR) levels were measured using ELISA (Ramco Laboratories, TX, USA). Iron deficiency was defined as a sTfR/log ferritin index of 5.6 or more. Malaria was defined as the presence of *Plasmodium falciparum* asexual parasites in the blood. Stool samples were examined for helminth infection by Kato-Katz and Polymerase Chain Reaction (PCR). Urine specimens were examined for *Schistosoma haematobium* using a semiquantitative concentration method. Bacterial cultures were carried out only on cases and HC according to a standard method using an automated BacT/Alert system (BioMérieux Industry, MO, USA) and cultured for 7 and 56 days for routine pathogens and mycobacteria respectively. Mixed growth or growth of Micrococi, Bacillus species or coagulase-negative staphylococci were considered contaminants. HIV testing was performed according to WHO guidelines using two rapid tests (Determine, Abbott-Laboratories, Japan; Unigold, Trinity-Biotech, Ireland). Discordant results and reactive results in children less than 18 months were resolved by PCR. For genetic analysis, DNA was extracted using a Nucleon extraction kit (Amersham biosciences, UK) and tested by Cytronomics and arms-PCR for a predefined set of polymorphisms including glucose-6-phosphate dehydrogenase deficiency (G6PD, variant A-202/376), sickle cell disease and single nucleotide polymorphism in the promoter region of the IL-10 and TNF-α genes.
Statistical analysis
Data were analyzed using SPSS for Windows © version 12 and STATA © version 9. Discrete data were analyzed by the chi-squared or Fisher’s exact test. Continuous data were analysed by independent samples Student’s t-tests. Survival times were recorded as the duration of follow-up from the date of recruitment or the date of discharge (if hospitalised at time of recruitment) until the date of a severe anaemia episode or post-discharge mortality. Severe anaemia was defined as [Hb] less than 5g/dl during the follow-up period and at least four weeks after a previous severe anaemia episode. Survival times were compared using Kaplan-Meier plots. Wasting was defined as a Z-score <-2 weight-for-height and stunting as a Z-score <-2 height-for-age.

Hazard ratios (HR) of predictors for post-discharge mortality and their 95% confidence intervals were estimated by using Cox Regression. Factors associated with post-discharge mortality in the univariate model were then included in a multivariate model if either \( p < 0.1 \) or if they were important confounders and effect modifiers.

A survey in Chikwawa of pre-school children found an annual incidence of severe anaemia of 7%. Samples of 380 cases and 380 controls provided power to detect factors increasing the incidence of severe anaemia from 7% in the controls to 13.5% in the cases (i.e. to detect a HR of 2.0) at an alpha level of 5%.

RESULTS
A total of 1138 children were recruited, of whom 381 (33.5%) were cases, 377 (33.1%) were HC and 380 (33.4%) were CC. Four children among the cases were excluded from the final analysis because of undeterminable discharge dates. Of all children included in the analysis, 53.4% (606/1134) were from the urban site (Blantyre). Over the 18 months study period 17.6% (195/1110) were lost to follow-up, the commonest reasons being emigration out of the study area and withdrawal of consent (Table 1). There were no significant differences in baseline characteristics of the children lost to follow-up compared to those that completed the study follow-up period.

There were a few differences in the baseline characteristics of the three groups. Cases had a higher prevalence of stunting, wasting and malaria compared to controls. Bacteraemia was more common among cases (15.2%, 54/355) than HC (4.0%, 14/353) with 72.1% (49/68) caused by non-typhoidal Salmonella. Blood cultures were not done on CC. HIV prevalence was significantly higher among the cases (12.7%, 45/553) than HC (8.1%, 27/335) or CC (4.0%, 14/347).
The overall mortality rate (in-hospital and post-discharge) was significantly higher among the \textit{cases}, 17.2\% (65/377), than the combined control groups 2.0\% (15/757, \(p<0.001\), Table 1). The overall incidence of death among \textit{cases} was 148 per 100 person-years (95\% CI...
116-189), and they were about ten times more likely to die than controls (HR 9.5, 95% CI 5.4-16.7). In-hospital mortality among the cases was 6.4% (24/377) while the post-discharge mortality after 18 months of follow-up was 12.6% (Kaplan-Meier estimates, adjusted for losses to follow-up). The post-discharge mortality was significantly higher (Log rank test, p<0.001) than that observed among the HC (2.9%) or CC (1.4%). Post-discharge deaths among cases occurred after a median time of 4.1 months (IQR 1.8-8.1), 70.7% (29) occurring within the first six months (Figure 1).

For cases, risk factors for post-discharge mortality are presented in Table 2 and Figure 2. Children who died were more commonly HIV-infected than survivors and after adjusting for confounders and effect modifiers, HIV was strongly associated with death in the follow-up period (HR 10.5, 95% CI 4.0-27.2). Overall mortality was 60.0% (27/45) among HIV infected as compared to 10.7% (33/308) among HIV uninfected severely anaemic children (p<0.001). Bacteraemia was associated with a non-significantly increased risk of post-discharge mortality (HR 2.2, 95% CI 0.8-5.6). Malaria, study site, sickle cell disease, G6PD or hookworm infection did not significantly predict post-discharge death.

Recurrence of severe anaemia was observed in 10.2% (Kaplan-Meier estimates, adjusted for losses to follow-up) of cases during the 18-month follow-up period, with a median time to event of 2.9 months (IQR 1.4-6.6) (Figure 3). Cases had a significantly higher rate of severe anaemic events (Log rank test, p<0.001) during the follow-up period than HC (1.1%) or CC (0.6%). Of these events 65.6% (21/32) occurred within the first six months after discharge. The incidence of severe anaemia in the follow-up period among the cases was 80 per 100 person-years (95% CI 57-113) compared to 5 per 100 person-years (95% CI 2-11) for the combined controls (p<0.001). The prevalence of HIV infection was not
significantly greater among cases that had recurrent severe anaemia than those that did not (16.7%, 5/30 versus 12.4%, 40/323 respectively, p= 0.5).

DISCUSSION

The in-hospital case-fatality rate of 6.4% in the present study is within the range of previously reported studies in Sub-Saharan Africa\textsuperscript{1,2,3,13}. In-hospital deaths usually occur shortly after admission and respiratory distress has been found to be an important predictor of a fatal outcome\textsuperscript{2}. Earlier identification of these children in the community and more timely transfusion may decrease these early deaths.

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**Table 2:** Baseline characteristics of severe anaemia cases by outcome

<table>
<thead>
<tr>
<th></th>
<th>All deaths (n= 65)</th>
<th>Post-discharge mortality (n= 41)</th>
<th>Survivors (n= 312)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urban site\textsuperscript{1}</td>
<td>63.1% (41/65)</td>
<td>61.0 % (25/41)</td>
<td>51.6% (161/312)</td>
</tr>
<tr>
<td>Age (in months)\textsuperscript{2}</td>
<td>19.6 (12.5)</td>
<td>17.8 (10.8)</td>
<td>20.5 (12.8)</td>
</tr>
<tr>
<td>Male\textsuperscript{1}</td>
<td>38.5% (25/65)</td>
<td>46.3% (19/41)</td>
<td>48.1% (150/312)</td>
</tr>
<tr>
<td>History of previous transfusion\textsuperscript{1}</td>
<td>14.1% (9/64)</td>
<td>12.2% (5/41)</td>
<td>15.4% (48/312)</td>
</tr>
<tr>
<td>Educated mother\textsuperscript{1}</td>
<td>86.2% (50/58)</td>
<td>81.6% (31/38)</td>
<td>88.6% (271/306)</td>
</tr>
<tr>
<td>Wasting\textsuperscript{1}</td>
<td>29.4% (15/51)</td>
<td>20.0% (7/35)</td>
<td>13.4% (37/277)</td>
</tr>
<tr>
<td>Stunting\textsuperscript{1}</td>
<td>62.7% (32/51)</td>
<td>65.7% (23/35)</td>
<td>51.4% (143/278)</td>
</tr>
<tr>
<td>Splenomegaly</td>
<td>58.7% (37/63)</td>
<td>50.0% (20/40)</td>
<td>64.5% (198/307)</td>
</tr>
<tr>
<td>Hb (g/dl)\textsuperscript{2}</td>
<td>3.4 (0.8)</td>
<td>3.5 (0.6)</td>
<td>3.6 (0.9)</td>
</tr>
<tr>
<td>Iron deficiency\textsuperscript{1}</td>
<td>50.0% (16/32)</td>
<td>52.0% (13/25)</td>
<td>45.7% (80/175)</td>
</tr>
<tr>
<td>Malaria\textsuperscript{1}</td>
<td>46.9% (30/64)</td>
<td>53.7% (22/41)</td>
<td>61.5% (192/312)</td>
</tr>
<tr>
<td>HIV\textsuperscript{1}</td>
<td>45.0% (18/39)</td>
<td>48.8% (18/31)</td>
<td>5.8% (2/36)</td>
</tr>
<tr>
<td>Bacteraemia\textsuperscript{1}</td>
<td>31.6% (18/57)</td>
<td>29.3% (12/41)</td>
<td>12.1% (36/298)</td>
</tr>
</tbody>
</table>

\textsuperscript{1} %, (n); \textsuperscript{2} mean (SD); \textsuperscript{3}p<0.0001 between Survivors and All deaths or post-discharge mortality
Figure 2: Adjusted Hazard Ratios of main risk factors for post-discharge mortality among severely anaemic children. Other factors included in the univariate analysis included: sickle cell, G6PD, hookworm.

Figure 3: Survival curve showing the time to severe anaemia during the follow-up period of severely anaemic children (cases) and hospital and community controls (see colour section). Log rank test, p<0.001

Of serious concern is the high post-discharge mortality observed in this study, with most deaths occurring within six months after admission for severe anaemia. Outside of the context of a research study, such deaths may remain undetected by the health services; even if recorded, the link with a previous episode of severe anaemia may be unrecognized.
Before this study, the longest follow-up of children after an episode of severe anaemia was eight weeks, in an investigation in rural Kenya. Even in that relatively short period, the post-discharge mortality rate was found to be 14%, which the investigators attributed to suboptimal medical care during admission to hospital (poor disease recognition and inadequate treatment). The present study has been the first attempt to validate these Kenyan findings in a different setting and with an extended follow-up period. We provided a good level of supervised medical care during the initial admission, and were therefore surprised to find that both post-discharge mortality and the rate of recurrence of severe anaemia, although better than in Kenya, remained high.

Several possible explanations for the high incidence of post-discharge mortality and recurrent severe anaemia should be considered. Firstly, it is possible that the underlying cause of the initial episode of severe anaemia was neither diagnosed nor treated, and therefore haemoglobin levels may have deteriorated again after discharge. Considering the extensive diagnostic investigations and frequent follow-up visits that were carried out by dedicated medical staff, this explanation appears unlikely.

Secondly, the aetiology of the severe anaemia episode may have been identified correctly, but treatment failure occurred as result of multi-drug resistant malaria or bacterial infections. It has been shown that recrudescent and new malaria infections during the first six months can negate the initial haematological improvement attained from receiving blood transfusion. Children with malaria in this study were treated with a combination of quinine for at least three days and sulfadoxine-pyrimethamine according to national policy. Although generally efficacious, this regimen is known to have an increasing failure rate in formal studies in Malawi, so that recrudescent malaria may have contributed to recurrent anaemia in some children. Similarly although sepsis or positive blood culture was treated with broad spectrum antibiotics, guided by drug sensitivity findings, un-eradicated or new bacterial infections may have developed and contributed to morbidity and mortality.

Thirdly, cases may have been seen in the end-stage of a chronic underlying disease, with severe anaemia being merely a marker of disease severity. This hypothesis is partly supported by the fact that HIV infection was found to be the most important independent risk factor for post-discharge mortality. Anaemia is not only a common paediatric manifestation of HIV, but has been shown to be strongly and consistently associated with the progression of HIV disease and death. At the time of this study, ART was not available to children in Malawi. Currently ‘unexplained moderate anaemia’ is a stage III criterion for commencing ART, but in practice this is often not used in deciding which children should be started on ART. If our finding of a very high HIV-related case fatality rate in severely anaemic children is confirmed in other studies, it may be appropriate to
consider adding severe anaemia to the criteria for stage IV disease in the WHO paediatric HIV staging system.

Finally, inadequate blood transfusion may explain the high rates of post-discharge mortality and recurrent severe anaemia, an explanation also suggested in the Kenyan study\(^3\). We have tried to prevent this happening by evaluating haemoglobin levels after transfusion and before discharge, and repeating transfusions if necessary. Nevertheless, in cases haemoglobin concentrations were still well below the normal range following transfusion – a situation that is likely to prevail in many health facilities in Africa – and a relatively minor additional haematological insult could have major consequences.

The importance of bacteraemia has been shown in a previous study in Malawi, where it was associated with severe anaemia\(^{15}\). Non-typhoidal salmonellae, principally \(S.\) \textit{typhimurium} and \(S.\) \textit{enteritidis}, are widely prevalent in Africa and are associated with malaria and HIV infection\(^{16}\). In our study 15% of children admitted with severe anaemia were bacteraemic, but bacteraemia was not an independent risk factor for post discharge mortality or recurrence of severe anaemia. Whether routine treatment with antibiotics should be given to all children with severe anaemia remains a matter of debate. Our data suggest that this would be a reasonable policy where blood cultures cannot be carried out.

Our analysis included only risk factors that could be ascertained at the time of admission. Causes of a post-discharge death, if it occurred in the community, could only be investigated retrospectively by using the verbal autopsy method. Verbal autopsies are known to be neither very sensitive nor specific; therefore the true causes of post-discharge mortality in the study population could not be clearly determined\(^{17}\).

This study provides disturbing evidence of consequences of severe anaemia on child health and survival. It is commonly thought that most deaths due to severe anaemia occur in-hospital\(^2\). This study shows that there is an even greater mortality post-discharge. This ‘hidden’ post-discharge mortality, calculated at 12.6% during 18 months (compared to 2% in the combined controls), is likely to be an underestimate, as it is based on the assumption that all children lost to follow-up survived. As severe anaemia is very common\(^1\), the impact on overall under-five mortality is likely to be considerable. Increased attention to the prevention and management of severe anaemia in African children is urgently needed if the fourth millennium development goal (MDG4) – a significant reduction in child mortality – is to be achieved.
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Chapter 5

HIV-associated anaemia in children; a systematic review from a global perspective

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AIDS in press
ABSTRACT

Objectives
To assess the importance of anaemia in HIV-infected children in western and tropical settings.

Design
A systematic review with a descriptive component.

Methods
Four databases were searched and reference lists of pertinent articles were checked. Studies that reported data on anaemia or haemoglobin levels in HIV-infected children were selected and grouped according to the location and the definition of anaemia.

Results
Thirty-six studies met the inclusion criteria. Mild (Haemoglobin<11g/dl) and moderate (Hb<9g/dl) anaemia were more prevalent with HIV infection (odds ratio 4.5; 95%-Confidence Interval 2.5-8.3 and 4.5; 95%-CI 2.0-10.3 respectively). Mean haemoglobin levels were lower (standardized-mean difference; 0.79; 95%-CI 0.47-1.10). These differences were observed in both western and tropical settings. Anaemia incidence ranged from 0.41-0.44 per person-year. There was limited data on more severe anaemia (Hb<7g/dl or<5g/dl). As anaemia was frequently identified as an independent risk factor for disease progression and death, we next reviewed the limited data to guide better strategies. Failure of erythropoiesis was the most important mechanism for anaemia in HIV-infected children. Therapeutic options include highly-active-antiretroviral-therapy and prevention or treatment of secondary infections. Erythropoietin can improve anaemia in children, but has not been evaluated in tropical settings. Micronutrient supplementation may be helpful in individual children. The potential benefits or risks of iron supplementation in HIV-infected children requires evaluation.

Conclusions
Anaemia is a very common complication of pediatric HIV infection, associated with a poor prognosis. With the increasing global availability of HAART, more data on the safety and efficacy of possible interventions in children is urgently needed.
INTRODUCTION

Early in the HIV-pandemic anaemia appeared to be the most common haematological complication in HIV-infected adults\(^1\) and a positive association had been reported between the prevalence of anaemia and severity of clinical disease\(^2\). Subsequently anaemia was repeatedly identified as a strong, independent and reversible predictor of mortality in large studies in western settings\(^3\)-\(^5\).

The highest prevalence of both HIV and anaemia occurs in tropical countries where over 60% of cases of both conditions occur in women and children\(^6\),\(^7\). However, current knowledge on HIV-related anaemia is predominantly based on studies in western men. This knowledge may not be applicable to children, especially for those living in resource poor settings, as the epidemiology and management of HIV infection and anaemia, and aetiological factors differ between the two settings\(^6\),\(^7\). In children it may present with weakness, fatigue, tachypnea and congestive cardiac failure and is associated with poor mental, motor, social-emotional, and neurophysiologic functioning\(^8\). In combination with HIV infection, anaemia may have an enhanced effect on the quality of life\(^8\).

The pathogenesis of anaemia in HIV-infected adults, although multi-factorial, relates primarily to a reduced production of erythrocytes\(^9\)-\(^12\). This reduction is influenced by several aetiological factors including infection and neoplasms, drugs such as zidovudine, a direct effect of HIV on erythropoiesis, a blunted response to erythropoietin and nutritional deficiencies\(^9\)-\(^12\). Compared to adults there is very little information available about the association between HIV infection and anaemia in children. This situation is not likely to improve because in western countries, which generate most of the research on this topic, pediatric HIV infection is declining\(^7\).

The aim of this study is to systematically review the prevalence and incidence of anaemia in HIV-infected compared to uninfected children in both western and tropical settings. We use the results of the review to discuss anaemia pathogenesis, aetiology and interventions in these children at a time of rapidly increasing global availability of HAART.

METHODS

Search strategy

The following databases were searched for primary studies reporting on anaemia and haemoglobin/haematocrit levels in HIV-infected children: PubMed (1950 to September 2006), Embase (1980 to 2006), African Index Medicus (1960 to 2006), and African
Selection criteria
All studies that met the following criteria were included: presented data on prevalence and/or incidence and/or mean haemoglobin/haematocrit levels in HIV-infected children (<18 years) or provided sufficient information to calculate these numbers; and had a well-defined definition for anaemia. If a study presented both haemoglobin and haematocrit data for a single population, the former was used. The following studies were excluded: case reports and reviews; those assessing restricted or possibly biased populations such as intervention trials with prophylactic or therapeutic regimens against Pneumocystis carinii Pneumonia (PCP) or HIV; studies on children with a specific (secondary) infection only; and duplicated studies. For the section on pathogenesis and aetiology in the discussion we used all articles on anaemia in HIV-infected children. Data was extracted by the first author and when there were doubts about whether studies should be included in the analysis these were resolved by discussion between the authors.

Definitions and Sub-group analysis
AIDS was defined as the presence of CDC stage C or in the older classification system as having P2 disease other than P2A. Studies were grouped by origin of study population: tropical (Africa, Central and South America, Asia with the exemption of the former Soviet Union, China and Mongolia) or western settings (Europe and North America) and the cut-off used to define anaemia. Haemoglobin cut-offs closest to 11g/dL (range 10.0-12.0) were used to define mild anaemia; 9g/dL (range 8.0-9.9) for moderate anaemia; 7g/dL (range 6.0-7.9) for marked anaemia; and 5g/dL (<5.9) for severe anaemia. If studies used haematocrit values to define anaemia these values were divided by three to provide an approximate equivalent haemoglobin level. Both HIV-unexposed and children who were HIV-uninfected, but transiently expressed maternal antibody against HIV (seroreverted children), were accepted as control groups in the meta-analysis and combined if appropriate. Studies that did not recruit a control group of uninfected children are reported in tables but were not included in the meta-analysis.

