Anaemia, iron deficiency and infections: new perceptions of the interaction between hepcidin, iron biomarkers, anaemia and inflammation in Malawian children
Jonker, F.A.M.

Citation for published version (APA):
Jonker, F. A. M. (2012). Anaemia, iron deficiency and infections: new perceptions of the interaction between hepcidin, iron biomarkers, anaemia and inflammation in Malawian children

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Anemia, Iron deficiency and Infections
Anaemia, Iron deficiency and Infections

New perceptions of the interaction between hepcidin, iron biomarkers, anaemia and inflammation in Malawian children
Anaemia, Iron deficiency and Infections, new perceptions of the interaction between hepcidin, iron biomarkers, anaemia and inflammation in Malawian children

Thesis, University of Amsterdam, the Netherlands

ISBN 9789491456213

Copyright © 2012 F.A.M. Jonker

All rights are preserved. No part of this thesis may be reproduced, stored of transmitted in any form or by any means, without prior permission of the author.

Cover illustration: Bao is a traditional mancala board game played in East Africa. The bone marrow slides on the board reflect a Bao game played at lake Malawi under the African sun, the ‘golden reference’ until clouds appear. With thanks to Annemiek Tuinhof de Moed.

Printed by EZbook, publications for professionals

This thesis was supported by Numico Research Foundation, Janivo Foundation, Stichting Emma Steun, Welcome Trust, AIGHD, Rotary Leusden, Rotary Den Helder, Johnson & Johnson, Imtech Projects B.V., ABN AMRO, De Greef Transport, Interfurn B.V., AV Werving en selectie, Emma Kinderziekenhuis, Robbe B.V., Opera Amsterdam, and Refero B.V.

The printing of this thesis was made possible by support of the AMC, Beckman Nederland, Hemocue, Nutricia Nederland, Pfizer B.V.
Anaemia, Iron deficiency and Infections

New perceptions of the interaction between hepcidin, iron biomarkers, anaemia and inflammation in Malawian children

Academisch Proefschrift

ter verkrijging van de graad van doctor
aan de Universiteit van Amsterdam
op gezag van de Rector Magnificus
prof. dr. D.C. van den Boom
ten overstaan van een door het college voor promoties ingestelde commissie,
in het openbaar te verdedigen in de Agnietenkapel
op donderdag 4 oktober 2012, te 12.00 uur

door

Femkje Annechien Maartje Jonker
geboren te Leiden
Promotiecommissie

Promotor
Professor dr. Bernard J. Brabin, hoogleraar Tropische Kindergeneeskunde, Emma Kinderziekenhuis AMC, Universiteit van Amsterdam en Liverpool School of Tropical Medicine, Verenigd Koninkrijk

Co-promotoren
Dr. Michael Boele van Hensbroek, universitair hoofddocent, afdeling tropische kindergeneeskunde Emma Kinderziekenhuis, Academisch Medisch Centrum, Universiteit van Amsterdam
Dr. Job C.J. Calis, kinderarts, Emma Kinderziekenhuis, Academisch Medisch Centrum, Universiteit van Amsterdam

Overige leden
Prof dr. Frank G.J. Cobelens, Amsterdam Institute for Global Health and Development, Academisch Medisch Centrum, Universiteit van Amsterdam
Prof. dr. Hugo S.A. Heymans, Emma Kinderziekenhuis, Academisch Medisch Centrum, Universiteit van Amsterdam
Prof. dr. Victor Mwapasa, Department of Epidemiology and Public Health, College of Medicine, Universiteit van Malawi
Dr. Marjolein Peters, universitair hoofddocent, afdeling kinderhematologie Emma Kinderziekenhuis, Academisch Medisch Centrum, Universiteit van Amsterdam
Prof. dr. André J.A.M. van der Ven, Department of International Health, Radboud Universiteit, Nijmegen

Faculteit der Geneeskunde
Contents

Chapter one  Introduction and outline of the thesis  7

Chapter two  Severe acquired anaemia in Africa: new concepts  21
M. van Hensbroek, F.A.M. Jonker, I. Bates
*British Journal of Haematology, 2011 Jun 28; 156:2141*

Chapter three  Real-time PCR Demonstrates *Ancylostoma duodenale* is a key factor in the aetiology of severe anaemia and iron deficiency in Malawian pre-school children  33
*PloS Neglected Tropical Diseases 2012; 6(3)*

Chapter four  Iron status predicts malaria risk in Malawian preschool children  47
*PloS ONE, 7(8): e42670*