Statistical analysis
Data on prevalence, incidence and mean haemoglobin/haematocrit were summarized using descriptive statistics. The effect of the different anaemia cut-offs on prevalence were depicted using exponential curves based on weighted data. In both the prevalence and incidence studies the risk of anaemia in HIV-infected children was assessed by
meta-analyses on studies that included a control group. Effect sizes of binary data were expressed in (pooled) Relative Risks (RR) or Odds Ratios (OR) estimates, when appropriate. Continuous data (mean haemoglobin/haematocrit values) were pooled using standardized mean difference (SMD). Additionally, planned sensitivity analyses exploring the consistency of the findings across western and tropical settings were performed. In case study results were heterogeneously distributed ($\chi^2$ analysis: p<0.20) a random-effects models (e.g. random odds ratio) were used. Fixed-effects models were used if no heterogeneity could be shown. Statistical uncertainty was expressed in 95% Confidence Intervals (CI). Publication bias was visually assessed using funnel plots. All analyses were performed in Review Manager 4.2.7 (The Cochrane Collaboration) and SPSS 12.0 (SPPS inc., USA).

RESULTS

Selection of articles
The combined search retrieved 1027 hits. 802 articles remained after exclusion of duplicate citations and of these, 226 concerned anaemia in HIV-infected children. For the meta-analysis articles were excluded if they were case reports or reviews (74), did not present anaemia incidence or prevalence data (29), presented data on children enrolled in trials using PCP, PMTCT or ARV (34), focused on children that had a specific co-infection (9), or described the same study twice (6). A final set of 36 articles, 14 longitudinal and 22 cross-sectional studies remained. Fourteen studies were performed in a western and 22 in a tropical setting.

For the prevalence section 15 of these 36 studies were used as 11 did not provide extractable data14-24 and 10 did not define anaemia25-34. The incidence section consisted of seven studies since four studies did not provide extractable data23,35-37 and three did not define anaemia30,31,34. The section on mean haemoglobin was written using 12 articles; the remaining 24 did not provide extractable data14-21;23;25-30;32-34;38-43.

The meta-analysis represents data on 903 HIV-infected and 3441 HIV-uninfected children that were enrolled in controlled studies. Haemoglobin and anaemia data from an additional 1170 HIV-infected children recruited in uncontrolled studies is also presented. Not all studies provided data on gender and/or age. For those controlled studies that did provide data the overall age range was 0.0-18 years and the overall male:female ratio was 1.00:1.13.

Prevalence of anaemia in HIV-infected children
Fifteen studies were included in the analysis of anaemia prevalence in HIV-infected children (Table 1). Seven studies included controls of non-HIV-infected children.
The overall prevalence of mild or moderate anaemia in HIV-infected children varied between 22-94% and 3-82% respectively. One study presented data on marked and none on severe anaemia. All studies with a control group except one reported a significantly higher prevalence of anaemia in HIV-infected than in non-infected children (Figure 1a). The pooled random effect odds ratio for mild anaemia in HIV-infected children was 4.5 (95%-CI 2.5-8.3; Figure 1a), and for moderate anaemia 4.5 (95%-CI 2.0-10.3, Figure 1b). Studies which included a control group were further analyzed using a weighted curve

**Figure 1.** Risk of mild, moderate anaemia and mean haemoglobin / haematocrit levels in HIV-infected and uninfected children.

Random effect models of the risk of mild (2a) and moderate anaemia (2b) using prevalence data and mean haemoglobin/haematocrit levels; (2c) per location. Original data is given in table 1 (prevalence) and 3 (mean haemoglobin). In figure 2c two studies could not be displayed as the data was incomplete. OR: Odds Ratio; SMD: Standardized Mean Difference; Hb: Haemoglobin

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### 1a. Risk of mild anaemia (Hb<10.0-12.0)

<table>
<thead>
<tr>
<th>Study or sub-category</th>
<th>OR (random) 95% CI</th>
<th>OR (random) 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Miller et al &lt;11.0</td>
<td>3.93 [2.92, 5.41]</td>
<td>3.93 [2.92, 5.41]</td>
</tr>
<tr>
<td>Shaffer et al &lt;13%</td>
<td>2.78 [1.56, 4.27]</td>
<td>2.78 [1.56, 4.27]</td>
</tr>
<tr>
<td>Subtotal (95% CI)</td>
<td>3.60 [2.76, 4.71]</td>
<td>3.60 [2.76, 4.71]</td>
</tr>
<tr>
<td>Test for heterogeneity: CH² = 5.86, df = 3 (P = 0.15)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test for overall effect Z = 9.98 (P = 0.00001)</td>
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<td></td>
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### 1b. Risk of moderate anaemia (Hb<8.0-9.9)

<table>
<thead>
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<th>Study or sub-category</th>
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<th>OR (random) 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Miller et al &lt;9.0</td>
<td>3.18 [2.32, 4.42]</td>
<td>3.18 [2.32, 4.42]</td>
</tr>
<tr>
<td>Shaffer et al &lt;13%</td>
<td>2.78 [1.56, 4.27]</td>
<td>2.78 [1.56, 4.27]</td>
</tr>
<tr>
<td>Test for heterogeneity: CH² = 0.63, df = 2 (P = 0.81)</td>
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<td></td>
</tr>
<tr>
<td>Test for overall effect Z = 0.26 (P = 0.00001)</td>
<td></td>
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</table>

### 1c. Mean haemoglobin & haematocrit levels

<table>
<thead>
<tr>
<th>Study or sub-category</th>
<th>SMD (random) 95% CI</th>
<th>SMD (random) 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Miller et al &lt;11.0</td>
<td>-6.32 [-1.70, 0.49]</td>
<td>-6.32 [-1.70, 0.49]</td>
</tr>
<tr>
<td>Shaffer et al &lt;13%</td>
<td>-6.53 [-1.82, 0.63]</td>
<td>-6.53 [-1.82, 0.63]</td>
</tr>
<tr>
<td>Test for heterogeneity: CH² = 1.11, df = 2 (P = 0.00001)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test for overall effect Z = 10.36 (P = 0.00001)</td>
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<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Study or sub-category</th>
<th>SMD (random) 95% CI</th>
<th>SMD (random) 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Miller et al &lt;11.0</td>
<td>-0.32 [-1.29, 0.66]</td>
<td>-0.32 [-1.29, 0.66]</td>
</tr>
<tr>
<td>Shaffer et al &lt;13%</td>
<td>-0.32 [-1.29, 0.66]</td>
<td>-0.32 [-1.29, 0.66]</td>
</tr>
<tr>
<td>Test for heterogeneity: CH² = 4.00, df = 1 (P = 0.00001)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test for overall effect Z = 4.00 (P = 0.00001)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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The non-overlapping 95% confidence intervals indicate that anaemia was significantly more prevalent in HIV-infected children.

The prevalence of mild and moderate anaemia in western settings ranged between 22-94% and 11-82% respectively. In tropical settings the equivalent ranges were 50-91% for mild anaemia and 3-38% for moderate anaemia. In the latter setting pooled odds ratios for mild and moderate anaemia were 3.6 (95%CI 2.8-4.7) and 3.0 (95%CI 2.3-3.8) respectively. To compare the prevalence of anaemia in HIV-infected children living in western or tropical settings the prevalences were plotted against the different cut-offs used (Figure 2b). The 95% confidence intervals for this comparison widely overlapped and did not suggest a significant effect of location on the prevalence of anaemia in HIV-infected children.

**Figure 2a.** Prevalence of anaemia in HIV-infected and uninfected children (only studies that simultaneously included both groups, n=7). (see colour section).

Curves display weighted exponential trendlines (thick lines) and 95% confidence intervals (thin lines) for HIV-infected (R²=0.83) and HIV-uninfected children (R²=0.84). For the analyses data for 12 cut-offs were used reflecting 700 HIV-infected and 2387 uninfected children.

**Figure 2b.** Prevalence of anaemia in HIV-infected children in tropical vs. western (Europe & North America) settings. (see colour section).

Curves display weighted exponential trendlines (thick lines) and 95% confidence intervals (thin lines) for HIV-infected children in tropical (R²=0.77) and western settings (R²=0.63). The analyses included 15 studies presenting data for 23 cut offs in 1085 HIV-infected children.
Incidence of anaemia in HIV-infected children

Seven longitudinal studies reported anaemia incidence including two studies with a control group of HIV-uninfected children (Table 2). Of these seven studies five reported a mean or median duration of follow up and ranged from 0.6-2.3 years. The incidence of *mild* and *moderate* anaemia in HIV-infected children varied between 0.41-0.44 and 0.07-0.37 per person-year of follow-up respectively. No study reported data for *marked* anaemia and one only for *severe* anaemia. The two studies with control groups showed that anaemia was more frequent in HIV-infected children (Table 2). Although both studies presented relative risks with 95% significance, the pooled random effect relative risk for mild anaemia was 1.35 (95%CI: 0.85-2.27, data not graphically presented).

In western settings mild and moderate anaemia was reported in 74-87% and 16-37% of HIV-infected children studied. In tropical settings these numbers ranged between 73-100% and 29-58%. Too little data was available in these subgroups to compare per person-year incidence rates.

*Figure 3.* Mean haemoglobin (Hb) levels of cohorts of HIV-infected and uninfected infants in Zimbabwe\(^\text{45}\) (upper left), Kenya\(^\text{23}\) (upper right) and Italy\(^\text{36}\) (bottom left). (see colour section)

The curves in the bottom right graph compare the mean haemoglobin levels in HIV-infected children in these settings. Reproduced with permission from MF Miller; The American Society of Tropical Medicine and Hygiene; and Mary Ann Liebert Inc. Publishers respectively.
Mean haemoglobin and haematocrit levels

Three studies presented longitudinal data (Figure 3) and 12 studies presented cross-sectional data on mean/median haemoglobin or haematocrit levels in HIV-infected children (Table 3).

In the cross-sectional studies mean haemoglobin levels for HIV-infected children ranged from 8.0-13.0 g/dL. All seven studies with a control group reported lower values in HIV-infected children. In two studies these differences were not significant, one of these had a very small sample size (n=14) and the other showed borderline significance (p=0.07). Five studies which reported means and standard deviations could be included in the meta-analysis. The pooled SMD was -0.78 (95%CI -0.47 – -1.10), the equivalent to -1.1 g/dL haemoglobin (Figure 1c).

In western studies mean haemoglobin levels for HIV-infected children varied between 9.8 and 13.0 g/dL (one with a mean haematocrit value of 27.5%). In tropical areas mean values ranged from 8.0-10.4 g/dL (one with a mean haematocrit value of 28.9%). In both settings the pooled SMD indicated a significantly decreased haemoglobin level in HIV-infected children (Figure 1c).

The three longitudinal studies reported mean haemoglobin levels in infants living in Zimbabwe45, Kenya23 and Italy36 (Figure 3). The mean haemoglobin levels from birth to one month of age were not different between HIV-infected and uninfected children in all studies. From six to twelve weeks mean haemoglobin levels of HIV-infected infants were significantly lower than uninfected infants and this difference increased slowly during infancy. In HIV-infected Italian, but not in African infants, the haemoglobin levels did recover from the physiological nadir at six weeks, but then decreased slowly over time. Seroreverting infants had similar haemoglobin curves to infants born to mothers not infected with HIV (Kenya and Zimbabwe only).

DISCUSSION

In this first review on children we assessed if anaemia was more common in HIV-infected compared to uninfected children in both western and tropical settings. Mild anaemia (Hb< 11g/dl) appeared to be a common complication of HIV in children in both settings and occurred in 73-100% of cases. A difference in mean haemoglobin between infected and non-infected children was present from the age of 6-12 weeks with lower haemoglobin levels in HIV-infected children.

Prevalence rates for mild and moderate anaemia were higher, and the mean haemoglobin and haematocrit levels lower, in HIV-infected compared to uninfected children. These differences were identified in both tropical and western settings. This combined with the
Table 1. Prevalence of anaemia in HIV-infected children by use of a control group of HIV-uninfected children and location.

<table>
<thead>
<tr>
<th>Country</th>
<th>Period</th>
<th>Definition</th>
<th>Hb (g/dL)</th>
<th>Prevalence (%)</th>
<th>Odds Ratio (95% CI)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Controlled Studies -Tropical settings</strong></td>
<td></td>
<td></td>
<td></td>
<td>HIV+</td>
<td>HIV-</td>
<td>HIV+</td>
</tr>
<tr>
<td>Zimbabwe</td>
<td>1997-2000</td>
<td>&lt;11</td>
<td>84</td>
<td>56</td>
<td>3.93</td>
<td>(2.77-5.59)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&lt;10.5</td>
<td>75</td>
<td>40</td>
<td>4.46</td>
<td>(3.29-6.02)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&lt;9</td>
<td>32</td>
<td>13</td>
<td>3.14</td>
<td>(2.30-4.29)</td>
</tr>
<tr>
<td>Uganda</td>
<td>1995-1998</td>
<td>&lt;11</td>
<td>91</td>
<td>77</td>
<td>3.00</td>
<td>(1.05-8.09)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&lt;9</td>
<td>35</td>
<td>21</td>
<td>2.10</td>
<td>(0.85-5.33)</td>
</tr>
<tr>
<td>Malawi</td>
<td>prior to 2001</td>
<td>&lt;11</td>
<td>78</td>
<td>50</td>
<td>3.62</td>
<td>(1.90-6.98)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&lt;9</td>
<td>30</td>
<td>13</td>
<td>2.78</td>
<td>(1.43-5.41)</td>
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<td>Zaire (Congo)</td>
<td>1987</td>
<td>&lt;30% b</td>
<td>50</td>
<td>27</td>
<td>2.70</td>
<td>(1.19-6.13)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&lt;20% b</td>
<td>18</td>
<td>9</td>
<td>2.26</td>
<td>(0.65-6.38)</td>
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<tr>
<td><strong>Controlled Studies -Western settings</strong></td>
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<td></td>
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<td>n/a</td>
<td></td>
</tr>
<tr>
<td>Austria</td>
<td>prior to 1990</td>
<td>&lt;12</td>
<td>22</td>
<td>20</td>
<td>1.14</td>
<td>(0.04-84.07)</td>
</tr>
<tr>
<td>USA</td>
<td>prior to 1990</td>
<td>&lt;33% b</td>
<td>94</td>
<td>32</td>
<td>33.4</td>
<td>(11.0-110.8)</td>
</tr>
<tr>
<td>Italy</td>
<td>prior to 1995</td>
<td>&lt;9</td>
<td>82</td>
<td>5</td>
<td>82.5</td>
<td>(14.2-563.4)</td>
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<tr>
<td><strong>Uncontrolled Studies -Tropical settings</strong></td>
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<td></td>
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</tr>
<tr>
<td>Zimbabwe</td>
<td>prior to 1994</td>
<td>&lt;11</td>
<td>85</td>
<td>-</td>
<td>-</td>
<td>46</td>
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<tr>
<td>South Africa</td>
<td>prior to 2002</td>
<td>&lt;11</td>
<td>72</td>
<td>3</td>
<td>-</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&lt;8</td>
<td>72</td>
<td>-</td>
<td>-</td>
<td>n/a</td>
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<tr>
<td>South Africa</td>
<td>1988-1989</td>
<td>&lt;10</td>
<td>89</td>
<td>-</td>
<td>-</td>
<td>9</td>
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<tr>
<td>Thailand</td>
<td>1994</td>
<td>&lt;10</td>
<td>65</td>
<td>-</td>
<td>-</td>
<td>43</td>
</tr>
<tr>
<td>Nigeria</td>
<td>2003</td>
<td>&lt;10</td>
<td>78</td>
<td>-</td>
<td>-</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&lt;8</td>
<td>38</td>
<td>-</td>
<td>-</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&lt;6</td>
<td>6</td>
<td>-</td>
<td>-</td>
<td>n/a</td>
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<tr>
<td>South Africa</td>
<td>1989-1990</td>
<td>&lt;8</td>
<td>35</td>
<td>-</td>
<td>-</td>
<td>23</td>
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<td><strong>Uncontrolled Studies -Western settings</strong></td>
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<td></td>
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<td>n/a</td>
<td></td>
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<tr>
<td>Italy</td>
<td>prior to 1995</td>
<td>&lt;11.5</td>
<td>66</td>
<td>-</td>
<td>-</td>
<td>61</td>
</tr>
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<td>USA</td>
<td>1984-1994</td>
<td>&lt;8</td>
<td>11</td>
<td>-</td>
<td>-</td>
<td>65</td>
</tr>
</tbody>
</table>

*Inter Quartile Range. *Haematocrit values. *Cross-section of an age group in a cohort study.

Hb: Haemoglobin. CI: Confidence interval. n/a: Not available. ARV: on antiretroviral medication.
<table>
<thead>
<tr>
<th>Description</th>
<th>Age range (y)</th>
<th>AIDS (%)</th>
<th>ARV (%)</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infants enrolled in a cohort study; Controls were exposed or unexposed</td>
<td>1.0 c</td>
<td>n/a</td>
<td>0</td>
<td>Miller47</td>
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<tr>
<td>Infants at an outpatient clinic</td>
<td>0.8 c</td>
<td>n/a</td>
<td>0</td>
<td>Totin42</td>
</tr>
<tr>
<td>Infants enrolled in a cohort study</td>
<td>1.0 c</td>
<td>n/a</td>
<td>0</td>
<td>Semba46</td>
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<tr>
<td>Children at an outpatient department</td>
<td>1.0-14</td>
<td>n/a</td>
<td>n/a</td>
<td>Shaffer47</td>
</tr>
<tr>
<td>Children with haemophilia</td>
<td>3.0-18</td>
<td>0</td>
<td>n/a</td>
<td>Fuchs44</td>
</tr>
<tr>
<td>Children with symptomatic infection; Controls were exposed or unexposed</td>
<td>0.2-8.0</td>
<td>n/a</td>
<td>n/a</td>
<td>Ellaurie48</td>
</tr>
<tr>
<td>Infants enrolled in a cohort study Controls were exposed</td>
<td>0.2 c</td>
<td>19</td>
<td>0</td>
<td>Galli36</td>
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<tr>
<td>Admitted symptomatic children with positive HIV serology</td>
<td>0.3-7.0</td>
<td>n/a</td>
<td>0</td>
<td>Adewuyi38</td>
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<td>Clinically stable infected children attending an HIV clinic</td>
<td>1.1-3.2 a</td>
<td>20</td>
<td>0</td>
<td>Eley49</td>
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<td>Admitted symptomatic children with positive HIV serology</td>
<td>0.3-2.5</td>
<td>78</td>
<td>0</td>
<td>Bobat50</td>
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<tr>
<td>Children with AIDS</td>
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<td>Children attending an HIV clinic</td>
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<td>n/a</td>
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<td>n/a</td>
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<td>Symptomatic children</td>
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<td>n/a</td>
<td>Castaldo59</td>
</tr>
<tr>
<td>Cohort of children; analyzed at time of AIDS diagnosis</td>
<td>0.3-26.2</td>
<td>100</td>
<td>32</td>
<td>Al-Attar35</td>
</tr>
</tbody>
</table>
Table 2. Incidence of anaemia in HIV-infected children in longitudinal analyses by use of a control group of HIV-uninfected children and location.

<table>
<thead>
<tr>
<th>Country</th>
<th>Period</th>
<th>Definition Haemoglobin (g/dL)</th>
<th>Incidence (%)</th>
<th>Relative Risk (95% CI)</th>
<th>n</th>
<th>Description</th>
<th>Incidence</th>
<th>ppY</th>
<th>AIDS (%)</th>
<th>ARV (%)</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Controlled Studies - Tropical settings</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brazil</td>
<td>1996-97</td>
<td>&lt;11.5</td>
<td>73</td>
<td>42</td>
<td>1.76</td>
<td>(1.19-2.62)</td>
<td>53</td>
<td></td>
<td>26</td>
<td>53</td>
<td>Silva20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(3-6m: &lt;9.5; 6m-24m: &lt;10.5)</td>
<td></td>
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</tr>
<tr>
<td><strong>Controlled Studies - Western settings</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>USA</td>
<td>1990-99</td>
<td>&lt;10.5</td>
<td>87</td>
<td>78</td>
<td>1.11</td>
<td>(1.04-1.19)</td>
<td>955</td>
<td></td>
<td>163</td>
<td>955</td>
<td>Paul19</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0m &lt;13.5; 1m 10.7, 2m &lt;9.4)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td><strong>Uncontrolled Studies - Tropical areas</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uganda</td>
<td>1995-98</td>
<td>&lt;11</td>
<td>100</td>
<td></td>
<td>168</td>
<td>n/a</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>&lt;9</td>
<td>58</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malawi</td>
<td>2002-03</td>
<td>&lt;8</td>
<td>29</td>
<td></td>
<td>45</td>
<td>n/a</td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
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<td>4</td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td><strong>Uncontrolled Studies - Western settings</strong></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>USA</td>
<td>1990-91</td>
<td>&lt;11</td>
<td>92</td>
<td></td>
<td>74</td>
<td>n/a</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>&lt;10</td>
<td>74</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>&lt;8</td>
<td>30</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>USA</td>
<td>1991-93</td>
<td>&lt;9.5</td>
<td>37</td>
<td></td>
<td>75</td>
<td>n/a</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Italy</td>
<td>prior to 1991</td>
<td>&lt;8a</td>
<td>16</td>
<td></td>
<td>433</td>
<td>n/a</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Children were excluded from the analysis from the first dose of zidovudine onwards. *Median Follow-up. n/a: Not available. CI: Confidence Interval. PPY: incidence of anaemia in HIV-infected children per person-year of follow up. ARV: on antiretroviral medication.
<table>
<thead>
<tr>
<th>Description</th>
<th>Incidence ppy</th>
<th>Age range (y)</th>
<th>AIDS (%)</th>
<th>ARV (%)</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV-infected vs. seroreverted birth cohort</td>
<td>0.41</td>
<td>0.1-12</td>
<td>0</td>
<td>65</td>
<td>Silva²⁰</td>
</tr>
<tr>
<td>HIV-infected vs. seroreverted birth cohort</td>
<td>0.44</td>
<td>0.0-2.0</td>
<td>34</td>
<td>76</td>
<td>Paul¹⁹</td>
</tr>
<tr>
<td>Symptomatic children with positive serology</td>
<td>n/a</td>
<td>0.8-4</td>
<td>n/a</td>
<td>0</td>
<td>Clark⁵⁴</td>
</tr>
<tr>
<td>Children at Voluntary Counseling and Testing (VCT) clinic</td>
<td>0.48</td>
<td>2.0-15</td>
<td>n/a</td>
<td>0</td>
<td>Laufer²⁴</td>
</tr>
<tr>
<td>Children admitted with AIDS</td>
<td>n/a</td>
<td>0.3-9.5</td>
<td>100</td>
<td>79</td>
<td>Suarez²¹</td>
</tr>
<tr>
<td>Infants followed from birth</td>
<td>0.37</td>
<td>0.0-1.0</td>
<td>12</td>
<td>6.6</td>
<td>Forsyth¹⁶</td>
</tr>
<tr>
<td>Children followed from birth or HIV diagnosis</td>
<td>0.07&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.0-6.0</td>
<td>50</td>
<td>0</td>
<td>Tovo²²</td>
</tr>
</tbody>
</table>

<sup>a</sup>Children were excluded from the analysis from the first dose of zidovudine onwards. <sup>b</sup>Median Follow-up. n/a: Not available. CI: Confidence Interval. PPY: incidence of anaemia in HIV-infected children per person-year of follow up. ARV: on antiretroviral medication.
<table>
<thead>
<tr>
<th>Country</th>
<th>Period</th>
<th>Mean Hb (g/dL)</th>
<th>p-value</th>
<th>n</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HIV+</td>
<td>HIV-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controlled Studies -Tropical settings</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zaire (Congo)</td>
<td>1987</td>
<td>28.9%</td>
<td>32.5%</td>
<td>0.01</td>
<td>28 673 Children presenting at an outpatient department 1-14 y</td>
</tr>
<tr>
<td>Zimbabwe</td>
<td>1997-00</td>
<td>9.5</td>
<td>10.6</td>
<td>&lt;0.001</td>
<td>273 1319 Infants enrolled in a cohort study Controls were exposed or unexposed 1.0-3.0 y</td>
</tr>
<tr>
<td>Malawi</td>
<td>prior to 2001</td>
<td>9.8</td>
<td>10.9</td>
<td>&lt;0.001</td>
<td>73 246 Infants enrolled in a cohort study 1.0 e n/a 0</td>
</tr>
<tr>
<td>Burkina Faso</td>
<td>2002-03</td>
<td>8.0</td>
<td>8.5</td>
<td>0.07</td>
<td>44 46 Malnourished children 1.0-5.0 n/a 0</td>
</tr>
<tr>
<td>Controlled Studies -Western settings</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Austria</td>
<td>prior to 1990</td>
<td>13.0 a</td>
<td>14.7 a</td>
<td>&gt;0.05^c</td>
<td>9 5 Children with haemophilia 3-18 y</td>
</tr>
<tr>
<td>USA</td>
<td>prior to 1990</td>
<td>27.5%</td>
<td>33.5%</td>
<td>&lt;0.001</td>
<td>100 47 Children with symptomatic infection; Controls were exposed or unexposed 0.2-8.0 y</td>
</tr>
<tr>
<td>Italy</td>
<td>prior to 1995</td>
<td>9.8</td>
<td>10.7</td>
<td>&lt;0.001</td>
<td>22 58 Infants enrolled in a cohort study. Those uninfected were exposed 0.2-19 y</td>
</tr>
<tr>
<td>Uncontrolled Studies -Tropical settings</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>South Africa</td>
<td>1988-89</td>
<td>8.1</td>
<td>-</td>
<td>-</td>
<td>9</td>
</tr>
<tr>
<td>Thailand</td>
<td>1994</td>
<td>9.1</td>
<td>-</td>
<td>-</td>
<td>43</td>
</tr>
<tr>
<td>South Africa</td>
<td>prior to 2002</td>
<td>10.4 a</td>
<td>-</td>
<td>-</td>
<td>60</td>
</tr>
<tr>
<td>Nigeria</td>
<td>2003</td>
<td>8.3</td>
<td>-</td>
<td>-</td>
<td>68</td>
</tr>
<tr>
<td>Uncontrolled Studies -Western settings</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>USA</td>
<td>1984-94</td>
<td>10.0^a</td>
<td>-</td>
<td>-</td>
<td>65</td>
</tr>
</tbody>
</table>

^aMedian Values. ^bHaematocrit Values. ^cMann Whitney U test. ^dInter Quartile Range; ^eCross-section of an age group in a cohort study. Hb: Haemoglobin. n/a: Not applicable. ARV: on antiretroviral medication.
### Table 3. Mean Haemoglobin and Haematocrit levels in HIV-infected children by use of a control group of HIV-uninfected children and location.

<table>
<thead>
<tr>
<th>Description</th>
<th>Age range (y)</th>
<th>AIDS (%)</th>
<th>ARV (%)</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Children presenting at an outpatient department</td>
<td>1.0-14</td>
<td>n/a</td>
<td>n/a</td>
<td>Shaffer47</td>
</tr>
<tr>
<td>Infants enrolled in a cohort study Controls were exposed or unexposed</td>
<td>1.0 e</td>
<td>n/a</td>
<td>0</td>
<td>Miller37</td>
</tr>
<tr>
<td>Infants enrolled in a cohort study</td>
<td>1.0 e</td>
<td>n/a</td>
<td>0</td>
<td>Semba46</td>
</tr>
<tr>
<td>Malnourished children</td>
<td>1.0-5.0</td>
<td>n/a</td>
<td>0</td>
<td>Simpore31</td>
</tr>
<tr>
<td>Children with haemophilia</td>
<td>3.0-18</td>
<td>0</td>
<td>n/a</td>
<td>Fuchs44</td>
</tr>
<tr>
<td>Children with symptomatic infection; Controls were exposed or unexposed</td>
<td>0.2-8.0</td>
<td>n/a</td>
<td>n/a</td>
<td>Ellaurie48</td>
</tr>
<tr>
<td>Infants enrolled in a cohort study. Those uninfected were exposed</td>
<td>0.2 e</td>
<td>19</td>
<td>0</td>
<td>Galli36</td>
</tr>
<tr>
<td>Admitted symptomatic children with positive HIV serology</td>
<td>0.3-2.5</td>
<td>78</td>
<td>0</td>
<td>Bobat50</td>
</tr>
<tr>
<td>Children with AIDS</td>
<td>0.3-4.0</td>
<td>100</td>
<td>n/a</td>
<td>Tienboon51</td>
</tr>
<tr>
<td>Clinically stable infected children attending an HIV clinic</td>
<td>1.1-3.2d</td>
<td>20</td>
<td>0</td>
<td>Eley49</td>
</tr>
<tr>
<td>Children attending an HIV clinic</td>
<td>0.3-13</td>
<td>32</td>
<td>n/a</td>
<td>Adetifa52</td>
</tr>
<tr>
<td>Cohort of infected children analysed at AIDS diagnosis</td>
<td>0.3-26</td>
<td>100</td>
<td>32</td>
<td>Al-Attar35</td>
</tr>
</tbody>
</table>
overlapping anaemia prevalence for HIV-infected children living in tropical compared to western settings (Figure 2b), indicate that mild anaemia can be highly prevalent in all HIV-infected children irrespective of the region.

Only two incidence studies included a control group and these showed that mild anaemia was more common in HIV-infected children, but the combined difference did not reach significance. The only controlled study from a tropical setting was performed in Brazil. No controlled data was available for Africa where the greatest burden of HIV lies. Two uncontrolled studies from Africa reported that anaemia occurred in almost all children with HIV infection\textsuperscript{24, 54}, which stresses the magnitude of the anaemia problem.

A limitation of this review was that the number of studies in the meta-analysis was small and heterogeneous. We partly addressed the heterogeneity by performing sub-group analyses by setting. Publication bias, commonly seen in diagnostic studies, could have affected our results but the funnel plots were not suggestive of bias (not displayed). Most studies that were published after 2000 needed to be excluded as they were clinical trials of antiretroviral regimens. These studies commonly excluded anaemic children at enrolment. Despite this selection, anaemia was still commonly reported\textsuperscript{55-62} suggesting that even after the introduction of highly active antiretroviral therapy (HAART), anaemia continues to be an important, and largely neglected, complication of pediatric HIV.

Studies mainly from tropical\textsuperscript{41, 49, 54} and to a lesser extent for western settings\textsuperscript{36, 48} have reported that severity of the anaemia was related to disease progression. We were unable to calculate incidence rates of (mild) anaemia according to disease progression as only two studies reported these data. Little comparative data on the prevalence and incidence of more severe anaemia in HIV-infected children was reported. One case-control study from Kenya reported HIV prevalence in children with and without severe anaemia (Hb<5 g/dL) and found no difference (7.6 vs. 9.6% respectively)\textsuperscript{63}. This study did not control for confounding factors and contrasts to other studies that have shown an association between HIV and severe anaemia in adults\textsuperscript{64} and children with malaria\textsuperscript{65}. More data on severe anaemia is needed as these conditions occur frequently in children in tropical countries, and are associated with a high morbidity and mortality\textsuperscript{66}.

Disease progression

As in adults\textsuperscript{3-5}, higher anaemia prevalences were reported in children with more advanced disease\textsuperscript{41, 49, 52}. Anaemia (Hb<8 or <9 g/dL) was identified as an independent risk factor for disease progression and death in four of five longitudinal studies in children\textsuperscript{16, 22, 36, 54, 67}. The CDC and WHO criteria currently include anaemia (Hb<8 g/L) as an indicator of moderate disease based on data from an Italian study\textsuperscript{13, 22, 68}. Only recently this marker of disease progression was shown to be of similar importance in children living in a tropical area\textsuperscript{69}. Since the determination of haemoglobin is relatively robust and cheap,
compared to other tests used to monitor HIV disease, haemoglobin may prove to be a useful tool to guide decisions on when to start or alter antiretroviral treatment. This would be of additional use of HIV-infected children living in areas where CD4-counts and viral load determinations are not available.

Morphology and Pathogenesis

It is unclear if reversal of the anaemia in children increases life expectancy as has been shown for adults. Although anaemia reversal appears to be an attractive option to reduce morbidity and mortality in HIV-infected children, it requires an understanding of the pathogenesis and aetiology of HIV-associated anaemia in children. The current pathological data on children suggests that, as in adults, insufficient production of erythrocytes is the most important pathogenetic mechanism for anaemia, since blood loss or haemolysis are not prominent features in HIV-infected children with anaemia. In addition these studies suggest that a positive Coombs, or direct antiglobulin test, is merely a reflection of hyperglobulinemia than actual haemolysis.

In eight studies reporting peripheral blood findings in HIV-infected children microcytic and hypochromic abnormalities appeared to be common with prevalences ranging from 12-100% and 20-100% respectively. Though microcytic and hypochromic abnormalities appeared to be prevalent in HIV-infected children and might even increase during disease progression, the only study that compared peripheral blood findings in HIV-infected and uninfected children concluded that mean MCV and MCH curves were comparable during the first two years of life. In addition no difference in the pooled prevalence of the cellular appearance was noticed between tropical and western settings. Pancytopenia was found in 11-20% of HIV-infected anaemic children, which further suggest that the bone marrow might be restricted in these children. One study reported pancytopenia was associated with ultimately fatal opportunistic infections.

Bone marrow examinations have rarely been carried out in HIV-infected children and most data come from retrospective analyses of hospital records which did not include a control group. No specific morphological findings have been identified, but bone marrow abnormalities, especially dyserythropoiesis, were generally noted to be more common in the later stages of HIV disease. As in HIV-infected adults, hypocellularity is uncommon in children with HIV infection. Data on more accessible markers of bone marrow function that could be used to study erythropoiesis in HIV-infected children, such as reticulocyte counts, have not been reported.
Aetiology
The aetiology of this reduction in (effective) erythropoiesis is diverse and includes (1) The direct effect of HIV on erythropoiesis; (2) HIV-associated infections and neoplasms; (3) Medications; (4) Micronutrient deficiencies.

In vitro data suggested that HIV itself may diminish erythropoiesis through apoptosis of erythroid precursors or infection of auxiliary cells, by altering cytokine and erythropoietin responses. Children may be more vulnerable to these mechanism because they differ from adults in their haematopoiesis, increased cytokine responses, or viral loads. Erythropoietin has been used to improve erythropoiesis in HIV-infected children in small studies and appeared to be successful, safe and cost-effective in western settings. Costs and the need for regular subcutaneous injections make the use of erythropoietin challenging in tropical settings. More information is needed about the cost-effectiveness, feasibility and usefulness of erythropoietin compared to blood transfusions in HIV-infected children in developing countries.

HIV-associated conditions predominantly infections and, to a lesser extent, neoplastic diseases are the largest group of factors associated with anaemia. Mycobacterial infections, such as M. Tuberculosis and especially Mycobacterium avium-intracellulare, were found to be common in HIV-infected children worldwide and often associated with severe anaemia and pancytopenia. Other bacterial infections associated with anaemia included non-typhoid salmonella bacteraemias which were mainly found in African children. Viral causes of anaemia included hepatitis C, cytomegalovirus (CMV), Epstein-Barr virus and parvovirus B19. Although persistent parvovirus B19 infections have been identified as a cause of severe anaemia in HIV-infected adults, this was not confirmed in the only study performed in HIV-infected children. Penicillium marneffei is a fungal infection, mainly found in South-East Asia and China, and in HIV-infected children commonly presented with severe anaemia. Other fungi were rarely associated with anaemia with the exception of Histoplasma capsulatum. Although Pneumocystis jiroveci (previously carinii) was commonly found in African infants infected with HIV, haematological data on this co-infection is lacking. The incidence of opportunistic infections may be different in HIV-infected children living in tropical or western settings. Mycobacterium avium and CMV are more common in western settings, whilst tuberculosis and malaria more often affect those living in tropical settings, PCP appears to occur equally in both settings. It is less clear if children living in tropical settings are affected earlier during HIV disease with specific opportunistic infections than their western counterparts.

There were no data suggesting that parasites commonly associated with anaemia, such as schistosomiasis or hookworm, occurred more frequently in HIV-infected children. Other parasites including Leishmaniasis have been associated with both anaemia and
paediatric HIV infections⁹⁶. The most well recognized cause of anaemia in tropical settings, falciparum malaria, was not found more frequently²３, ⁹⁷, ⁹⁸, or with higher parasite densities in HIV-infected than uninfected children⁶⁵, ⁹⁷-⁹⁹. However children with HIV and malaria were found to be more anaemic, and to have more severe anaemia (Hb<5g/dL), than children with either infection alone²³, ⁶⁵. Whether this is simply the additional effect of two risk factors for anaemia or the result of a biological interaction, such as increased viral loads¹⁰⁰, or the introduction of other co-infections such as non-typhoid salmonella⁸⁹, remains unclear and requires further evaluation.

Several drugs given during the course of HIV disease could cause anaemia in children including antiretrovirals¹⁰¹, ¹⁰² (especially AZT¹⁰³-¹⁰⁶), other antiviral agents⁹⁰, ¹⁰⁷; antibiotics¹⁰⁸, tuberculostatic⁸⁴; and cytostatic agents¹⁰⁹. HAART was reported to increase haemoglobin levels in adults, despite the haematotoxic effects of some individual agents³, and this may also apply to children¹¹⁰. Prophylactic regimens against Pneumocystis jiroveci pneumonia commonly include antifolate agents. Several combinations that were tested in children however were not associated with anaemia¹¹¹-¹¹³. The current WHO-recommended regimen of cotrimoxazole was proven to be protective against malaria in adults¹¹⁴, ¹¹⁵. The effect of this regimen on malaria incidence and immunity in these children requires further studies as the evidence is limited¹¹⁶ and conflicting¹¹⁷.