Chapter five  Iron deficiency anaemia in children with HIV-associated anaemia: systematic review and meta-analysis  65
M.O. Esan, F.A.M. Jonker, M. Boele van Hensbroek, J.C.J. Calis, K.S. Phiri
*Transactions of the Royal Society of Tropical Medicine and Hygiene, 2012 Jul 28.*
*(e-publication ahead of print)*

Chapter six  Unexpected low hepcidin levels in severely anaemic Malawian children with high incidence of infectious diseases and bone marrow iron deficiency  81
*Submitted for publication*

Chapter seven  Conventional and novel peripheral blood iron markers compared against bone marrow in Malawian children  95
*Submitted for publication*

Chapter eight  Conclusions and Recommendations  111

Nederlandse samenvatting  121
Acknowledgements  129
Resume  134
Abbreviations  136
Colour section  138
Chapter one

Introduction and outline of the thesis
Anaemia, Iron deficiency and Infections

Anaemia, iron deficiency and infections are three major causes of child morbidity and mortality throughout the world. These mostly prominently occur in resource poor settings such as sub-Saharan Africa. As the three conditions may have the same underlying aetiologies, they often occur simultaneously and might interact. For example, iron deficiency may increase susceptibility to infection by suppressing the immunological response to pathogens, conversely treatment of the deficiency with supplementation has been associated with an increased incidence of infection. In order to highlight some outstanding research questions on such complex interactions, background information on childhood anaemia, iron deficiency and infection risk will be discussed.

Anaemia

Anaemia is defined as a reduction of the haemoglobin concentration causing a decrease in oxygen carrying capacity. Anaemia is prevalent globally and constitutes a major public health problem affecting especially children in sub-Saharan Africa. Since iron is essential for synthesis of haemoglobin, iron deficiency is often considered the primary cause of anaemia. As a consequence the terms anaemia, iron deficiency and iron deficiency anaemia are often used interchangeably. Besides confusing definitions, this approach is often incorrect as a number of other important causes, contribute to the development of anaemia, and anaemia can occur with sufficient iron stores. Moreover iron deficiency does not necessarily lead to anaemia, as the initial stages of iron deficiency do not restrict erythropoiesis. For this reason anaemia and iron deficiency may occur concurrently, thought should be considered as distinct conditions and requiring their own management.

Anaemia | Pathogenesis and aetiology

The pathogenesis of anaemia in African children may be classified as secondary to: a) increased red blood cell destruction; b) impaired red blood cell production, or c) acute or chronic blood loss. Anaemia has multiple aetiologies and may be a symptom of several underlying conditions rather than a specific disease. These aetiological conditions include: infections including malaria, hookworm and HIV infection; drugs (antibiotics, tuberculostatics and antiretrovirals; genetic disorders (G6PD, alpha-thalassemia, sickle cell trait and haemoglobin C); and micronutrient deficiencies (deficiency of iron, vitamin B12, folate, vitamin A).

Despite its multifactorial causes, the management of anaemia in sub-Saharan Africa is still mainly focused on treatment and prevention of iron deficiency whilst alternative treatment options related to other causes of anaemia are often neglected.

Anaemia | Severe anaemia

Severe childhood anaemia in Africa is not just the extreme part of the anaemia spectrum as it has a distinctive pattern of causes. It is defined by a haemoglobin <5 g/dL, or <6 g/dL), and is associated with major morbidity and mortality. In contrast to moderate anaemia which often presents without clinical symptoms, severe anaemia usually presents with fatigue, weakness, dizziness or drowsiness, although chronic severe anaemia also may be masked by lack of acute symptoms. Hospital and post-discharge mortality are of worrisome major concern, which suggests that the common treatment recommendations, including blood transfusion and iron supplementation, are insufficient. Current guidelines for preventing and treating severe
anaemia are not adequately evidence-based\textsuperscript{18}. Recently new findings have been published on the aetiology; surprisingly, severe anaemia was associated with a lower prevalence of iron deficiency as compared to non-severely anaemic children\textsuperscript{14}. These and other new findings about the mechanisms and causes of acquired severe anaemia in Africa may provide a basis for generating novel approaches for its treatment and prevention.

**Iron deficiency**

Iron deficiency is considered to be the most common and widespread nutritional disorder worldwide, with children and pregnant women living in resource poor settings forming the main risk groups\textsuperscript{8}. Iron deficiency is defined as a state in which there is a shortage of non-utilisable iron storage. This condition is a consequence of disturbance of the normally stable cycle of iron metabolism (Figure 1).