The micronutrient deficiencies that have been associated with HIV-infection and which could lead to anaemia are iron, folate, vitamin B12, vitamin A and zinc. Although iron deficiency appeared to be common in HIV-infected children in studies that assessed bone marrow⁴⁸, ⁷¹, ⁷² and peripheral markers²⁰, ²⁸, ³⁸, ³⁹, ⁴¹, ⁴², ⁴⁵, ⁴⁸, ⁴⁹, ⁵¹, ⁵³, ⁵⁴, ⁷¹-⁷³, studies with a control group suggested that iron deficiency may not have been more common than in uninfected children²⁰, ⁴², ⁴⁵. Bone marrow iron status was found to be unrelated to anaemia in these children⁴⁸. Definitive evidence on the contribution of iron deficiency to the anaemia of HIV-infected children and the effects and possible harm of iron supplementation¹¹⁸-¹²⁰ was lacking as intervention trials have not been undertaken in children or adults infected with HIV. Such information is urgently needed⁹⁵ as presumptive supplementation is recommended for most children living in tropical countries¹²¹.

Folate and vitamin B12 deficiency were not common in paediatric HIV infection¹⁴, ⁴⁹, ⁷¹, ⁷², ¹²²-¹²⁴ and the haematopoietic effect of supplementation with these haematinics has not been assessed in HIV-infected children. Deficiencies of vitamin A and zinc appeared to occur more frequently in HIV-infected children than those without infection¹²⁵-¹²⁸ and zinc supplementation increased mean haemoglobin in a placebo controlled trial in South African children with HIV infection¹²⁹. Data on the effect of antenatal vitamin A supplementation on haemoglobin levels in HIV-infected newborns³⁷, ¹³⁰ and on vitamin
A supplementation in older HIV-infected children is limited and conflicting\textsuperscript{131, 132} and needs further research.

**CONCLUSIONS**

Worldwide anaemia occurred in 73-100\% of HIV-infected children studied. Prevalence of mild and moderate anaemia is higher in HIV-infected children compared to those without HIV infection in both tropical and western settings. There is very limited data on severe anaemia, a common diagnosis in tropical areas, which is associated with high morbidity and mortality. The role of HIV in the development of severe anaemia should be a focus for future research.

As in adults, an association between anaemia and disease progression was reported in children regardless of geographical location. The use of haemoglobin to predict and monitor disease progression, and the effect of anaemia reduction for reversing disease progression in children infected with HIV in resource poor settings are neglected and important areas for research.

HAART is rapidly becoming available worldwide and has altered HIV from a rapidly fatal to a chronic disease. Therefore the importance of and possibilities for prevention of anaemia are increasing. Failure of erythropoiesis was the most important mechanism for anaemia in HIV-infected children and adults. The conditions leading to failing erythropoiesis were diverse and complex and included secondary infections. HAART (especially without AZT) and the prevention or treatment of secondary infections, appeared to be the most effective therapies for anaemia reduction. Erythropoietin can improve anaemia in children with HIV infection, but has not been evaluated in tropical settings. Micronutrient supplementation may be helpful in individual children with HIV infection but data to support mass supplementation are lacking. The potential benefits or risks of iron supplementation in HIV-infected children requires evaluation.
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Chapter

Erythropoiesis in HIV-infected and uninfected Malawian children with severe anaemia

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Submitted
ABSTRACT

Introduction
Anaemia is a common complication and independent predictor of mortality in HIV-infected adults and children. In adults, anaemia results primarily from reduced production of erythrocytes, mediated by various factors, including inflammation, and resulting in an increased dyserythropoiesis and apoptosis of erythroid cells. Little is known about the pathophysiology and erythropoietic abnormalities of HIV-associated anaemia, in children.

Methods & Results
We compared haematological abnormalities among 329 severely anaemic (haemoglobin <5g/dL) Malawian children with (n=40) and without HIV (n=289) infection. Bone marrow flow cytometry revealed that HIV-infected children had fewer CD34+ haematopoietic progenitors (1.0% vs. 1.5%, p=0.04) and erythroid progenitors (0.2% vs. 0.3%, p=0.05). Dyserythropoiesis and apoptosis of red cell precursors were not more common in HIV-infected children than uninfected children (2.8% vs. 3.8%, p=0.12 and 9.3% vs. 12.3 %, p=0.23). Polychromatic erythroblasts (37% vs. 36%, p=0.69), reticulocyte counts (58.6 vs. 52.7 10⁹/L, p=0.85) and peripheral blood erythrocytic indices were similar in both groups.

Conclusions
Among severely anaemic Malawian children HIV infection was associated with decreased numbers of CD34+ haematopoietic progenitors and early progenitor cells. Despite this deficiency in the earliest stages of erythropoiesis, and contrary to previous studies, we could find no evidence of an effect of HIV status on the subsequent viability and maturation of erythrocytes.
INTRODUCTION

Early in the HIV pandemic anaemia was identified as the most common haematological complication in HIV-infected adults and a positive association had been reported between the prevalence of anaemia and severity of clinical disease. Subsequently anaemia has been repeatedly identified as a strong, independent and reversible predictor of mortality in large studies of adults in western settings.

The highest prevalence of HIV and anaemia occurs in tropical countries where over 60% of individuals with these conditions are women and children. Current knowledge about HIV-related anaemia is predominantly based on studies in western men and may not be applicable to children in resource poor settings because of differences in epidemiology and aetiology.

Anaemia in HIV-infected adults results primarily from reduced production of erythrocytes. This reduction is believed to be mediated by alterations in the secretion of cytokines and erythropoietin, and to be influenced by infection, neoplasms, drug toxicity and nutritional deficiencies. There is also a direct effect of HIV on erythropoiesis. In vitro data suggest that increased dyserythropoiesis and apoptosis of erythroid cells caused by HIV may play an important role.

Compared to adults there is very little information available about HIV-associated anaemia in children. Only a few studies have assessed erythropoiesis and haematological findings in HIV-infected children and these studies were either retrospective or lacked a control population for comparison. Published studies used light microscopy but not flow cytometry which can provide information about the early stages of erythropoiesis, and none was undertaken in sub-Saharan Africa.

We have previously reported that HIV infection was more common among severely anaemic Malawian children than in a carefully selected control population (13% vs. 6%, p<0.001). HIV was strongly associated with severe anaemia after adjusting for confounding factors and red cell production failure appeared to be the primary mechanism. The aim of the present study was to determine in severely anaemic Malawian children if HIV infection was associated with reduced erythroid precursor cells, or increased rates of apoptosis and dyserythropoiesis. A further objective was to study the role of cytokines, erythropoietin and plasma vitamin A related to these cellular mechanisms.
MATERIALS & METHODS

Population
The study was conducted in southern Malawi at Queen Elizabeth Central hospital in Blantyre (urban site) and Chikwawa District hospital (rural site) between July 2002 and July 2004. All children with a primary diagnosis of severe anaemia, defined as a blood haemoglobin concentration (Hb) less than 5 g/dl, were consecutively recruited into a prospective study. Inclusion criteria were: age 6-60 months, no blood transfusion within the previous month and informed consent from a parent or guardian. The study was approved by the Ethics Committees of the College of Medicine, Malawi, and the Liverpool School of Tropical Medicine, UK.

Procedures
This study was part of a large case-control study investigating the aetiology of severe anaemia in Malawian children and the admission and clinical management procedures have been described elsewhere. In summary, on enrolment, a standardized study questionnaire and physical examination were completed, and peripheral blood samples were collected. If the clinical condition permitted, a fine needle bone marrow aspirate was obtained under local anaesthesia from the posterior superior iliac spine. Patients were treated in a study ward and all conditions were managed according to standard protocols. Nutritional Z-scores (weight-for-height) were calculated in EPI info and ‘wasting’ was defined as a Z-score <-2.

Laboratory measurements
Routine laboratory tests (haematology, bacteriology and parasitology) were performed within 24 hours and serum and plasma aliquots were stored at -80°C for later analysis. Laboratory staff were unaware of children’s HIV status.

a) Haematology
Whole blood haemoglobin concentration was measured on site using the HaemoCue system (Angelholm, Sweden). A full blood count, including reticulocytes, was performed by Coulter counter analyzer (Beckman Coulter, Durban, South Africa). Bone marrow aspirate smears were stained with May-Grüwald-Giemsa for differential counting and myeloid:erythroid ratio assessment. Dyserythropoiesis was defined by the presence of one or more of the following features: (a) multinucularity; (b) karyorrhexis; (c) intercellular chromatin bridging; (d) incomplete mitoses. Two hundred cells were counted and the number of dyserythropoietic cells were expressed as a percentage of all red cell precursors.

b) Chemistry, microbiology and parasitology
C-reactive protein (CRP) and erythropoietin were determined using a Roche p800/e170 system (Roche, Switzerland). Inflammatory cytokine profiles were measured
by Cytometric Bead Array on a FACS-Calibur flow-cytometer (FACS-Calibur, BD Biosciences, Franklin Lakes, USA). Serum vitamin A (retinol) was measured using high performance liquid chromatography\textsuperscript{22}. Malaria slides were read by two independent readers, with a third reader resolving discrepant results. Malaria infection was defined as the presence of any asexual \textit{P. falciparum} parasites in a thick blood film. HIV testing was performed using two rapid tests (Determine, Abbott-Laboratories, Japan; Unigold, Trinity-Biotech, Ireland). Reactive results in children less than 18 months and all discordant results were resolved by PCR\textsuperscript{23}.

c) Flow cytometry

Bone marrow aspirates were processed within 24 hours, an automated cell count (Coulter counter) was followed by flow cytometry to determine the proportion of erythropoietic cells at different stages of maturation and to quantify the percentage of apoptotic cells. Bone marrow cells were separated using Ficoll-Paque (Pharmacia Biotech Uppsala, Sweden) and incubated with different combinations of the following antibodies and dyes: CD14-PE-Cy5 (Tük4), CD34-FITC and PE (QBEND/10), CD36-PE (CLB-IVC7), CD235a-FITC (CLB-AME-1) (Sanquin Reagents, Amsterdam, The Netherlands), Laser Dye Styril-751 (LDS, Applied Laser Technology Ltd, Maarheeze, The Netherlands), and Annexin V and Propidium iodide (IQ products, Groningen, the Netherlands)\textsuperscript{24}. Cells were read by a four colour flow cytometer adapted with a 488-nm argon laser and a 633-nm red diode laser (FACSCalibur, BD Biosciences, Franklin Lakes, USA). Cell Quest Pro\textsuperscript{TM} software (BD Biosciences, Franklin Lakes, USA), was used for cell acquisition and analysis. Acquisition was stopped after 50,000 events were detected within a pre-determined gate for mononuclear cells, and information on cells was collected for analysis. For each patient, gates were applied twice according to a predefined protocol, by a reader (JCJC) who was blinded for patient characteristics.

\textit{CD34}+ haematopoietic progenitors were defined as all mononuclear bone marrow cells expressing CD34 (Figure 1); \textit{Erythroid progenitor cells} were defined as mononuclear bone marrow cells expressing CD34 and CD36 but not CD14; the more mature \textit{Erythroid precursor cells} were defined as all mononuclear bone marrow cells positively stained with LDS-751 (a DNA dye) and expressing CD235a (Glycoprotein A). Apoptosis was assessed in mononuclear bone marrow cells expressing CD235a. Early apoptosis was defined if cells expressed phosphatidylserine but not propidium iodide whilst in late apoptosis cells expressed both phosphatidylserine expression and propidium iodide. In viable cells neither of these markers was detected\textsuperscript{24}.

Statistical methods

Patient characteristics and haematological variables were compared between HIV-infected and uninfected children using Chi-square and Fisher exact test for categorical data, or student t (normal distribution) and Mann-Whitney U tests (skewed distribution)
for continuous variables. Correlations were assessed using Pearson or Spearman correlation coefficients, when appropriate. A two-sided significance level was set at $p=0.05$. In view of the explorative nature of this study we did not statistically adjust for multiple comparisons\(^\text{25}\). All analyses were preformed in SPSS 12 (SPSS, IL).

![Figure 1: Maturation of erythroid cells and surface marker expression. (see colour section)](image)

Pluripotent stem cells/CD34+ haematopoietic progenitors and erythroid progenitor cells can only be differentiated using surface marker expression. Light microscopy can differentiate between erythroid precursor cells. CFU-GEMM: Colony forming units-granulocyte/erythrocyte/macrophage/megakaryocyte, BFU-E: Burst Forming Units-Erythrocyte, CFU-E: Colony Forming Units-Erythrocyte, GPA: Glycophorin-α (CD235a); LDS: Laser Dye Styrol-751, a DNA dye.

Children aged 6-60 months with Hb<5g/dl

![Figure 2: Flowchart of selection of cases](image)

381 recruited (100%)

- 23 no Bone marrow
- 19 no HIV result

329 included (86%)

- HIV+ 40 Cases
- HIV - 289 Controls

Reasons for not collecting bone marrow samples or performing HIV tests were: severe clinical condition of the child, death prior to collection of samples and no consent (HIV testing only).
RESULTS

381 severely anaemic children were enrolled in the study and complete data were available for 329 (Figure 2). Children were excluded if bone marrow samples or HIV tests were not available or bone marrow quality was poor which was not different amongst HIV-infected and uninfected children (p=0.37). Reasons for not collecting these data were: severe clinical condition of the child, death prior to collection of samples and no consent.

Table 1. Characteristics of HIV-infected and uninfected children with severe anaemia.

<table>
<thead>
<tr>
<th></th>
<th>HIV+</th>
<th>HIV-</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>40</td>
<td>289</td>
<td></td>
</tr>
<tr>
<td>Age median, (IQR) in months</td>
<td>24.9 (15.6-38.4)</td>
<td>15.8 (10.2-25.5)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>n=40</td>
<td>n=289</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Boys</td>
<td>17/40</td>
<td>144/289</td>
<td>0.39</td>
</tr>
<tr>
<td>(43%)</td>
<td>(50%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wasting</td>
<td>6/33</td>
<td>37/261</td>
<td>0.54</td>
</tr>
<tr>
<td>(18%)</td>
<td>(14%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malaria parasitaemia</td>
<td>23/39</td>
<td>170/289</td>
<td>0.99</td>
</tr>
<tr>
<td>(59%)</td>
<td>(59%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRP median, IQR in mg/L</td>
<td>117 (47-193)</td>
<td>95 (42-153)</td>
<td>0.73</td>
</tr>
<tr>
<td>n=38</td>
<td>n=269</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Basic data, disease prevalence and red cell statistics are presented per study group. Wasting was defined as a weight for height Z-score of less than -2. IQR: Inter-Quartile Range; CRP: C-Reactive Protein.

Table 2. Haematological parameters in HIV-infected and uninfected children with severe anaemia using automated counts.

<table>
<thead>
<tr>
<th></th>
<th>HIV+</th>
<th>HIV-</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUTOMATED COUNT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Haemoglobin concentration</td>
<td>3.6 ±0.7</td>
<td>3.6 ±0.8</td>
<td>0.67</td>
</tr>
<tr>
<td>mean ±SD in g/dL</td>
<td>n=40</td>
<td>n=289</td>
<td></td>
</tr>
<tr>
<td>MCV</td>
<td>81.1 ±13.7</td>
<td>83.3 ±15.6</td>
<td>0.27</td>
</tr>
<tr>
<td>mean ±SD in fL</td>
<td>n=35</td>
<td>n=247</td>
<td></td>
</tr>
<tr>
<td>MCHC</td>
<td>32.4 ±3.1</td>
<td>32.5 ±7.2</td>
<td>0.68</td>
</tr>
<tr>
<td>mean ±SD in g/dL</td>
<td>n=35</td>
<td>n=245</td>
<td></td>
</tr>
<tr>
<td>RDW</td>
<td>25.2 ±8.7</td>
<td>24.4 ±7.4</td>
<td>0.50</td>
</tr>
<tr>
<td>mean ±SD in %</td>
<td>n=35</td>
<td>n=246</td>
<td></td>
</tr>
<tr>
<td>Reticulocytes</td>
<td>58.6</td>
<td>52.7</td>
<td>0.85</td>
</tr>
<tr>
<td>median and IQR in 10⁹/L</td>
<td>(30.3-88.2)</td>
<td>(30.2-91.7)</td>
<td></td>
</tr>
<tr>
<td>n=32</td>
<td>n=209</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

MCV: Mean Corpuscular Volume; MCHC: Mean Corpuscular Haemoglobin Concentration; RDW: Red cell Distribution Width. IQR: Inter-Quartile Range, SD: Standard Deviation.
Table 3. Haematological parameters in HIV-infected and uninfected children with severe anaemia using light microscopy and flow cytometric counts.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HIV+ n=40</th>
<th>HIV- n=289</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LIGHT MICROSCOPY</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>MYELOID:ERYTHROID RATIO</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Decreased (&lt;2.0:1)</td>
<td>29/34 (85%)</td>
<td>194/261 (74%)</td>
<td>0.38</td>
</tr>
<tr>
<td>Normal (2.0-4.9:1)</td>
<td>4/34 (12%)</td>
<td>54/261 (21%)</td>
<td></td>
</tr>
<tr>
<td>Increased (&gt;5.0:1)</td>
<td>1/34 (3%)</td>
<td>13/261 (5%)</td>
<td></td>
</tr>
<tr>
<td><strong>ERYTHROID CELLS</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pro-erythroblasts</td>
<td>0.8 (0.0-1.6)</td>
<td>0.4 (0.0-1.5)</td>
<td>0.33</td>
</tr>
<tr>
<td>Basophilic erythroblast</td>
<td>0.8 (0.0-2.4)</td>
<td>0.8 (0.0-1.6)</td>
<td>0.55</td>
</tr>
<tr>
<td>Ortho &amp; Polychromatic erythroblast</td>
<td>37 ±15</td>
<td>36 ±16</td>
<td>0.69</td>
</tr>
<tr>
<td><strong>DYSEREYTHROPOIESIS</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dyserythropoietic cells</td>
<td>2.8 ±2.2</td>
<td>3.8 ±3.0</td>
<td>0.12</td>
</tr>
<tr>
<td><strong>COULTER COUNTER</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CELLULARITY</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nucleated bone marrow cells</td>
<td>64.4</td>
<td>76.8</td>
<td>0.37</td>
</tr>
<tr>
<td>Cellularity</td>
<td>(43.2-112.8)</td>
<td>(45.6-119.6)</td>
<td></td>
</tr>
<tr>
<td><strong>FLOW CYTOMETRY</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CELLS</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All CD34+ haematopoietic progenitors</td>
<td>CD34+ 1.0%</td>
<td>1.5%</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>(0.5-2.0%)</td>
<td>(0.7-3.0%)</td>
<td></td>
</tr>
<tr>
<td>Erythroid progenitor cells</td>
<td>CD34+ 0.22%</td>
<td>0.34%</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>CD36+ (0.08-0.44%)</td>
<td>(0.15-0.66%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CD14- n=27</td>
<td>n=210</td>
<td></td>
</tr>
<tr>
<td>Erythroid precursor cells</td>
<td>CD235+ 17.9%</td>
<td>25.6%</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>LDS+ (13.0-30.8%)</td>
<td>(14.9-38.3%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n=35</td>
<td>n=248</td>
<td></td>
</tr>
<tr>
<td><strong>APOPTOSIS OF ERYTHROID PRECURSORS</strong></td>
<td>CD235+ 87%</td>
<td>85%</td>
<td>0.25</td>
</tr>
<tr>
<td>Viable cells</td>
<td>Annexin- PI-</td>
<td>(73-94%)</td>
<td>(68-91%)</td>
</tr>
<tr>
<td></td>
<td>n=15</td>
<td>n=78</td>
<td></td>
</tr>
<tr>
<td>Early apoptotic</td>
<td>CD235+ 9.3%</td>
<td>12.1% (6.3-22.6%)</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>Annexin+ PI-</td>
<td>(4-19.8%)</td>
<td>n=78</td>
</tr>
<tr>
<td>Late apoptotic</td>
<td>CD235+ 2.1%</td>
<td>2.6% (1.0-5.5%)</td>
<td>0.67</td>
</tr>
<tr>
<td></td>
<td>Annexin+ PI+</td>
<td>(1.2-4.9%)</td>
<td>n=78</td>
</tr>
</tbody>
</table>

Dyserythropoiesis was defined as: (a) multinucularity; (b) karyorrhexis; (c) intercellular chromatin bridging; and (d) incomplete mitoses. Early apoptosis refers to the expression of Phosphatidylserine only, whilst in late apoptosis also Propidium iodide was detected. In viable cells neither of these dies were detected. IQR: Inter-Quartile Range, LDS: Laser Dye Styril-751, stains DNA, PI: Propidium Iodide.
Of the remaining 329 children, 40 (12%) were infected with HIV and their median age was 25 months compared to 16 months for HIV-uninfected children (p<0.01). No significant differences between HIV-infected and uninfected children with regard to gender, wasting, malaria infection and a raised CRP (>10mg/L, Table 1). There were no differences in mean haemoglobin levels (p=0.67) or other erythrocytic indices between both groups (Table 2).

Bone marrow microscopy identified a decreased myeloid/erythroid ratio (<2.0:1) in 85% of HIV-infected and 74% of HIV-uninfected children (p=0.16). Flow cytometric analysis revealed that HIV-infected children had fewer bone marrow CD34+ haematopoietic progenitors, erythroid progenitor cells and erythroid precursor cells when compared with HIV-uninfected children (Table 3). In HIV-infected and uninfected children, nucleated bone marrow cells identified by microscopy as pro-erythroblasts, basophilic erythroblasts and polychromatric erythroblast were similar in the two groups (Table 3). Peripheral blood reticulocyte counts were also similar between HIV-infected and uninfected children (median 58.6 and 52.6 x10⁹/L, p=0.85). Although HIV-infected children were on average 9 months older than uninfected children in this study, correction for age did not alter the associations found (data not displayed).

Dyserythropoietic features were observed in 2.8% and 3.8% of erythroid precursors in HIV-infected and uninfected children respectively (p=0.12, Table 3). The proportions of erythroid precursor cells that were viable and those that were in various stages of apoptosis were similar between the two groups of children (Table 3). The percentage of dyserythropoietic cells was positively correlated with the percentage of red cells undergoing early apoptosis (r= 0.34, p=0.01). There were no correlations (range r=-0.14 – +0.15) between both the proportion of dyserythropoietic or apoptotic cells, and the peripheral blood levels of cytokines TNF-α (p=0.90 and 0.28), IFN-γ (p=0.15 and 0.36), IL-10 (p=0.74 and 0.19), erythropoietin (p=0.22 and 0.83), or vitamin A (p=0.83 and 0.22).

**DISCUSSION**

This study is the first detailed prospective analysis of erythropoiesis using bone marrow samples and flow cytometry in HIV-infected children with severe anaemia. HIV infection was associated with decreased numbers of CD34+ haematopoietic progenitors and early progenitor cells, but normal numbers of later erythropoietic cells.

Although both HIV and anaemia occur frequently in children in tropical countries, and have been found to be associated with a high morbidity and mortality, there have been few published studies of the pathogenesis of anaemia in HIV-infected children.
Circumstantial evidence suggests that, as in adults, red cell production failure is a more important cause of anaemia in HIV-infected children than blood loss or haemolysis. Bone marrow examinations have rarely been carried out in HIV-infected children and most data about anaemia have been derived from retrospective analyses of hospital records which did not include a control group.

In this group of severely anaemic children, HIV-infected individuals were found to have fewer CD34+ haematopoietic progenitors and erythroid progenitor cells in the bone marrow than children without HIV. This supports the hypothesis that red cell production failure is an important cause of severe anaemia in HIV-infected children and may be caused by a reduced CD34+ haematopoietic progenitor capacity. Despite the difference in CD34+ haematopoietic progenitors and erythroid progenitors the proportion of more mature erythroid precursor cells in bone marrow or peripheral blood (reticulocytes) did not differ between the two groups. Hence HIV-uninfected children with severe anaemia appeared to be less productive or efficient in their (subsequent) erythropoiesis than HIV-infected children. This explanation is supported by the trend towards less dyserythropoiesis and apoptosis in HIV-infected children. A finding which is in contrast to other studies which reported dyserythropoiesis more commonly in later stages of HIV disease and concluded that anaemia may related to increased dyserythropoiesis.

HIV infection affects haematopoietic processes, including CD34+ haematopoietic progenitor differentiation, possibly through abnormal expression of cellular genes and cytokines that influence haemopoiesis and especially the African strain of HIV (1C) can directly infect CD34+ haematopoietic progenitors. In this study dyserythropoiesis or apoptosis were not associated with altered cytokine levels in contrast to other reports. Vitamin A deficiency has been associated previously with apoptosis or dyserythropoiesis, although this association was not observed in the present study despite the fact that 90% of children were deficient. More intensive investigations might identify cytokines that may affect regulatory signals which could be therapeutic targets to reduce haemopoietic inhibition in HIV patients.

There were no differences in peripheral blood erythrocytic indices or bone marrow microscopy in HIV-infected compared to non-infected children. This confirms previous reports for Italian children and American children as well as adults. The absence of differences using these common diagnostic tools may relate to the anaemia in these HIV-infected and uninfected children being multi-factorial in aetiology, as is so commonly observed in African children.

The study results were not effected by anti-retroviral therapy (ART), known to exacerbate blood and bone marrow abnormalities, as ART was not routinely available in Malawi at the time of the study. The fact that not all tests were done on all children is a study limitation.
limitation, although the large sample size available increases confidence that the study sample was representative.

In conclusion the findings in these severely anaemic Malawian children indicate that despite an HIV-associated deficiency in early red-cell precursors, subsequent erythropoiesis is at least as efficient in HIV-infected as in HIV-uninfected children with severe anaemia.
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Chapter

Severe anaemia is not associated with HIV-1 env gene characteristics in Malawian children

Job CJ Calis, Hellen P Rotteveel, Antoinette C van der Kuyl, Fokla Zorgdrager, David Kachala, Michaël Boele van Hensbroek, Marion Cornelissen

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ABSTRACT

Background
Anaemia is the most common haematological complication of HIV and associated with a high morbidity and a poor prognosis. The pathogenesis of HIV-associated anaemia is poorly understood and may include a direct effect of HIV on erythropoiesis. In vitro studies have suggested that specific HIV strains, like X4 that uses the CXCR4 co-receptor present on erythroid precursors, are associated with diminished erythropoiesis. This co-receptor affinity is determined by changes in the hypervariable loop of the HIV-1 envelope genome. In a previous case-control study we observed an association between HIV and severe anaemia in Malawian children that could not be fully explained by secondary infections and micronutrient deficiencies alone. We therefore explored the possibility that alterations in the V1-V2-V3 fragment of HIV-1 were associated with severe anaemia.

Methods
Using peripheral blood nucleic acid isolates of HIV-infected children identified in the previous study we assessed if variability of the V1-V2-V3 region of HIV and the occurrence of X4 strains were more common in HIV-infected children with (cases, n=29) and without severe anaemia (controls, n=30). For 15 cases bone marrow isolates were available to compare against peripheral blood. All children were followed for 18 months after recruitment.

Results
Phylogenetic analysis showed that HIV-1 subtype C was present in all but one child. All V1-V2-V3 characteristics tested: V3 charge, V1-V2 length and potential glycosylation sites, were not found to be different between cases and controls. Using a computer model (C-PSSM) four children (7.8%) were identified to have an X4 strain. This prevalence was not different between study groups (p=1.00). The V3 loop characteristics for bone marrow and peripheral blood isolates in the case group were identical. None of the children identified as having an X4 strain developed a (new) episode of severe anaemia during follow up.

Conclusions
The prevalence of X4 strains in these young HIV-1-subtype-C-infected children that were most likely vertically infected and naïve to anti-retroviral therapy can be considered high compared to previous results from Malawi. It is unlikely that V1-V2-V3 fragment characteristics and HIV co-receptor affinity is an important feature in the development of severe anaemia in Malawian children.
BACKGROUND

Anaemia is the most common haematological complication of HIV in adults and children worldwide 1-3 and is associated with a reduced quality of life and a high morbidity 4. Inadequate erythropoiesis is generally considered to be the main pathophysiological mechanism of HIV-associated anaemia 1;5;6. Despite the obvious medical importance of anaemia the aetiology of this erythropoietic failure is still not well understood. Several possible pathways have been investigated including opportunistic infections, micronutrient deficiencies and the more recently identified direct effect of HIV on erythropoiesis 3;7. The induction of anaemia by specific strains of HIV is an example of this direct effect of HIV 8.

Early in infection the HIV-1 population usually consist of a strain that has the capacity to bind to both CD4 and the co-receptor CCR5 (R5 strain)9;10. Later in infection a broadening or switch occurs and HIV evolves to infect cells expressing CD4 and the co-receptor CXCR4 (R4 strains) 10;11. This switch is thought to occur in 50% of infections and is associated with an accelerated loss of CD4+ T-cells and progression to AIDS 12.

Like the T-helper cells, erythropoietic stem cells express both CD4 and CXCR4 on their membrane13;14. Although productive infection is uncommon in erythroid precursor cells 3, several in vitro studies have associated X4 strains with cell death in erythroid and other cell lines 7;15-17. Another similarity between T-helper cells and erythroid cells is the decline in both cell types during disease progression 3;7. Large studies have suggested that anaemia might even be a better predictor of mortality than loss of CD4 cells or HIV load increase 5;18;19. A decline of 1 g/dL in the haemoglobin concentration was associated with a greater increased hazard of death than a halving of the absolute CD4 count or a log increase in viral load 18. The reversal of anaemia, again similar to an increase in T-helper cells, was associated with a better life expectancy 18;19. Despite these similarities to T cells, no study has evaluated if the decrease in erythrocytes might be a direct or indirect consequence of an alternated co-receptor affinity 16.

Co-receptor affinity is a consequence of changes in the variable loops (V1-V2-V3) of the envelope protein (env) of HIV-1. Especially a high V3 amino acid charge was found to be associated to X4 co-receptor affinity 20-24 and several models have been published to predict co-receptor affinity using V3 data 25-28. Changes to the V1-V2 fragment appear to be more indirectly linked to X4 affinity and are increasingly associated with a neutralizing antibody escape 29-37. These changes include an increased number of potential N-linked glycosylation sites in V1-V3 30-35 and possibly an extended length of the (V1-)V2 fragment 29;36;37.
In a recent case-control study we found a strong association between HIV and severe anaemia in African children. This association could partly be explained by opportunistic infections with micronutrient deficiencies playing only a modest role. After correction for this an independent association remained which can be explained by a direct effect of HIV on erythropoiesis. We hypothesized that the occurrence of X4 strains and other alterations of the hypervariable loops V1-V2-V3 of the HIV-1 *env* genome would be associated with severe anaemia. Using nucleic acid isolates of HIV-infected children with (cases) and without (controls) severe anaemia we assessed the prevalence of: (1) high V3 and total V1-V2-V3 amino acid charge; (2) an extended V1-V2 length; (3) an increase of potential N-linked glycosylation sites of the V1-V3 and V3 fragments; (4) a HIV-1 subtype C position specific scoring matrix (C-PSSM) that predicts co-receptor usage.

**METHODS**

In two hospitals in southern Malawi we recruited two groups of children aged 6-60 months into a case-control study on severe anaemia (Haemoglobin concentration < 5 g/dL) as previously described. In short, a severely anaemic child requiring a blood transfusion (Case, Haemoglobin concentration < 5 g/dL) was recruited at presentation to hospital alongside two controls (Haemoglobin concentration ≥ 5 g/dL). One control was recruited from apparently healthy residents living within proximity of the case-patient (Community Control, CC) the other randomly selected at the outpatient department (Hospital Control, HC). On enrolment, a standardized study questionnaire and physical examination were completed, and blood samples were collected. In cases only, a bone marrow aspiration was performed under anaesthesia if the clinical condition permitted. Nutritional Z-scores were calculated in EPI info 2000. ‘Wasting’ (weight-for-height), applied to children with Z-scores < -2. Children requiring admission were treated in a study ward. All conditions were managed according to standard protocols. All three study groups (cases, HC and CC) were actively followed at 1, 3, 6, 12 and 18 months. In addition, children were passively followed by asking guardians to return to study clinics whenever the child was sick. During follow-up visits clinical data was collected and a peripheral blood haemoglobin concentration was determined. Fully informed consent was obtained from a parent or guardian in all three study groups. HIV testing was discussed after transfusion for the cases and on a follow-up visit for controls. The study was approved by the ethics committees of the College of Medicine, Malawi, and the Liverpool School of Tropical Medicine, UK.

**Laboratory tests on site**

Haemoglobin was measured on site using a Hemocue system (Angelholm, Sweden). A full blood and reticulocyte count was performed by Coulter counter (Coulter, Hialeah, Fla).
C-reactive protein (CRP) was analyzed in heparin plasma on a Roche p800/p170 system (Roche, Switzerland). HIV testing was performed using two rapid tests (Determine, Abbott-Laboratories, Japan; Unigold, Trinity-Biotech, Ireland). Reactive results in children less than 18 months and discordant outcomes were resolved by PCR. Lymphocyte subsets including CD4 cell counts were measured, during the second year of recruitment, by adding 50µl of whole blood to TRUECOUNT absolute count tubes (Becton Dickinson, USA) and incubated with 20µl MultiTest reagent. After incubation and red cell lysis, the cells were analysed on a Becton Dickinson FACSCalibur flow cytometer and analysed using MultiSet software (Becton Dickinson, USA). CD4 expressing T-cells were expressed as percentage of the total lymphocyte population and age adjusted cut-offs were used to define immunodeficiency. Peripheral blood samples were separated and aliquots of serum and plasma were stored at -80°C for later testing.

**DNA extraction and polymerase chain reaction (PCR)**

DNA was isolated from the blood and bone marrow samples with a silica-guanidiniumthiocyanate based method. The target DNA (env gene) was amplified using polymerase chain reaction (PCR) with a pair of target-specific sense and antisense primers (Figure 1) in a 96-well 9700 thermocycler (Applied Biosystems, USA). First a reverse transcriptase (RT)-PCR was performed using 10 µl of the eluated nucleic acid solution and Avian Myeloblastosis Virus reverse transcriptase (AMV-RT, Boehringer Mannheim) to generate cDNA. Five microliter of the PCR product was used with Amplitaq (Applied Biosystems, USA) to amplify the V1-V2-V3 env (810 bp) gene fragment, or in case this failed a combination of nested PCRs was performed to amplify this region as fragments (primers Figure 1).

**Sequencing and cloning**

PCR products were directly sequenced with the ABI Prism Big-dye Terminator v 1.1 Cycle Sequencing Kit in an ABI Prism 377 DNA sequencer using multiple fluorescent dyes (Applied Biosystems, USA). PCR fragments showing evidence of recombinants or a dual infection were cloned with TA TOPO cloning kit (Invitrogen, USA). For each sample at least eight clones were sequenced and from these a consensus sequence was made. All sequences were manually analysed and assembled with CodonCode Aligner (version 1.5.2.). Multiple sequences were aligned with CLUSTAL W and optimised manually with BioEdit Sequence Alignment Editor (version 7.0.1).

**Viral Load determination**

The HIV-1 viral load in plasma was determined with an in-house real-time PCR assay, with primers located in the HIV-1 pol gene. Primer/probe sequences were: upstream primer 5’TGC ATT YAC CATACC TAG T 3’, downstream primer 5’ATT GCT GGT GAT CCT TTC CA 3’, and probe 5’AAA CAA TGA GAC ACC AGG GAT TAG ATA 3’.
The probe was 6-FAM labelled. The detection limit of this assay was 5 HIV-1 RNA copies per reaction.

Phylogenetic analysis

Genetic subtypes were determined by phylogenetic analysis. The env gene sequence fragments were aligned with the corresponding reference sequences obtained from the Los Alamos HIV sequence database. Phylogenetic reconstruction was carried out with Molecular Evolutionary Genetics Analysis (MEGA) software version 3.0. A model based upon the Kimura two-parameter model with pair wise deletion was used. Bootstrap values were based on a generation of 1000 replicate trees. Newly found recombinant forms were analysed using Simplot (version 3.5.1.0.) comparing recombinant sequences against a background of reference sequences.
Potential N-linked glycosylation sites and Amino acid charges
All protein fragments containing Asparagine (N) and Serine (S) or Threonine (T) that were separated by any third amino acid (X) other than Proline (P) were counted as a glycosylation site (N-X-S/T)\(^4^4\). All possible glycosylation sites and the amino acid charges were counted for the V3 and V1-V2-V3 fragment separately.

Subtype C-specific phenotype predictor (C-PSSM)
A C-PSSM predictor is available online\(^4^5\). This predictor is based on the computational techniques presented in the paper from Jensen and colleagues\(^2^5\).

Statistics
We compared characteristics of HIV-infected cases to HIV-infected controls using the Fisher exact and Chi-square test (categorical data) and the t-test or Wilcoxon rank sum test (continuous data). For 2XN contingency tables the Fisher-Freeman-Halton exact test was used. For all tests a two-sided alpha of <0.05 was used to assess significance. The tested sample size would be able to detect an odds ratio of 8 or more assuming an X4 strain prevalence of 8% in the control population, a power of 80% and an alpha of 0.05. Analyses were performed using SPSS 12.0 (SPSS inc, USA) and StatsDirect 2.6 (StatsDirect ltd, UK).

RESULTS

Patient Characteristics
The current study was embedded into a larger case-control study that followed children for an additional 18 months. That study recruited 381 children with severe and 757 children without severe anaemia over a two year period. Overall 1039 (91%) parents consented to HIV testing, 57 (5.0%) refused and 42 (3.7%) were lost to follow up before Table 1.