There are different factors that may disturb iron homeostasis and induce iron deficiency (Figure 2). Physiological causes of iron deficiency include periods of increased requirements as well as nutritional iron deficiency. Iron requirements are increased during periods of rapid growth such as during the first years of life and pregnancy. During infancy iron stores present at birth are usually exhausted by the age of 6 months. Limited iron bioavailability in weaning foods may compound the risk of nutritional iron deficiency, which occurs when the diet is unable to cover physiological requirements\textsuperscript{19}. This is an important cause of iron deficiency in sub-Saharan Africa, where limited bioavailability of iron from staple foods is common as a consequence of a low socio-economic status\textsuperscript{8}. Pathological iron deficiency includes increased blood loss e.g., gastrointestinal blood loss due to enteric parasitic infections including hookworm\textsuperscript{20}.
In addition to actual iron shortage, normal physiological systems for transporting iron into target tissues may be impaired in the presence of adequate iron stores\textsuperscript{21}. This condition is called *functional iron deficiency* and is caused by cytokine release during the acute phase response to infection\textsuperscript{22}. In sub-Saharan Africa, as infectious pressure is high, this may contribute to the high prevalence of iron deficiency in these areas.

**Assessment of iron deficiency**

In sub-Saharan Africa, high prevalence of infectious diseases and the various nutritional deficiencies, complicate measurement of iron status using serum iron biomarkers. However in African children these markers have not been validated against bone marrow assessment of iron status, the reference standard for iron status. As a result, no consensus on the use of iron biomarkers exists. A wide variety of markers including haematological markers (haemoglobin, mean corpuscular haemoglobin concentration (MCHC), mean corpuscular volume (MCV), and biochemical markers (ferritin, serum transferrin receptor (sTHR), serum iron, Total iron-binding capacity (TIBC), serum transferrin, transferrin saturation), have been used to define iron deficiency. This has lead to inconsistency in selection of iron markers, and inconsistency in definitions used for iron deficiency, across different studies. As a consequence, studies assessing iron status are often difficult to compare and results are difficult to translate into clinical practice. There is therefore an urgent need for validated iron biomarkers, applicable in African paediatric populations and suitable for large scale use. Hepcidin, a recently discovered key iron regulating hormone\textsuperscript{23-25}, and the reticulocyte haemoglobin content\textsuperscript{26, 27} are two novel potential iron markers, although both have not yet been validated against bone marrow iron in African children. Validating these novel markers, as well as the currently used iron biomarkers, against bone marrow assessment of iron in African children, may contribute to the development of a reliable assessment of iron status in one the most important risk groups for iron deficiency and anaemia.
Assessment of iron deficiency | **Haemoglobin content**

The haemoglobin content of erythrocytes (MCHC and MCH) and of reticulocytes (RSF) has been explored as biomarkers for diagnosing iron deficiency\(^28,29\). These measures reflect the haemoglobinization of the early reticulocytes and mature erythrocytes respectively\(^30\). Reduced haemoglobin content indicates that the iron supply for the bone marrow is too low to allow normal haemoglobinization. Since these parameters are not directly affected by inflammation\(^31\) they may reliably detect iron deficiency in conditions of inflammation.

Assessment of iron deficiency | **Hepcidin**

It has now generally been accepted that the recently discovered hormone hepcidin\(^23-25\), a small 20-25 amino acid peptide, plays a crucial role in the iron regulation. It is predominantly expressed in hepatocytes in the liver \(^28,32\). Hepcidin is also produced by other cells than hepatocytes, although at much lower levels. These include kidney tubule, heart, retina, monocytes, neutrophils, fat cells, alveolar cells, pancreatic cells, and myocardial cells\(^33-40\). Through binding to the cellular iron transporter, ferroportin, in the small intestine, macrophages and bone marrow, hepcidin induces internalization and degradation of ferroportin and regulates cellular iron efflux\(^41\). There are several factors that regulate hepcidin expression including systemic stimuli of iron levels, inflammation, erythropoietic drive and hypoxia\(^42\) (Figure 3). Hepcidin is down-regulated during iron deficiency, hypoxia and enhanced erythropoietic drive\(^43\); conversely it is up-regulated in the process of increased iron levels and during inflammation\(^44\).