<table>
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<tr>
<th>Characteristics</th>
<th>Cases</th>
<th>Controls</th>
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<tr>
<td>HIV-infected</td>
<td>45</td>
<td>41</td>
<td>n/a</td>
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<tr>
<td>Age (mean in months ± SD)</td>
<td>25.5 ± 14.1</td>
<td>28.8 ± 12.4</td>
<td>0.25</td>
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<tr>
<td>Sex (M:F)</td>
<td>18:27</td>
<td>23:18</td>
<td>0.14</td>
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<tr>
<td>Haemoglobin (mean in g/dL ± SD)</td>
<td>3.6 ± 0.7</td>
<td>9.3 ± 2.0</td>
<td>n/a</td>
</tr>
<tr>
<td>Reticulocytes (Median and IQR *10e9/L)</td>
<td>58.6 (30.3-93.1) n=35</td>
<td>55.7 (34.7-86.0) n=31</td>
<td>0.72</td>
</tr>
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<td>CRP&gt;10mg/L</td>
<td>38/42 (90%)</td>
<td>25/36 (69%)</td>
<td>0.02</td>
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Baseline characteristics of children with severe anaemia (cases, Hb<5.0 g/dL) as compared to those without severe anaemia (controls). SD: Standard deviation; IQR Inter Quartile Range; The number of children is only displayed if data was not available for all children.
counselling. Of all children tested 45 (13%) of severely anaemic cases and 41 (6%) non-severely anaemic controls were HIV-infected (p<0.001). These 86 children formed the current study population and their baseline characteristics are described in Tables 1 and 2. Haemoglobin levels were lower in cases than controls but reticulocyte counts were not different between the study groups (p=0.72). Of all HIV-infected children,

<table>
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<th>Table 2. Markers of disease progression and outcome study group.</th>
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<td>Wasting (Z-score weight for height &lt;-2)</td>
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<td>Previous hospital admission</td>
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<td>Mortality (18 months)</td>
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<td>During admission</td>
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<td>After discharge</td>
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<td>Plasma viral load (mean ± SD in Log10/mL)</td>
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<td>Severe immunodeficient (based on CD4*)</td>
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Markers of disease progression in children with severe anaemia (cases Hb<5.0 g/dL) as compared to those without severe anaemia (controls). SD: Standard deviation; Viral loads were performed using a in-house methods as described in the method section. *According to the revised WHO guidelines CD4 <25% (6-12.months); <20% (12–35 months); <25% (36–59 months).40.

cases were more likely to have been admitted to hospital in the past, were more wasted, immunodeficient for age and had higher plasma viral loads. Though the latter two results were only tested in a subset of children, none of these differences reached significance (Table 2). During follow-up, HIV-infected children with severe anaemia had a significant increased mortality (53%) compared to those without severe anaemia (15%).

Sample Processing
Overall 29 (64%) of HIV-infected cases and 30 (73%) of HIV-infected controls had samples available for further analysis of the env fragment (Table 3). For one patient the PCR failed, resulting in a recovery rate of 98%. For seven other samples the env fragment

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<th>Table 3. Variability and predictors of co-receptor affinity per study group</th>
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<td>Available for testing</td>
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<tr>
<td>PCR failed</td>
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<tr>
<td>Complete env product</td>
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<tr>
<td>Degenerate base counts (median, range)</td>
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<tr>
<td>Viral type C</td>
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<tr>
<td>V3 amino acid charge&gt;+5*</td>
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<td>X4 strains (C-PSSM)*</td>
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Presence of several indicators of co-receptor affinity in children with severe anaemia (cases, Hb<5.0 g/dL) as compared to those without severe anaemia (controls). * according to C-PSSM score by Jensen, available at45.
Figure 2. Phylogenetic tree based on the V1-V2 fragment of the HIV-1 *env* gene nucleotide sequences. Phylogenetic tree to assess if the HIV-1 genome of children with severe anaemia (cases, Hb<5.0 g/dL) is different from those without severe anaemia (controls). Subtype A and CRF-13 reference sequences are used as outgroup sequence.
Figure 3. Distribution of V3 amino acid charges per study group.

Distribution of the nett charges of the V3 fragment in children with severe anaemia (cases, Hb<5.0 g/dL) as compared to those without severe anaemia (controls). The distribution is expressed as a percentage of the total number of codons analysed per study group (Controls: n=28, Cases n=26). Error bars express 95% Confidence intervals. p=0.19.

Figure 4. Distribution of total amino acid charge of the V1-V2-V3 fragment per study group.

Distribution of the nett charges of the V1-V2-V3 fragment in children with severe anaemia (cases, Hb<5.0 g/dL) as compared to those without severe anaemia (controls). The distribution is expressed as a percentage of the total number of fragments analysed per study group (Controls: n=26, Cases n=25). Error bars express 95% Confidence intervals. p=0.36.

Figure 5. Distribution of potential N-linked glycosylation sites on V1-V2-V3 per study group.

Distribution of number of potential N-linked glycosylation sites on the V1-V2-V3 fragment in children with severe anaemia (cases, Hb<5.0 g/dL) as compared to those without severe anaemia (controls). The distribution is expressed as a percentage of the total number of fragments analysed per study group (Controls: n=25, Cases n=26). Error bars express 95% Confidence intervals. p=0.75.
could only be partially amplified (V3 failed: $n=5$ and C2 failed: $n=2$). Cloning was needed to determine consensus sequences for 14 samples. In one sample V1-V2 fragment the nucleotide differences were too large between clones, two consensus sequences were made and separately analysed. However V3 loops of these clones were found to be identical. Degenerate base counts were taken into account and were not different between cases and controls ($p=0.33$, Table 3).

**Phylogenetic analysis**

Phylogenetic analysis of V1-V2 nucleotide sequences showed that 57 of 58 isolates clustered with subtype C reference sequences (bootstrap value: 99%, Figure 2). Only one child, a child recruited as control, had a new circulating recombinant form that showed similarity with CRF13-cpx, which has genomic regions identified as subtypes A, G, and J 46.

**Additional File 1.** Frequency amino acid codon logos of the V3 loop per study group. (see colour section)

Sequence logos of HIV-1 V3 sequences found in children with severe anaemia (cases, Hb<5.0 g/dL, $n=25$) as compared to those without severe anaemia (controls $n=28$). The character and size of each logo represent the amino acid and its prevalence at the specific site.

**Amino acid charges V fragments**

Amino-acid charges of the V3 loop and the total V1-V3 fragment are displayed in Figure 3 and 4 respectively. The charges of the subtype C isolates ranged from +2 to +5 (V3) and from +1 to +7 (V1-V3) and were not significantly different between the study groups ($p=0.19$ and 0.36 respectively). Higher V3 charges (+5 or more) were found in four isolates tested (7.7%), all in controls (Table 3). Additional file 1 shows the amino acid logos for the V3 loop per study group. Alterations of the amino acid in positions, 5-9, 11 and 25 of the V3 loop were not different ($p>0.2$ for all).
Potential N-linked glycosylation sites
All isolates contained one potential N-linked glycosylation site in the V3 loop. The number of potential glycosylation sites on the V1-V3 fragment ranged between 10 and 17 (Figure 5) and was not different amongst cases and controls (p=0.75).

Length of the V1-V2 fragment
The distribution of the length of the V1-V2 fragment ranged from 177 to 255 base pairs and is displayed in Figure 6. Mean length for cases was not significantly different from controls (214 vs. 211 base pairs respectively, p=0.55).

C-Position Specific Scoring Matrix
C-PSSM scores were used to predict co-receptor affinity for HIV-1 subtype C strains using V3 loop data. Within our study X4 tropism was found in 2 of 25 (8.0%) cases and 2 of 27 controls tested (7.4%, p=1.0, Table 3). Both C-PSSM results for the child with two different clones suggested identical co-receptor affinity (CCR5). One of four children identified with an X4 strain according to this method had previously received a blood transfusion.

Bone Marrow isolates
Some studies have suggested different compartments of the body may contain different viral strains. For 15 cases bone marrow samples were available. Synonymous changes in the amino acid sequences of the V3 fragments between bone marrow and peripheral blood isolates were observed in three children (20%). These patients showed alterations of amino acid structure at position 25 (n=2, both e->d), and 29 (n=1, n->d), changes that did not effect the C-PSSM prediction.
Follow-up
After recruitment all children were followed during an 18 month follow-up period as part of this study. During this follow-up period none of the four children (two cases and two controls) that were infected with an R4 virus were diagnosed with a (new) episode of severe anaemia. The two cases died during follow up at 65 and 182 days from recruitment from other causes than severe anaemia.

DISCUSSION
Severe anaemia is major cause of morbidity and mortality in African children. We previously reported that in an urban environment as many as 21% of children admitted with severe anaemia were HIV-infected which was more than twice as prevalent as for the control population. This association could only partly be explained by more prevalent secondary infections and an additional modest contribution of nutrient deficiencies. A direct effect of HIV on erythropoiesis was suspected and we assessed if HIV-1 envelope characteristics and co-receptor affinity were associated with the occurrence of severe anaemia in these children. In this first study to assess this association using a case-control design, we found no variations in the genetic domain of HIV env or the predicted prevalence of X4 strains between children with and without severe anaemia.

HIV-1 subtype C appeared to be the most prevalent subtype in our sample. Previous reports on adults and children in the region have confirmed that in Malawi HIV-1 subtype C is the most prevalent variant. A new recombinant form was identified in one of the control children and showed similarities to CRF13-cpx, recently found in Cameroon. Since this strain is very different from all other isolates found in Malawi, it might have spread from Central Africa by the major transports route running through the rural area in which this was found.

The phylogenetic tree based on the data from the env protein did not show clustering of cases as compared to controls and argues against our hypothesis that specific strains of HIV would predispose to the development of severe anaemia. More specifically we assessed V3 amino acid charge, overall V1-V2-V3 fragment charge, potential N-linked glycosylation sites on the V1-V2-V3 and the V3 fragments, and V1-V2 length and did not identify any significant difference between HIV-infected children with and without severe anaemia.

Although much has been published on co-receptor affinity for HIV-1 subtype B, the strain commonly found in western settings, relatively little is known on HIV-1 subtype C and co-receptor affiliation. Recently Jensen et al. published a validated algorithm to
predict co-receptor usage in subtype C. This C-PSSM score predicted X4 affinity in four strains, two in each study group.

The X4-strain prevalence of 7.7% in the case and control groups combined was higher than expected in this predominantly HIV-1 subtype C infected population. Both studies reporting on co-receptor usage in Malawian populations did not identify a single X4-strain in both an adult and paediatric population. Others have published higher prevalences of X4-strains in HIV-1 subtype C-infected patients, however these reports came from Zimbabwe and South Africa and concerned adults in end-stage HIV infection (17-36%). Furthermore, this prevalence is remarkable since most children will have been vertically infected which is commonly considered to occur by R5 variants. Previous studies have suggested that the occurrence of X4 strains in subtype C infections was associated with the use of anti-retroviral therapy (ART). No child was receiving ART at the time of this study.

The link between the occurrence of an X4 strain and disease progression is well studied in HIV-1 subtype B-infected persons. Little data is available concerning this association in HIV-1 subtype C-infected individuals. Our results suggest X4 variants are not uncommon in a HIV-1 subtype C-infected population and their survival, especially in the presence of anaemia, may be limited. Therefore more attention should be given to the clinical importance of the occurrence of this variant in HIV-1 subtype C infected children and adults.

A limitation of our study is that we used indirect measures to define co-receptor affinity rather than to assess actual infectivity of cell-lined expressing CXCR4 or CCR5. Since the C-PSSM method applied had a 94% specificity and 75% sensitivity, we might have underestimated X4 tropism in our entire population. Since this underestimation would have affected both our cases and controls it is unlikely to have had a major impact on our findings. We therefore did not pursue assessing our hypothesis using the more costly and laborious exercise of infecting cell lines expressing CXCR4 and CCR5. Although our data cannot fully refute an association between X4 strains and severe anaemia, X4 tropism is not a major cause of severe anaemia.

The study had several strengths including the case-control design and the long term follow-up period. This allowed a cross-sectional analysis of X4 infected children with a longitudinal assessment. We hypothesized that the occurrence of an X4 strain would predispose to severe anaemia. The cross-sectional design used may have been underpowered to detect a difference finding only four X4-infected children. However none of these four children developed a new episode of severe anaemia in the longitudinal study. This may be considered additional evidence against our hypothesis. Another strong point of the study was the availability of bone marrow samples in a subgroup
of patients. The similarity of these isolates to those obtained from the peripheral blood argues against compartmentalisation in the bone marrow and strengthens our findings.

CONCLUSIONS

In summary, we assessed whether HIV-1 env characteristics and CXCR4 co-receptor affinity were associated with the occurrence of severe anaemia in Malawian children. In this first study assessing clinical relevance we were unable to find any differences either by phylogenetic analysis and several tests used to assess co-receptor usage. We identified a relatively high prevalence of X4 strains in these HIV-1 subtype C-infected children that were young, most likely vertically infected and naive to anti-retroviral therapy. More attention should be given to the clinical importance of the occurrence of this variant in HIV-1 subtype C infected children and adults.
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Severe anaemia
Severe anaemia is a major public health problem in sub-Saharan African children, responsible for many hospital admission and deaths. The aetiology is complex and as a consequence poorly studied. HIV may be amongst these causes, however little data on the association between severe anaemia and HIV in children has been published. The aim of this thesis is to investigate these causes and to understand their pathogenesis in order to identify possible targets to prevent and treat (HIV-associated) severe anaemia.

Severe anaemia aetiology
In Chapter 2, a large case control study identifying potential aetiological factors for severe anaemia in Malawian children is described. Bacteraemia, malaria, hookworm, HIV infection, G6PD, deficiency of vitamin A or vitamin B12 were significantly associated with severe anaemia. Folate deficiency, sickle cell disease, Parvo B19 infection and laboratory indicators of an abnormal inflammatory response were uncommon. Iron deficiency occurred significantly less frequently in case-patients and was inversely associated with bacteraemia. The majority of hookworm infections were found in children aged less than two years.

These results provide new insights into the aetiology and of severe anaemia in children living in this area of sub-Saharan Africa. Current WHO guidelines focus on treatment of malaria and hookworm, in children over two years of age, and supplementation with iron and folic acid. These guidelines are mostly derived from data in adults or studies in children which have focussed on investigation of single aetiologies. Our results suggest that folate supplementation is less important than treatment of other causes or potentially harmful co-morbidities such as vitamin B12 deficiency, bacteraemia or hookworm infections in young children. The importance of these findings has not been previously recognised in children and the study emphasises the need to complete comprehensive assessments of anaemia co-morbidities. The data presented in this thesis is the first study in African children to undertake such a comprehensive assessment.

An unexpected and important finding which provides a critical new insight was the negative association between iron deficiency and severe anaemia. The structural equation model in Chapter 2 helps to explain this finding by indicating that iron deficiency was negatively associated with bacteraemia. This supports the hypothesis that iron deficiency protects against infection by creating an unfavourable environment for bacterial growth. It is also in agreement with observations of increased morbidity and mortality in children receiving iron supplementation in areas where bacterial infections are common. Although iron supplementation does help to prevent anaemia, it has recently been associated with increased morbidity and mortality in children in a population based supplementation trial in Pemba island Tanzania which was stopped prematurely by the Data Safety Monitoring Board because of the increased mortality risk. In severe malaria
anaemia iron supplementation has been shown to have no haematological benefits as well as increase morbidity risk⁶. It is likely that routine iron supplementation in children living in sub-Saharan Africa can prevent mild anaemia⁷; however its role in preventing live-threatening severe anaemia in populations with a high infectious pressure is less clear and may incur substantial health risk.

To assess these associations of causation of severe anaemia and its consequences, or comorbidities, properly powered intervention studies are essential. As several of these potential aetiological factors interact methodologies are required to unravel their associations (Chapter 2, structural equation model), and as many children suffer from more than one condition at a time (Chapters 2 and 3) longitudinal studies are also required to describe the natural history of anaemia. Perhaps because of the complexity of undertaking such studies several investigators have focussed on supplementation trials to prevent severe anaemia focussing on a single aetiology⁸⁻⁹. An example of this is the vitamin A supplementation trial in Papua New Guinea. Children receiving vitamin A supplements had reduced incidence of malaria without any improvement in haemoglobin⁸. Our results suggest that preventing anaemia using a single intervention may be an oversimplification of the biological processes underlying the condition and as a consequence will have limited success. The way forward to develop preventive strategies for severe anaemia is to evaluate combined intervention strategies based on a detailed understanding of pathogenesis and natural history.

We have concluded that current recommendations promoting iron and folate-supplementation and ignoring bacteraemia and vitamin B12 deficiency may not be applicable in severely anemic children. Even in the presence of malaria parasites, additional or alternative causes of severe anaemia should be considered, and without a more comprehensive approach limited effectiveness will be achieved with the current policy of prevention.

Pathogenesis of severe anaemia

The pathogenesis of anaemia is complex because several distinct mechanisms may lead to a reduced number of circulating red cells. In African children, mechanisms known to contribute to severe anaemia include haemolysis (intra or extra vascular), acute or chronic blood loss, and red cell production failure¹⁰. Each of these mechanisms may be activated by a variety of aetiological factors and single aetiologies may affect more than one mechanism¹¹⁻¹². Furthermore, a single aetiology may predominate in some patients, while in others multiple aetiologies and mechanisms may combine to result in severe anaemia.

In Chapter 3 we have applied a new approach to the assessment and description of severe paediatric anaemia by identifying both the mechanisms and aetiologies associated with
severe anaemia in Malawian children. Red cell production failure was the most important mechanism and was identified in 48% of cases, haemolysis occurred in 22% and direct or indirect evidence of blood loss was found in 7%. Infections were common as 59% had one, and 17% had two or more of the four infections which have previously been associated with severe anaemia (HIV, *P.falciparum* malaria, hookworm or bacteraemia). No differences in the prevalence of these mechanisms were observed amongst children suffering from either infections or those with micronutrient deficiencies.

These findings support the view that malarial anaemia is not primarily a consequences of increased destruction of red blood cells\textsuperscript{13}. They also demonstrate that, especially in HIV-infected children with severe anaemia, co-infections are common (84%). This is the first report to confirm that in children HIV-associated anaemia may be a consequence of decreased red cell production as has previously been reported in adults\textsuperscript{14-17}.

We have produced an assessment of the syndrome of severe anaemia in these children which shows that red cell production failure is the principle underlying mechanism which occurs in the presence of these various co-morbidities. Preventive strategies need to take this finding into consideration in order to promote ways of enhancing red cell production which, in severely anaemic children in this population, are unlikely to be based on single (micronutrient) initiatives.

**Long-term outcome of severe anaemia**

In different geographical settings in sub-Saharan Africa the in-hospital mortality of paediatric severe anaemia ranges between 8-17%\textsuperscript{12,18,19}. Investigators in Kenya reported an unexpectedly high post-discharge mortality and recurrence of severe anaemia in children within two months of their severe anaemia episode\textsuperscript{20}. There is a need to confirm these findings as the excess risk of death in this large group could contribute an important and potentially preventable component of the high mortality of young African children.

In **Chapter 4** we described the long term outcome of children recruited in the case-control study of severe anaemia. In severely anaemic children the in-hospital mortality was 6.4%. In severely anaemic children who were discharged from hospital the additional mortality during the 18-month follow-up period was 12.6%, which was significantly higher than in the hospital controls (2.9%) or community controls (1.4%), (p<0.001). The incidence of a (new) episode of severe anaemia during the follow-up period among the cases was 80 per 100 person-years, which was significantly higher than the rate of 5 per 100 person-years in both control groups combined (p<0.001).

HIV infection had the highest risk estimate for mortality (HR 10.5, 95% CI 4.0-27.2). Mortality was 60% among HIV-infected compared to 11% among HIV-uninfected severely anaemic children (p<0.001). Severe anaemia was significantly associated with
increased mortality in HIV-infected cases compared to controls (60% vs. 15%, p<0.001, Chapter 4). Currently ‘unexplained moderate anaemia (Hb<8g/dL)’ is a stage III criterion for commencing ART (WHO)\textsuperscript{21}, but in practice this is often not used in deciding which children should be started on ART. If the finding of the very high HIV-related case fatality rate in severely anaemic children is confirmed in other studies, it may be appropriate to consider including severe anaemia in the criteria for stage IV disease in the WHO paediatric HIV staging system.