![Figure 3. Regulatory pathways of hepcidin.](image)

Hepcidin has been shown to be a potential marker to define iron deficiency\(^44,45\) though it has not yet been validated against bone marrow iron status. Furthermore hepcidin has been suggested as a marker guide iron supplementation by predicting the effect of intervention with iron supplementations\(^46\). Hepcidin accuracy may be negatively affected, in areas where multiple conditions regulating hepcidin are simultaneously prevalent. Validating hepcidin against bone marrow iron status and exploring the hierarchy of the hepcidin signalling pathways in different African paediatric sub-populations may contribute to the use of hepcidin in the iron deficiency management in African children.
Iron deficiency and infection

In the late 1970s a positive association between iron supplementation and increased infection risk in malaria endemic areas had already been observed. Nevertheless the benefit of iron supplementation was considered to outweigh possible increased infection risk. When in 2006, a large trial in Zanzibar reported that iron supplementation increased (malaria related) morbidity and mortality in iron replete children, the World Health Organization restricted its recommendations to supplementation with iron only to iron deficient children. Nevertheless, a consensus on the safety of iron supplementation in children in malaria endemic areas has yet to be reached as important research questions remain to be answered. For instance, current evidence is based on studies which lack important data such as adequate descriptions of baseline iron status. In recent years the concerns of health authorities have focused mainly on the possible harmful effects of treating iron deficiency rather than on the effects of iron status itself on subsequent infection risk. There are little conclusive data that support a protective effect of iron deficiency against malaria. The reliability of currently available data on iron status assessment in relation to infection risk is very limited due to a lack of reliable or valid assessments of iron status. This makes currently available data difficult to interpret and compare. In addition, the complexity of factors influencing both malaria susceptibility and iron status are not always taken into account, which may lead to unexplained confounding. There is a need for reliable data on the influence of iron status on malaria risk, using well validated biomarkers, in order to improve control and management strategies for both malaria and iron deficiency.

As on the one hand iron deficiency may protect against infections, on the other hand certain infections may induce iron deficiency. This is either indirect, through increased hepcidin/cytokine production in response to inflammation; the subsequent inhibition of absorption or release of respectively dietary iron or stored iron may withhold iron from pathogens; a condition called ‘functional iron deficiency’. A direct effect may arise through increased blood loss caused by helminthic or other enteric infections. An important but easily neglected cause of iron deficiency and eventually (even severe) anaemia, is hookworm (Ascaris duodenale and Necator americanus), a helminthic infection prevalent in Sub-Saharan Africa. Especially the subspecies A. duodenale may cause substantial intestinal blood loss which may lead to iron deficiency and (severe) anaemia. In areas where hookworm is prevalent, de-worming may significantly contribute to prevention and treatment of anaemia and iron deficiency. However due to the insensitive and time-consuming detection methods, the prevalence and disease burden of the different hookworm species is mostly unknown. Recently a real-time PCR test for hookworm was developed, a highly sensitive test, capable of species differentiation and determination of infection density. The potential use of this novel method to detect and treat hookworm infected children in sub-Saharan Africa requires further research.

Iron deficiency and infection | HIV

HIV often presents with malnutrition and anaemia, and as iron deficiency is generally considered the most important micronutrient deficiency causing HIV-associated anaemia, iron supplementation is often provided to these children. However, the contribution of iron deficiency to anaemia in HIV-infection is unclear. Poor dietary iron intake and reduced intestinal absorption, possibly due to infections, could theoretically result in a low iron status. On the other hand, there is evidence that iron metabolism is altered in HIV-infection, resulting in an immune-mediated increase in iron stores. Increased iron levels have been associated with advanced stages of HIV disease and mortality; and iron supplementation in this context may be detrimental. There is a need to determine the prevalence of iron deficiency in HIV-infected children in order to determine the need for iron supplementation.
Study setting

Malawi is a land-locked country in the south-eastern part of Africa of which 20% is covered by Lake Malawi (Figure 4). The climate is tropical, but prevalence of malaria and other infectious diseases vary with proximity to the lake and altitude. Estimates of iron deficiency prevalence vary from 20-40% \(^{8, 21}\). Study participants were recruited in two settings; Blantyre district (study sites: Queen Elizabeth Central Hospital and CURE Orthopedic Hospital), and Chikwawa District (study site: Chikwawa District Hospital), both in the southern region of Malawi. Blantyre is the main commercial town of Malawi with a predominantly urban population of half a million. At an altitude of 800m above sea level malaria is mainly seasonal (approximately 1 infectious bite per person per year\(^{20}\)). Chikwawa District Hospital, which caters for a predominantly rural population of approximately 400 thousand people, is situated in the lower Sire Valley, 50 km south of Blantyre. With an altitude of 250 m above sea level malaria transmission is year round (approximately 170 infectious bites per person per year) (Milahowa T, personal communication).

Study Design

The first part of this thesis concerns secondary analyses of a large data set from a case control study investigating the aetiology, pathophysiology and outcome of severe anaemia in southern Malawi \(^{14, 70}\) (Figure 4). For this study three groups of children were recruited between 2002 and 2004 in an urban and rural setting in Southern Malawi. Cases were children (aged 6-60 months) presenting with severe anaemia (haemoglobin <5.0 gram per decilitre). For each case, two control children were enrolled, one community control living within 100 to 1000 meters of the investigated case, and one hospital control, presenting at the same hospital or outpatient facility as the case. Controls were eligible for recruitment if aged 6-60 months and if their haemoglobin level was at least 5.0 gram per decilitre. Cases, hospital and community controls were recruited in a ratio of 1:1:1. Before enrolment in the study, written informed consent was obtained from the parent or guardian of all study participants. At recruitment a detailed medical and socio-economic history was recorded and a physical examination was performed. Prior to blood transfusion samples of blood, stool and urine were collected. In cases only a bone marrow aspirate was performed under general anaesthesia. Children were treated if indicated using local treatment guidelines. Clinical malaria was defined as a positive blood slide with concurrent fever (axillary temp >37.5\(^{\circ}\) C), or history of fever (caregiver recall of fever in the last week). Severe malaria was defined as a positive blood slide with either severe anaemia (haemoglobin<5.0 g/dl), or coma\(^{71}\). Malaria was treated with sulfadoxine-pyrimethamine (SP) and if the child was unable to take oral medication parenteral quinine was administered. As a standard procedure at recruitment all study participants received presumptive malaria treatment (25.0/1.25 mg/kg SP), and according to local guidelines all children received iron supplementation 2 mg/kg/day for 28 days. Follow-up procedures included further assessment of medical history, physical examination and a blood sample to determine haemoglobin and malaria parasitaemia, and if indicated, children were treated using local treatment guidelines. Deaths were recorded, and if they occurred outside the study clinics they were investigated as completely as possible using a verbal autopsy procedure. This study was approved by the Ethics Committees of the College of Medicine, Malawi and the Liverpool School of Tropical Medicine, United Kingdom.

The second part of the thesis concerns a study that was conducted between March and October 2011. Study participants were identified from children aged 6–66 months, scheduled for elective surgery at Queen Elizabeth Central Hospital and Beit CURE Orthopedic Hospital. Exclusion criteria were: blood transfusion within the previous 4 weeks, signs of infection (axillary temperature >37.5 \(^{\circ}\)C or current infectious diagnosis, suspected neoplasm, known haemoglobinopathy, or a hemoglobin level below 8.0 g/dl. (local guidelines for elective surgery). Prior to enrolment, written informed consent was obtained from the parent or guardian
of each child. The recruitment procedure included a detailed history and physical examination, a venous blood sample and a bone marrow aspiration. Both samples were collected during generalized anesthesia and prior to surgical intervention. The study was approved by the Ethical Committees of the College of Medicine, Malawi and of the Academic Medical Centre of Amsterdam, the Netherlands.

Figure 4. Malawi

Aims and outline of the thesis

The aim of this thesis is to provide insights into the complex factors which affect iron metabolism and their relation with (severe) anaemia and infection.

The specific objectives were as follows:

• To review currently available guidelines for management of severe anaemia
• To assess prevalence of hookworm in Malawian children using the novel real-time PCR test and to determine its contribution to severe anaemia and iron deficiency.
• To study the potential protective effect of iron deficiency on susceptibility to malaria in Malawian children
• To review the prevalence of iron deficiency in HIV-infected children.
• To assess the performance of hepcidin as potential iron marker in severely anaemic Malawian children, and to improve the understanding of the other signalling pathways related to infection and anaemia.
• To compare peripheral iron biomarkers, including hepcidin, against bone marrow iron assessment in a healthy population of Malawian children
References


4. WHO. Conclusions and recommendations of the WHO Consultation on prevention and control of iron deficiency in infants and young children in malarialendemic areas. 2007.


15. WHO. Management of the child with a serious infection or severe malnutrition. 2000.


42. Steele TM, Frazer DM, Anderson GJ. Systemic Regulation of Intestinal Iron Absorption. 2004. Iron Metabolism Laboratory, Queensland Institute of Medical Research and, School of Medicine, University of Queensland.
Chapter two

Severe acquired anaemia in Africa: new concepts

M. van Hensbroek, E.A.M. Jonker, I. Bates

British Journal of Haematology, 2011 Jun 28; 1365-2141
Abstract

Severe anaemia is common in Africa. It has a high mortality and particularly affects young children and pregnant women. Recent research provides new insights into the mechanisms and causes of severe acquired anaemia and overturns accepted dogma. Deficiencies of vitamin B12 and vitamin A, but not of iron or folic acid, are associated with severe anaemia. Bacterial infections and in very young children, hookworm infections, are also common in severe anaemia. Irrespective of the aetiology, the mechanism causing severe anaemia is often red cell production failure. Severe anaemia in Africa is therefore a complex multi-factorial syndrome which, even in an individual patient, is unlikely to be amenable to a single intervention. Policies and practices concerning anaemia diagnosis, treatment and prevention need to be substantially revised if we are to make a significant impact on the huge burden of severe anaemia in Africa.
Chapter two | Severe acquired anaemia in Africa