These data provide disturbing evidence of the consequences of severe anaemia on child health and survival. It is commonly thought that most deaths due to severe anaemia result from in-hospital mortality\textsuperscript{22}. This study shows that there is an even higher mortality post-discharge. As severe anaemia is very common\textsuperscript{19}, the impact on overall under-five mortality is likely to be considerable. Increased attention to the prevention and management of severe anaemia in African children is urgently needed if the fourth Millennium Development Goal – a significant reduction in child mortality – is to be achieved. In HIV-infected children severe anaemia should be considered an important predictor of mortality and future research should focus on the potential use of severe anaemia as a stage IV criterion in the WHO paediatric HIV staging system.

**HIV and severe anaemia**

Early in the HIV-pandemic anaemia appeared to be the most common haematological complication of HIV in infected adults\textsuperscript{23}, and a positive association had been reported between prevalence of anaemia and severity of clinical disease\textsuperscript{24}. Subsequently anaemia has been repeatedly identified as a strong, independent and reversible predictor of mortality in large studies in western settings\textsuperscript{25-27}. In contrast to adults there is very little information available about the association between HIV infection and anaemia in children. This situation is not likely to improve because in western countries, which generate most of the research on this topic, paediatric HIV infection is declining\textsuperscript{28}.

In Chapter 2 we outlined results which showed that HIV infection occurred in 13% of cases and 6% of controls. The attributable risk of HIV for severe anaemia was 6.2% for both settings combined and 15% in the urban setting. To further study the association between HIV and (severe) anaemia in children, a systematic review was undertaken on this topic. In Chapter 5 an overview is provided of the data on HIV-associated anaemia in children worldwide. This meta-analysis of anaemia prevalence and incidence data suggested that mild (haemoglobin <11 g/dl) and moderate (Hb <9 g/dl) anaemia were more prevalent with HIV infection and mean haemoglobin levels were lower. These differences were observed for both western and tropical settings. Anaemia incidence ranged between 0.41-0.44 per person-year. There was limited data available for severe categories of anaemia (Hb <7 g/dl or <5 g/dl).
We further reviewed data available relevant to the development of curative or preventive strategies. Failure of erythropoiesis appeared to be the most important mechanism causing anaemia in HIV-infected children. This conclusion is based on descriptive evidence showing that other pathogenetic mechanisms (blood loss\textsuperscript{29-35} or hemolysis\textsuperscript{31,35-38}) were not prominent features in HIV-infected children with anaemia. Only a few studies have directly assessed erythropoiesis and haematological findings in HIV-infected children\textsuperscript{31,33-35} and these studies were either retrospective\textsuperscript{31,34,35} or lacked a suitable control population for comparison\textsuperscript{33-35}.

Therapeutic options include highly-active-antiretroviral-therapy\textsuperscript{25,39}, and prevention or treatment of secondary infections. Erythropoietin can improve anaemia in children\textsuperscript{40}, but this has not been evaluated in tropical settings, although low values are reported in African children\textsuperscript{41,42}. Little data was available on the preventive effectiveness of supplementation with micronutrients other than iron. However, the available data does not suggest that routine supplementation may be an effective intervention to reduce anaemia in HIV-infected children though it may be helpful in individual children.

Definitive evidence on the contribution of iron deficiency to the anaemia of HIV-infected children and the effects and possible harm of iron supplementation\textsuperscript{5,43,44} was lacking as intervention trials have not been undertaken in children or adults infected with HIV. Given the data in Chapter 2 showing that a substantial number of severely anaemic children were not iron deficient, and the fact that presumptive supplementation is currently recommended for most children living in tropical countries\textsuperscript{45}, studies addressing the safety and efficacy of iron supplementation in HIV-infected children are urgently needed. The situation is complex as it is likely some children could benefit, whereas for others it would be detrimental. This dilemma might be resolved by improved understanding of how these distinct categories of children can be identified, and it may be necessary to take a much more cautious approach to blanket supplementation with iron, or to consider only very low dose strategies.

We have concluded that anaemia is a very common complication of paediatric HIV infection worldwide and is associated with a poor prognosis.

Areas for future research and which have been partly addressed in this thesis include:
The association between HIV and severe anaemia, a common diagnosis in tropical areas, which is associated with high morbidity and mortality (Chapter 2).
The pathogenesis of anaemia in HIV-infected children (Chapters 3 and 6).
The role of viral characteristics of HIV in the development of anaemia (Chapter 7)
The use of haemoglobin to predict and monitor disease progression, and the effect of anaemia reduction for reversing disease progression in children infected with HIV in resource poor settings (Chapter 4 and ongoing work).
The safety and efficacy of possible nutritional intervention strategies in children, including iron supplementation (Chapter 3 and ongoing work).

**Haematology of HIV-associated severe anaemia**

In the previous section we suggested that the unexplained association between HIV and severe anaemia in Malawian children (Chapter 5) may result from an increased cell death of erythroid precursors. In Chapter 6 results comparing haematological abnormalities among severely anemic children with and without HIV infection are described.

Bone marrow flow cytometry showed that HIV-infected children had fewer CD34+ haematopoietic progenitor cells (1.0% vs. 1.5%, \( p=0.04 \)), erythroid progenitors (0.2% vs. 0.3%, \( p=0.05 \)) and erythroid precursor cells (18% vs. 26%, \( p=0.06 \)). Dyserythropoiesis and apoptosis of red cell precursors were not more common in HIV-infected than uninfected children (2.8% vs. 3.8%, \( p=0.12 \) and 9.3% vs. 12.3 %, \( p=0.23 \)). There were no significant correlations between the proportion of dyserythropoietic or apoptotic cells, and peripheral blood levels of the cytokines TNF-\( \alpha \), IFN-\( \gamma \), IL-10, erythropoietin, or vitamin A. Polychromatic erythroblasts, reticulocyte counts and peripheral blood erythrocytic indices were similar in both groups.

The finding that HIV-infected children had fewer CD34+ haematopoietic progenitor and erythroid progenitor cells in the bone marrow than uninfected children supports the hypothesis that red cell production failure is an important correlate of severe anaemia in HIV-infected children which may be caused by a reduced erythroid progenitor capacity. Despite the difference in CD34+ haematopoietic progenitors and erythroid progenitors the proportion of more mature erythroid precursor cells in bone marrow or peripheral blood (reticulocytes) did not differ between the two groups. Hence HIV-uninfected children with severe anaemia appeared to be less productive or efficient in their (subsequent) erythropoiesis than HIV-infected children. This explanation is supported by the trend, (although not significant), towards less dyserythropoiesis and apoptosis in HIV-infected children. This finding contrasts with other studies which reported that dyserythropoiesis was more common in the later stages of HIV disease leading to the conclusion that anaemia may be related to increased dyserythropoiesis.

In this study dyserythropoiesis or apoptosis were not associated with altered cytokine levels or Vitamin A deficiency which is in contrast to other reports. It is possible that more intensive investigations might identify cytokines which affect regulatory signals and could be therapeutic targets to reduce haemopoietic inhibition in HIV patients.

In conclusion the findings in these Malawian children indicate that despite an HIV-associated deficiency in early red-cell precursors, subsequent erythropoiesis is at least as efficient in HIV-infected as in uninfected children with severe anaemia. Apoptosis and
dyserythropoiesis were not more prominent mechanisms in HIV-infected than in HIV-uninfected children.

**Infections and HIV-associated severe anaemia**

Infections, as described in Chapter 5, were identified as potential contributory causes of anaemia in HIV-infected children. The contribution of opportunistic infections to severe anaemia in HIV-infected children in sub-Saharan Africa remains unclear. The role of malaria is unclear as, although in adults there is evidence in HIV-infected women that the prevalence of malaria parasitaemia is increased\(^ {50}\), in children there is no evidence to support this finding\(^ {51-55}\). However children suffering from both infections could have more severe anemia than children with either infection alone\(^ {51,54}\).

We found that the acute phase reactant CRP was raised (>10 mg/L) in HIV-infected children with severe anaemia (90%). In **Chapter 2** we described that in severely anemic children, the prevalence of EBV (50% vs. 31% \(P=0.03\)), or bacteraemia (26% vs. 13% \(P=0.02\)) was increased in HIV-infected compared to uninfected children. The prevalence of malaria parasitaemia did not differ between these groups (59% and 59%, \(p=0.96\)) and hyperparasitaemia was less common (5% vs. 13%, \(P=0.09\)). CMV, Parvovirus B19 infection, hookworm and invasive mycobacterial infections were uncommon in severely anaemic HIV-infected children (0%, 3%, 6% and 0% respectively).

Our data does not identify malaria as a more important factor contributing to the development of severe anaemia in HIV-infected compared to uninfected children, which contrasts hypotheses from other publications\(^ {51,54}\). This finding is in agreement with observations that malaria does not occur more frequently in HIV-infected children\(^ {51-55}\). Parvo B19 was not identified as contributory to the development of severe anaemia in HIV-infected children in this setting, which agrees with previous studies in children\(^ {56}\), but not adults\(^ {15}\). Invasive mycobacterial infections were not identified from blood or bone marrow isolates, which conflicts with data on Malawian adults\(^ {57}\), and other studies in HIV-infected children\(^ {36,57-63}\).

EBV has been considered as a possible aetiological factor in HIV-associated anaemia in adults, although data on children are lacking\(^ {14}\). It is uncertain whether this is a true aetiological factor or a co-morbidity. Bacteraemia, especially with nontyphoidal salmonella has been associated with both HIV infection and severe anaemia\(^ {64,65}\). As this is a treatable and preventable factor, future studies should aim to identify if salmonella bacteraemia is a causal factor, or rather a consequence possibly resulting from increased availability of iron which occurs during haemolysis (Chapter 3)\(^ {3}\).

We conclude that infections occur frequently in HIV-infected children with severe anaemia, including bacteraemias (with nontyphoidal salmonella) and EBV infection.
Future studies need to elucidate the role of these pathogens in the development (and prevention) of severe anaemia in these children.

**Viral aspects of HIV-associated severe anaemia**

In a Chapter 2, we observed an association between HIV and severe anaemia that could not be fully explained by secondary infection or micronutrient deficiency alone. The association may be largely explained by a direct effect of the virus (HIV) on erythropoiesis (Chapter 5). *In vitro* studies have suggested that specific HIV strains, such as X4 which uses the CXCR4 co-receptor on erythroid precursors, are associated with diminished haemo- and erythropoiesis\(^{66-70}\). This co-receptor affinity is determined by changes in the hypervariable loop of the HIV-1 envelope genome\(^{71}\). We therefore explored the possibility that alterations in the V1-V2-V3 fragment of HIV-1 were associated with severe anaemia and these results are described in Chapter 7.

Phylogenetic analysis showed that HIV-1 subtype C, the predominant subtype in South-East Africa\(^{71,72}\), was present in all but one child. All V1-V2-V3 characteristics tested: V3 charge, V1-V2 length and potential glycosylation sites, did not differ between cases and controls. Using a computer model (C-PSSM)\(^{73}\) four children (7.8%) were identified to have an X4 strain. This prevalence was not different between study groups (\(p=1.00\)). The V3 loop characteristics for bone marrow and peripheral blood isolates in the case group were identical. None of the children identified as having the X4 strain developed a (new) episode of severe anaemia during follow up.

Early in an infection the HIV-1 population usually comprises a strain with the capacity to bind to both CD4 and the co-receptor CCR5 (R5 strain)\(^{74,75}\). Later in infection a broadening, or switch, occurs and HIV evolves to infect cells expressing CD4 and the co-receptor CXCR4 (R4 strains)\(^{74,76}\). This switch is thought to occur in 50% of infections and is associated with an accelerated loss of CD4+ T-cells and progression to AIDS\(^{77}\). Like the T-helper cells, erythropoietic stem cells express both CD4 and CXCR4 on their membrane\(^{78,79}\). Until recently productive infection was considered uncommon in erythroid precursor cells\(^{16}\), though a recent study reported that in contrast to HIV-1 subtype B\(^{16}\), the predominant subtype in western settings, subtype C may actually infect red cell precursors\(^{46,80}\). As this is a new observation, these results will require confirmation in settings and populations like ours.

A further explanation for the association of HIV infection with severe anaemia could be increased cell death of erythroid precursors. Viral proteins, such as *tat*, erythropoietin and cytokines may play a crucial role in this\(^{66,68-70}\). Our results related to these hypotheses are discussed in the section above titled ‘haematology of HIV-associated severe anaemia’.
We conclude from these results that the prevalence of X4 strains was high in these young HIV-1 subtype C infected children who were most likely vertically infected and naïve to anti-retroviral therapy. It is unlikely that V1-V2-V3 fragment characteristics and HIV co-receptor affinity was an important feature influencing their development of severe anaemia. Further research on the possibility of direct infection of erythroid progenitors in HIV-1 C-infected children is an area for future research.

**Micronutrient deficiencies and HIV-associated severe anaemia**

The contribution of micronutrient deficiencies and malnutrition to the development of severe anaemia in these children is a critical area for assessment. Micronutrient deficiencies which have been associated with HIV-infection and which could lead to anaemia include iron, folate, vitamin B12, vitamin A and zinc. Controlled studies do not indicate that iron deficiency is more common in HIV-infected children\(^ {41;81;82}\). Iron intervention studies have not been undertaken and bone marrow iron status has not been associated with anaemia in HIV infected children\(^ {31}\). Folate and vitamin B12 deficiency were also not more frequent in paediatric HIV infection\(^ {30;33;34;83-86}\). The hematopoietic effect of supplementation with these hematinics has not been assessed in HIV-infected children. Data on vitamin A and zinc deficiency or supplementation is available but the results are conflicting\(^ {87-90 91-95}\). This is an area for further detailed research (Chapter 5).

In our non-severely anaemic control populations wasting occurred in 13% of HIV-infected and 6% of uninfected children (\(p=0.09\)). Deficiencies of vitamin B12 (20% vs. 15%, \(p=0.40\)), vitamin A (57% vs. 65%, \(p=0.70\)) or iron (52% vs. 71%, \(p=0.07\)) were not more common in HIV-infected than uninfected children. This is in line with other studies suggesting that in general micronutrient deficiencies were not more common in HIV-infected children\(^ {30;33;34;41;81-86}\).

Our data on severe anaemia (Chapters 2, 3 and 7) showed that wasting was present in 19% of HIV-infected cases and in 13% of HIV-infected controls (\(p=0.46\)). Vitamin B12 deficiency was found in 13% of HIV-infected cases compared to 20% of HIV-infected controls (\(p=0.40\)). Folate deficiency was not present in any child. Vitamin A deficiency was present in 96% of HIV-infected cases and in 57% of HIV-infected controls (\(p=0.01\)). Iron deficiency was found in 33% of HIV-infected cases and in 52% of HIV-infected controls (\(p=0.18\)).

The size of these associations with severe anaemia was similar for HIV-infected and uninfected children. Only vitamin B12 deficiency did not appear to be associated with severe anaemia in HIV-infected unlike in HIV-uninfected children. This could be a result from selection bias as severely anaemic children without HIV infection children may be more prone to have vitamin B12 deficiencies (possibly due to poor food intake), whereas the anaemia in HIV-infected children could be anaemic due to primarily due to other
conditions such as infectious diseases (as indicated in Chapters 2 and 5). This hypothesis requires further examination which would entail obtaining detailed dietary evaluation for these different categories of children.

The evidence from this thesis supports the conclusion that nutritional deficiencies do not play a more important role in the development of severe anaemia in HIV-infected children than in uninfected children. Randomised controlled trials are required to establish the validity of this conclusion and an approach using multi-micronutrient supplementation would be the most convenient choice in developing these studies.

**CONCLUSIONS**

This study was the first to comprehensively investigate the aetiology, pathogenesis and consequences of severe anaemia in sub-Saharan African children from the same study population. We found that several independent yet overlapping conditions were associated with severe anaemia in these children including bacteremia, malaria, hookworm, G6PD deficiency−/−, vitamin A and vitamin B12 deficiencies and HIV infection. Folate deficiency was uncommon and iron deficiency occurred less frequently in case-patients and was inversely associated with bacteremia.

The pathogenesis data suggests that in these populations, red cell production failure is the most important mechanism, with haemolysis and blood loss contributing relatively little to the development of severe anaemia. It should be stressed that these conditions were frequently overlapping and that, especially in HIV-infected children with severe anaemia, co-infections were common (84%).

We identified an unexpected high mortality after discharge from hospital in severely anaemic children (12%), and this mortality was 60% in they were HIV-infected, indicating that severe anaemia should be interpreted as an important predictor of mortality in HIV-infected children.

A meta-analysis of anaemia prevalence and incidence data suggested that mild (haemoglobin <11 g/dl) and moderate (Hb <9 g/dl) anaemia were more prevalent with HIV infection and that mean haemoglobin levels were lower. These differences were observed for both western and tropical settings. We concluded that anaemia was a very common complication of paediatric HIV infection worldwide and associated with a poor prognosis. There was limited data available on more severe anaemia (Hb <7 g/dl or <5 g/dl), and little data on the pathogenesis and aetiology of HIV-associated anaemia. The research in this thesis aimed to address these gaps in knowledge.
The pathogenesis study suggested that despite an HIV-associated deficiency in early red-cell precursors, subsequent erythropoiesis is at least as efficient in HIV-infected as uninfected children with severe anaemia. Apoptosis and dyserythropoiesis are not more prominent mechanisms in HIV-infected children than in HIV-uninfected children.

We found that infections occurred more frequently in HIV-infected children with severe anaemia, especially bacteraemias (with nontyphoidal salmonella) and EBV infection. Future studies should focus on the potential contributory role of these pathogens in the development (and prevention) of severe anaemia in these children.

Our study of HIV characteristics indicated that there was a high prevalence of X4 strains in young Malawian children with HIV-1 subtype C infection compared to previous results from Malawi. It is unlikely that V1-V2-V3 fragment characteristics and HIV co-receptor affinity is an important feature in the development of severe anaemia in Malawian children. Direct infection of erythroid progenitors in HIV-1 C-infected children may be an area for future research.

The data from the control population and case-control study showed that micronutrient deficiencies were not significantly associated with HIV infection, nor that nutritional deficiencies played a more important role in the development of severe anaemia in HIV-infected than uninfected children. Intervention studies would be required to prove the validity of these statements.