Introduction
Severe anaemia in developing countries is a common syndrome with a high mortality\(^1\) which has received surprisingly little research attention. In Africa anaemia affects 68% of pre-school children, 57% of pregnant women and 48% of non-pregnant women\(^2\) but young children and pregnant women are particularly at risk of severe anaemia. Little is known about the complex interactions between the many factors that lead to severe anaemia so recommendations for preventing and treating severe anaemia are often not underpinned by evidence\(^3\). In this article we highlight recent research findings that provide new insights into the mechanisms and causes of acquired severe anaemia in Africa and which have the potential to generate novel approaches for its treatment and prevention.

Search strategy and selection of articles
There is no universally accepted definition of severe anaemia\(^2,4\) so for this review, unless otherwise stated, we have used the most frequently quoted haemoglobin threshold of <50 g/l. With assistance from a specialist clinical librarian, a search of the following databases and websites was conducted for information on ‘severe anaemia’, ‘sub-Saharan Africa’, ‘humans’ and ‘Africa (including the specific names of 57 African countries, nationalities and sub-regions: MEDLINE (National Library of Medicine, Bethesda, MD), EMBASE (Elsevier, Amsterdam, the Netherlands), the Cochrane Central Register of Controlled Trials (CENTRAL) (Wiley InterScience, Hoboken, NJ), ‘ClinicalTrials.gov’, “National Guideline Clearinghouse”, “National Library of Guidelines” and “Guideline International Network”, as well as World Health Organisation (WHO), the WHO regional office for Africa and UNICEF websites. Information from 1990 to February 20\(^{th}\) 2011 was collected and different spelling and languages of keywords were included if relevant.

Nine hundred and nineteen articles were identified through MEDLINE and 1110 through Embase. One thousand articles were retained for review after excluding 1029 articles because they were duplicate studies, case reports, letters or published prior to 1990. Using additional filters these articles were sub-categorised into “Epidemiology” (72 articles) “Aetiology/etiology” (425 articles), “Diagnosis” (82 articles), “Treatment” (123 articles), “Prevention” (45 articles) and “General” (253 articles). The ‘Clinical trials.gov’ search identified 43 trials related to severe anaemia since 1990 in Africa of which 31 had been completed and 12 were still recruiting.

Epidemiology
Anaemia is a moderate or severe public health problem in almost all countries in sub-Saharan Africa and has a significant morbidity and mortality. Severe anaemia features in the global policies of many programmes including those dealing with maternal, child and adolescent health, malaria, HIV, helminth infections, nutrition and tuberculosis. However population data on severe anaemia prevalence is scarce because it is generally documented in the context of a specific disease or in sub-populations (e.g. hospital in-patients)\(^5,7\). In community studies in Tanzania and Malawi 1% of children aged 6-60 months had severe anaemia\(^8,9\). 4% of children in primary level facilities In Ethiopia\(^10\), 11% of children in a rural Zambian hospital and 27% of children admitted to a tertiary hospital in The Gambia\(^11,12\) were severely anaemic. In Kenya 17% of pregnant
women had severe anaemia (Hb <70 g/dl)13. It is surprising that despite the extremely high prevalence of anaemia in pregnancy, international ‘general standards of care’ indicators for healthy pregnancy and childbirth do not include haemoglobin levels or anaemia14.

Aetiology and pathogenesis

Little is known about the aetiology of severe anaemia in Africa from a public health perspective as most studies have investigated anaemia in relation to specific diseases (e.g. malaria, HIV infection) or individual factors (e.g. folate or iron deficiency)15, 16. However, many factors are potentially important in the development of severe anaemia and several factors may occur together in an individual patient.

Aetiology and pathogenesis | Infection
Malaria is considered to be the principal cause of severe anaemia in malaria-endemic areas in Africa. At the population level a clear association has been found between malaria and severe anaemia17-18. However, the relationship between malaria and severe anaemia in individuals is highly variable with odds ratios varying between 0.8 and 3.5 depending on the intensity of malaria transmission17. It is therefore important to consider non-malaria causes for severe anaemia in an individual even when their malaria test is positive.