There are multiple independent as well as overlapping causes of severe anaemia in Malawian children. The current preventive and therapeutic guidelines may not be applicable with respect to folate and iron supplementation in these children. HIV is a prevalent, independent and important factor associated with severe anaemia in these children and is associated with a very poor prognosis. Red cell production failure appeared to be the main pathogenetic mechanism in HIV-associated severe anaemia. This may result from a reduced number of erythroid progenitor cells rather than as a consequence of increased apoptosis or dyserythropoiesis in red cell precursors. Infectious diseases causing bacteraemia and EBV were especially prominent amongst HIV-infected children with severe anaemia in contrast to micronutrient deficiencies, or HIV V1-V2-V3 fragment characteristics.
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Samenvatting voor niet medici
Anemie

Bloedarmoede (anemie) is een tekort aan rode bloedcellen (erytrocyten). Rode bloedcellen zijn verantwoordelijk voor het zuurstoftransport van de longen naar de organen. Een (ernstig) tekort aan rode bloedcellen kan leiden tot een zuurstoftekort in de organen, een potentiële levensbedreigende situatie. Het lichaam beschikt echter over compensatiemechanismen om dergelijke tekorten op te vangen zoals een versnelde werking van het hart en een verhoogde aanmaak van nieuwe cellen. Milde of langzaam ontstane anemieën leiden daardoor tot beperkte klachten zoals moeheid, hartkloppingen en een verminderd vermogen tot inspanning. Wanneer het aantal rode bloedcellen daalt tot onder een kritische grens treden er levensbedreigende situaties op zoals hartfalen en een te lage zuurstofspanning in de weefsels.

Ernstige anemieën zijn zeldzaam in de Westerse wereld, maar in Derde wereldlanden komt dit frequent voor en treft vooral jonge kinderen en zwangere vrouwen. Veel verschillende ziektes kunnen in theorie leiden tot een tekort aan rode bloedcellen. Zij worden doorgaans in drie mechanismen ingedeeld:

- Een beperkte aanmaak van rode bloedcellen. Bijvoorbeeld door tekorten aan bouwstenen zoals ijzer, foliumzuur en vitamine B12 of door remming zoals optreedt bij verschillende infecties;
- Een verhoogde afbraak van rode bloedcellen (bij een vergrote milt of malaria);
- Verlies, zoals bij een bloeding of bloedverlies in de darm bij (mijn)worm infecties.

De gegevens over de bijdrage van deze mechanismen en ziektes zijn over het algemeen afkomstig van onderzoeken bij mensen met milde anemie, voornamelijk bij volwassenen en zijn vaak uitgevoerd in Westerse landen. Echter de data over de mogelijke oorzaken en daardoor behandelingen bij kinderen in Afrika zijn beperkt, terwijl juist zij veelvuldig (over)lijden aan de ernstige vormen van anemie.

Mogelijke oorzaken van ernstige anemie (ziektes)

De huidige Wereld Gezondheidszorg Organisatie (WHO) richtlijn suggereert dat malaria, tekorten aan ijzer en foliumzuur en, bij oudere kinderen mijnworm, de belangrijkste behandelbare oorzaken van ernstige anemie zijn. Dit is echter niet goed wetenschappelijk onderbouwd. Om een beter inzicht te verkrijgen in de mogelijke oorzaken van ernstige anemie hebben wij de prevalenties van alle mogelijke oorzaken vergeleken tussen 381 Malawiaanse kinderen met ernstige anemie (Hb<3.0 mmol/L) en 757 Malawiaanse kinderen zonder ernstige anemie (hoofdstuk 2).
De resultaten van dit onderzoek tonen aan dat de richtlijnen van de WHO wellicht niet kloppen. IJzergebrek kwam verbazingwekkend genoeg minder vaak voor bij kinderen met ernstige anemie. Uit ons onderzoek blijkt tevens dat dit verklaard zou kunnen worden door een beschermend effect van ijzergebrek tegen ernstige infecties. Dit sluit aan bij eerdere onderzoeken die aantoonden dat ook bacteriën ijzer nodig hebben om te vermenigvuldigen.

In ons onderzoek kwam foliumzuurdeficiëntie in het geheel niet voor bij de kinderen met ernstige anemie. In tegenstelling tot tekorten aan ijzer en foliumzuur lijken tekorten van vitamine A en B12, het hebben van een bacteriemie, malaria of mijnowminfectie (juist bij jonge kinderen) wel een rol te spelen bij het ontstaan van ernstige anemie en zijn mogelijk behandelbare en te voorkomen oorzaken. Naast de eerder genoemde infecties blijkt ook HIV vaker bij kinderen met ernstige anemie aanwezig te zijn (21% in de stedelijke populaties).

**Ontstaanswijze van ernstige anemie (mechanismen)**

Om een beter inzicht te verkrijgen in de ontstaanswijze van ernstige anemie hebben we onderzocht welk mechanisme het belangrijkst is. In hoofdstuk 3 wordt beschreven dat voornamelijk een verminderde aanmaak van rode bloedcellen verantwoordelijk lijkt te zijn voor het ontstaan van ernstige anemie in de bestudeerde groep kinderen. Bloedafbraak (hemolyse) en bloedverlies komen minder vaak voor. Kinderen die ernstige anemie hebben, hebben vaak meerdere ziektes tegelijkertijd (zoals HIV, malaria en tekort aan vitamine B12). Voor een effectieve preventie of behandeling van ernstige anemie zal een behandeling van één aandoening waarschijnlijk niet afdoende zijn. Bij de ontwikkeling van nieuwe strategieën zal dit in ogenschouw moeten worden genomen.

**Gevolgen van ernstige anemie**

Hoewel bekend is dat ernstige anemie kan leiden tot een hoge sterfte bij kinderen in Afrika is weinig bekend over de langetermijngevolgen. Een kleine studie in Kenia suggereerde dat ook na behandeling van ernstige anemie er een hoge sterfte zou zijn onder deze groep kinderen.

In hoofdstuk 4 worden de gevolgen van ernstige anemie beschreven. Gedurende de ziekenhuisopname blijkt de sterfte bij kinderen met ernstige anemie 6% te zijn. We vervolgd degenen die naar huis terugkeerden en vonden dat binnen zes maanden na ontslag uit het ziekenhuis nog eens 12% van de kinderen kwam te overlijden. Deze sterfte is veel hoger dan in de controlegroep van kinderen zonder ernstige anemie. Onze analyse
toonde verder aan dat HIV een van de belangrijkste verantwoordelijke factoren is. Bijna 60% van de kinderen met én HIV én ernstige anemie overleed binnen een jaar.

Dit geeft aan dat ernstige anemie een verborgen late sterfte heeft en moet worden beschouwd als een zeer ernstige aandoening, waarvan het onderliggend lijden moet worden behandeld. Gezien ernstige anemie veel voorkomt is het aantrekkelijk om door aanpak van dit probleem de kindersterfte in Afrika terug te dringen.

HIV
Onze analyse suggereert dat HIV een belangrijke factor is bij het ontstaan van ernstige anemie. Wanneer beide condities zich tegelijk voordoen leiden zij tot een sterk verhoogde sterftekans. Om een beter inzicht te krijgen in de frequentie, de oorzaken en de mogelijke behandeling van anemie bij kinderen met HIV, hebben we de beschikbare literatuur over anemie bij HIV-geïnfecteerde kinderen samengevat. Uit deze studie (hoofdstuk 5) blijkt dat (milde) anemie bij bijna alle HIV-geïnfecteerde kinderen optreedt gedurende hun ziekte, ongeacht of zij opgroeien in een Westerse of in een Tropische leefomgeving. De gegevens over ernstige anemie zijn echter beperkt. Net als bij onze studie, blijkt uit de literatuur dat het hebben van een (milde) anemie een slechte prognostische factor is voor kinderen met HIV. Het vaststellen van een anemie bij deze kinderen zou dus ook gebruikt kunnen worden als een indicator van een vergevorderd stadium van HIV.

Er zijn weinig gegevens over de mechanismen die verantwoordelijk zijn voor de (milde) anemie bij deze kinderen. Onderzoeken bij volwassenen suggereren dat de aanmaak van rode bloedcellen afneemt door infecties, ondervoeding en misschien zelfs door een direct effect van het HIV-virus. Mogelijke behandelingen zoals suppleties met vitamines, het geven van anti-HIV middelen (HAART) en erytropoëtine (EPO), een hormoon dat de aanmaak van rode bloedcellen stimuleert, worden beschouwd in dit hoofdstuk. Vooral deze laatste twee opties lijken veelbelovend, hoewel de kosten van deze medicijnen hoog zijn. Het geven van ijzer is mogelijk gevaarlijk door een toenemend risico op infecties. Er is echter behoefte aan goed onderzoek om dit te testen.

Ernstige anemie bij Malawiaanse kinderen met HIV
Zoals hiervoor al beschreven, is er weinig bekend over de oorzaken en ontstaanswijzen van (ernstige) anemie bij de HIV-geïnfecteerde kinderen. Uit ons onderzoek is gebleken dat ernstige anemie in deze groep kinderen vooral wordt veroorzaakt door een probleem in de productie van rode bloedcellen in het beenmerg (hoofdstuk 6). Vooral bacteriële en virale infecties spelen hierbij een belangrijke rol. We konden geen bewijs vinden dat bepaalde vitamintekorten of het voorkomen van subtypes van HIV (CXCR4) zouden leiden tot een verhoogd risico op ernstige anemie (hoofdstuk 7). Het verbeteren van de afweer en het voorkomen van bacteriële (en virale) infecties lijken daarom de meest
veelbelovende strategieën in de strijd tegen ernstige anemie bij HIV-geïnfecteerde kinderen.

Conclusies

Samengevat geeft dit proefschrift een goed overzicht van de ontstaanswijzen, oorzaken, en gevolgen van ernstige anemie bij kinderen in Malawi. IJzergebrek en foliumzuur lijken minder belangrijk dan gedacht terwijl malaria, gebrek aan vitamine A en B12, mijnworm, bacteriemie en HIV belangrijke factoren lijken. Dit komt voornamelijk door een gebrek in de aanmaak van nieuwe rode bloedcellen. De aanpak van dit probleem is belangrijk gezien de grote sterfte die vooral optreedt juist na behandeling van de anemie.

Zowel bij het ontstaan van ernstige anemie als de prognose op lange termijn speelt HIV een belangrijke rol. Dit komt overeen met gegevens uit de literatuur over milde anemie die vaak optreedt bij kinderen met HIV in zowel een Westerse als een Tropische leefomgeving. We bewezen als eerste dat een verminderde aanmaak van rode bloedcellen de belangrijkste factor is bij het ontstaan van ernstige anemie bij kinderen met HIV. Vooral bacteriële en virale infecties, maar niet tekorten aan vitamines en ijzer noch een subtype van HIV spelen hierbij een belangrijke rol.

Met behulp van deze resultaten moeten nieuwe strategieën ter voorkoming en genezing van ernstige anemie bij kinderen met en zonder HIV moeten worden getest.
Acknowledgements
The preparations for this PhD started in 2001. Now, seven years later, a big task lies behind me and many have contributed to the thesis you are currently reading. Because it is impossible to mention every person by name, I would like to express my gratitude to all who supported me during this period. I will try however to focus on those who have contributed to the coming about of this thesis in particular.

Firstly I would like to thank the parents and children who participated in the study. Without their contribution this study would not have been possible in the first place.

The recruitment and follow up of these children has been a major task, which has been done by a great group of people in Chikwawa and Blantyre: Alex Siyasiya, Andrew Naunje, Ben Nkumbira, Bridget Mangochi, David Kachala, Davis Kazembe, Ernest Nkhoma, Francis Munthali, Geoffrey Chipungu, Georgina Makuta, Harriet Khoﬁ, Inez Kawinga, Isaac Chirambo, Kate Sapanga, Linda Bwanali, Mary Kandeya, Mercy Kamdolozi, Miriam Nkhoma, Miriam Khoka, Tapika Mwafulirwa and William Kachina.

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The people working at the Wellcome Trust Research Laboratories, the (Malaria Project) research ward and the College of Medicine were essential in completing this research. One really learns to value facilities such as water, power, supplies, IT- and statistical support (special thanks to Pelani Malange, Lucia Nuka, Mavuto Mukaka and Sarah White), human resources (special thanks Mrs Grace Mapunda), ﬁnances, security, serviced equipment, transport, know-how and many more that were all facilitated by you. Even more important, especially during the few times that one of these facilities was lacking, was the great atmosphere and camaraderie. There are really too many names to mention here, but to all staff, colleagues and friends a big thanks. A special word of thanks should go out to Professor Malcolm Molyneux, not only for founding the WTRL, a great place to work, but also for the support and the extraordinary ability in helping to write proposals and manuscripts.

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Of course I thank all friends and family whom we met in Malawi, who visited us or who kept us up to date. I would like to thank Afitha Voeten for creating such a beautiful cover; all who helped revising the content of this thesis (especially Steve Graham, Maarten
Schim van der Loeff and Henriëtte Scherpbier) and those who financially supported the research (Nutricia research foundation and the Ter Meulen Fund, Royal Netherlands Academy of Arts and Sciences). A special word of thanks should go out to my parents, who supported me for the past 30 years, who showed their enthusiasm by visiting Malawi and will hopefully continue to do so in near future.

I thank both paranymfen whose contributions expand far beyond this day in May. Martijn, you have shown to be a great friend over the last years. We shared a great time doing our research electives in the district hospital in Phalombe. When I came back to do research you visited and did a clinical rotation in the paediatric department in Blantyre. I would not be surprised if, in future, our shared interest will make us work together once again. Kamija you have been a great colleague, sparring partner and friend. Much of the work presented is yours as well as mine. Although you have already obtained you PhD in Liverpool, I hope you will feel to have obtained another degree.

Of course I am thankful to professor Bernard Brabin, who supported me throughout this PhD, coping with all the deadlines for revisions of manuscripts, proposals and chapters. His enthusiasm and extensive knowledge of tropical paediatrics has been an encouragement throughout the years.

Obtaining a PhD is a big compliment to a co-promotor. It is needless to say that without Michaël Boele van Hensbroek I would not have started nor completed this PhD. Michaël you have been a great almost daily support during these seven years. Your endless enthusiasm and optimism, your trust and constructive criticism and your effort towards me and this study make you an ideal mentor. In addition I would like to stress that the warm-heartedness and hospitality of you and your family have made our stay in Malawi really enjoyable.

Lastly I would like to thank Brechje for the love, support and patience over the years. You gave up your job to move to Malawi but, gladly, never regretted doing so. You indirectly and directly contributed to the study in many ways and in return had to life with being Mrs Doctor Calis. I hope you will rightly and proudly share this new degree with me.
Curriculum Vitae

The author of this thesis was born in Laren, The Netherlands on 23 July 1977. In 1995 he finished his secondary education (VWO) at “Het Baarnsch Lyceum” in Baarn where he participated in exchange projects with foreign schools. He studied medicine at the University of Amsterdam and obtained his Masters degree in 2000. As part of this degree he followed a course in tropical medicine, performed a nine-month research elective towards the outcome of sputum smear negative TB- suspects in Phalombe, Malawi, and consecutively did a clinical rotation in this hospital. Returning to the Netherlands he finished his internships between 2000 and 2002.

In 2002 he successfully obtained a ‘Ter Meulen grant’ for international research in the field of paediatrics and left for Malawi to collaborate in a study on severe anaemia in Malawian children, with special attention to HIV. He worked for three years at the Wellcome Trust Research Laboratories and the Paediatric Department of the Queen Elizabeth Central Hospital combining research, laboratory work and clinical paediatrics.

When he obtained an independent ‘Nutricia research grant’ in 2005, he returned to Amsterdam to attend courses in statistics and epidemiology and to write the papers as presented in this thesis. In 2007 he started clinical work in the Paediatric Department of the Emma Kinderziekenhuis, Academic Medical Centre in Amsterdam and was awarded a training post in Paediatrics.

Together with dr. Peter Moons he was awarded a NWO-NACCAP grant in 2007, for a study which currently investigates the use of haemoglobin and other simple markers in staging children infected with HIV.

After completion of his paediatric training he would like to work in the field of infectious diseases and international child health.
Colour Section
CHAPTER 2

Figure 1. Adjusted odds ratios and 95% confidence intervals for factors associated with severe anaemia by study group and recruitment site.

* Cultures only performed in case-patients and hospital controls. ** Hookworm was not entered in the ‘urban’ model because the prevalence was <5%. CI: Confidence interval; Wasting was defined as a Z-score for weight-for-height <-2; Iron deficiency was defined as TfR/log(ferritin) ratio >5.6; Iron deficiency was defined as TfR/log(ferritin) ratio >5.6; Concentrations of vitamin B12 <200 ng/L and vitamin A <20 μg/dL were considered deficient; HIV: Human immunodeficiency virus; EBV: Epstein-Barr virus; The rs-classification for IL10 +4949:rs3024500. The model was corrected for possible confounders: age, sex, recent anti-malarial treatment, recent haematinic treatment, previous transfusions and death of a parent. Owing to the high correlation between the three IL-10 polymorphisms, only one (most strongly associated to severe anaemia) was included in the multivariate model. In the combined model interaction existed between malaria and site (p<0.001). The goodness-of-fit of the model was evaluated using the Hosmer and Lemeshow test (P=0.65).
CHAPTER 4

**Figure 1:** Survival curve showing the time to post-discharge death during the follow-up period of severely anaemic children (*cases*) and hospital and community controls. Log rank test, \( p<0.001 \).

**Figure 3:** Survival curve showing the time to severe anaemia during the follow-up period of severely anaemic children (*cases*) and hospital and community controls (see colour section). Log rank test, \( p<0.001 \).
CHAPTER 5

Figure 2a. Prevalence of anaemia in HIV-infected and uninfected children (only studies that simultaneously included both groups, n=7).

Curves display weighted exponential trendlines (thick lines) and 95% confidence intervals (thin lines) for HIV-infected ($R^2=0.83$) and HIV-uninfected children ($R^2=0.84$). For the analyses data for 12 cut-offs were used reflecting 700 HIV-infected and 2387 uninfected children.

Figure 2b. Prevalence of anaemia in HIV-infected children in tropical vs. western (Europe & North America) settings.

Curves display weighted exponential trendlines (thick lines) and 95% confidence intervals (thin lines) for HIV-infected ($R^2=0.77$) and western settings ($R^2=0.63$). The analyses included 15 studies presenting data for 23 cut-offs in 1085 HIV-infected children.
Figure 3. Mean haemoglobin (Hb) levels of cohorts of HIV-infected and uninfected infants in Zimbabwe, Kenya, and Italy. The curves in the bottom right graph compare the mean haemoglobin levels in HIV-infected children in these settings. Reproduced with permission from MF Miller; The American Society of Tropical Medicine and Hygiene; and Mary Ann Liebert Inc. Publishers respectively.
Pluripotent stem cells/CD34+ haematopoietic progenitors and erythroid progenitor cells can only be differentiated using surface marker expression. Light microscopy can differentiate between erythroid precursor cells. CFU-GEMM: Colony forming units-granulocyte/erythrocyte/macrophage/megakaryocyte, BFU-E: Burst Forming Units-Erythrocyte, CFU-E: Colony Forming Units-Erythrocyte, GPA: Glycophorin-α (CD235a); LDS: Laser Dye Styryl-751, a DNA dye.
**CHAPTER 7**

**Figure 1.** PCR Primers used to amplify V1-V2-V3 fragment.

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</table>

A display of the primer sequences used to amplify the V1-V3 fragment located on the viral genome of the HIV-1 *env* protein. External PCR: primers indicated with a blue star (*). Internal PCR primers are indicated with (•) for the V regions and with (•) for the C regions.
Additional File 1. Frequency amino acid codon logos of the V3 loop per study group.

Sequence logos of HIV-1 V3 sequences found in children with severe anaemia (cases, , Hb<5.0 g/dL, n=26) as compared to those without severe anaemia (controls n=28). The character and size of each logo represent the amino acid and its prevalence at the specific site.