Severe anaemia is common in individuals with HIV infection, particularly if they are co-infected with tuberculosis19. 19% of hospitalised adults in Malawi had severe anaemia; of these 79% were HIV positive and 28% died in hospital20. The association between HIV and severe anaemia can partly be explained by the potential of the HIV virus (especially X4 strains which use the CxCR4 co-receptor) to infect bone marrow stem cells and promote apoptosis17, 21. A strong association between bacteraemia, particularly non-typhoid salmonella, and severe anaemia has been described in both children and adults with a blood culture positivity rates of 15 and 27%, respectively17, 20. The association is strongest in areas where HIV infection is endemic probably because of increased susceptibility to bacterial infections.

In rural areas in Africa hookworm infection is a well recognised cause of severe anaemia. There are several interesting new findings concerning this association. In Kenya and Malawi, hookworm infection is common in children under 2 years old with severe anaemia. This is surprising as young children have traditionally not been thought to have a high burden of hookworm infection17, 18. In Malawi the less common but more virulent *Ascaris lumbricoides* hookworm species, occurs more frequently in children with severe anaemia compared to controls17 and in Malawian adults, heavy hookworm infections were found in 38% of HIV negative, but only 2% of HIV positive, individuals20.

Aetiology and pathogenesis | Nutrition
The role that iron deficiency plays in the aetiology of severe anaemia has been the subject of debate for decades. Iron deficiency thought to be responsible for around 50% of all cases of anaemia. However this figure may not be reliable because anaemia has been used as a proxy for iron deficiency22. It is difficult to determine the true prevalence of iron deficiency in Africa because serum ferritin is an acute phase protein and therefore not a helpful indicator where infectious diseases are common. Estimations of iron stores in bone marrow aspirates in Malawian children indicated that the threshold of ferritin should be raised almost 10-fold in order to detect iron deficiency23. Bone marrow sampling is not a practical investigation to determine iron status so, based on available evidence, WHO policies recommend that serum ferritin and transferrin receptor are used to gauge the iron status of populations24.
Chapter two | Severe acquired anaemia in Africa

Recently a negative association between iron deficiency and severe anaemia was found in Malawian children, which was partly explained by an inverse relationship between iron deficiency and bacteraemia\textsuperscript{17}. This supports the hypothesis that iron deficiency protects against infection by creating an unfavourable environment for bacterial growth\textsuperscript{25, 26}. Observations of increased morbidity and mortality in iron supplementation studies conducted where bacterial infections are common (e.g. in children in Tanzania) further corroborate this hypothesis\textsuperscript{27, 28}.

Folic acid deficiency is uncommon in children\textsuperscript{17, 29, 30} and pregnant women in Africa\textsuperscript{31}. In contrast, vitamin B12 deficiency occurs in up to 30\% of African children and adults with severe anaemia possibly due to a lack of dietary animal products\textsuperscript{17, 31}. Severe vitamin A deficiency occurs in about one third of severely anaemic African adults and children\textsuperscript{31, 32}. Interestingly, it has recently been shown that the association between Vitamin A and severe anaemia could partly be explained by the effect of vitamin A deficiency on susceptibility to malaria and bacterial infection\textsuperscript{17}. Although vitamin A supplementation can reduce the incidence of malaria it has surprisingly little effect on the incidence of severe anaemia in children\textsuperscript{33, 34}.

Aetiology and pathogenesis | New concepts
Several distinct mechanisms, including haemolysis, blood loss and red cell production failure (RCPF), may lead to anaemia and individual factors (e.g. iron deficiency, malaria) may contribute to more than one mechanism\textsuperscript{16,33}. In Malawian children with severe anaemia 48\%, 22\% and 7\% were found to have indications of RCPF, haemolysis and blood loss respectively; multiple aetiologies were common in individual children. RCPF, unlike haemolysis or blood loss, is likely to persist unless all of the factors contributing to its aetiology are corrected. The combination of factors that ultimately result in severe anaemia is likely to vary from region to region or between different age groups or populations within a region\textsuperscript{17,18,20,38,36}. For example, hookworm is an important cause of severe anaemia in rural but not urban areas, and HIV infection is important in adults in urban East Africa but not rural West Africa\textsuperscript{17}. This new evidence concerning the variability of factors that contribute to pathogenesis of severe anaemia, and particularly to RCPF, may explain why the current ‘blanket’ approach to prevention and treatment has not been effective in reducing the burden severe anaemia in Africa\textsuperscript{35}.

Diagnosis
There are inconsistencies in international strategies concerning the diagnosis of anaemia. For example policies concerning iron and folate supplementation in pregnancy, imply that clinical diagnosis is adequate to diagnose anaemia and monitor changes in individuals\textsuperscript{14, 37}, whereas HIV policies require a diagnosis of severe anaemia to be confirmed by a laboratory test\textsuperscript{38}. Clinical assessment remains the most commonly used method for diagnosis of anaemia but its effectiveness for identifying patients with severe anaemia (Hb <50 g/l) varies widely. In children the sensitivity and specificity of clinical examination for pallor (palms, conjunctivae, nail-beds and/or tongue), either alone or in combination with other clinical signs, vary from 53 to 96\% and 57 to 91\% respectively\textsuperscript{6-10}. In pregnant women with severe anaemia (Hb <70 g/l) the sensitivity and specificity of clinical diagnosis by midwives was 65\% and 82\% respectively\textsuperscript{39} though improvements were possible with training and close supervision.

Accurate diagnosis of severe anaemia is critical because the treatment may include blood transfusions which are a scarce resource, and risky and expensive in Africa. Clinical examination is not accurate enough for detecting the 10-20 g/l changes in haemoglobin level that are likely to occur in response to treatment and so
accurate laboratory test are required. The direct cyanmethaemoglobin method is the gold standard for haemoglobin measurement\textsuperscript{46} but it requires sample dilution, electrical power and cyanide buffer, which is increasingly difficult to obtain. Two other methods, the HemoCue and the Haemoglobin Colour Scale, have been used for field studies in Africa because they are simple and do not require sample dilution or a mains electricity supply\textsuperscript{46, 41}. The HemoCue system\textsuperscript{8} provides a reliable, rapid one-step haemoglobin determination with a sensitivity of 80-96.6\% compared to standard laboratory methods\textsuperscript{8, 22}. The main drawback is the cost of the disposable cuvettes which may make this technique expensive if the volume of tests is high. The HemoCue Hb301 version has been specifically designed to operate in humid conditions and at high temperatures, and uses cuvettes that are less expensive compared to earlier models. The Haemoglobin Colour Scale is a simple, inexpensive, semi-quantitative method but more studies are needed to evaluate whether it is superior to clinical diagnosis for detecting severe anaemia in routine use\textsuperscript{42}. Despite its variable sensitivity (24-63\%) for detecting severe anaemia, the Haemoglobin Colour Scale is reported to be able to improve treatment rates from <10\% to >65\% for children with severe anaemia\textsuperscript{43-46}.

**Management and prevention**

Many policies concerning the treatment and prevention of anaemia have been based on the assumption that iron deficiency is the most common cause of anaemia. For example the current WHO recommendation for preventing anaemia in pregnancy is daily supplementation with iron and folic acid\textsuperscript{5, 47}. However this strategy means that other potentially preventable or treatable co-morbidities, such as bacteraemia and other vitamin deficiencies may be missed \textsuperscript{48-50}. There is conflicting evidence about the value of the traditional approach of treating anaemia presumptively with iron and folate. In some studies, iron supplementation was effective for reducing anaemia, but in others it had no effect on haemoglobin levels and it has even been linked to increased mortality rates in children who are not iron deficient \textsuperscript{51-54}. Folic acid supplementation in Africa generally has very little effect on haemoglobin levels \textsuperscript{48, 55-57} and there is some evidence to suggest that the efficacy of anti-folate antimalarial treatment may be reduced by folic acid supplementation.

Blood culture facilities are often not available in Africa and the high prevalence of bacteraemia, mainly non typhoid salmonella, in severe anaemia raises the question of whether antibiotic treatment should be recommended presumptively for severe anaemia \textsuperscript{48, 58}. Currently in malaria endemic areas in Africa, severely anaemic patients are presumptively treated for malaria \textsuperscript{59}. Successful prevention of *Plasmodium falciparum* infections in endemic areas reduces the risk of severe maternal anaemia by 38\% \textsuperscript{60} and this has led to the recommendation that pregnant women in malarious areas who have severe anaemia should be treated presumptively for malaria \textsuperscript{61}. However current malaria policies recommend that anti-malarials should only be prescribed for proven infections such as those demonstrated by rapid diagnostic malaria tests\textsuperscript{72}. In areas with a high incidence of hookworm infections, anti-helminthic treatment may need to be considered for patients with severe anaemia, especially children <2 years of age and HIV-negative adults \textsuperscript{48, 58}.

Blood transfusion can be life-saving for patients with severe anaemia \textsuperscript{62, 63} and is recommended for pregnant women of >34 weeks gestation with a haemoglobin concentration <70 g/l \textsuperscript{61}. WHO guidelines for treating children with uncomplicated severe anaemia and a haemoglobin <40 g/l recommend using 20ml/kg of whole blood (or 10ml/kg packed cells) but transfusions are not recommended for children with a haemoglobin of 40-60 g/l \textsuperscript{64}. Strict adherence to these guidelines has resulted in under-transfusion of a significant proportion of severely anaemic Kenyan and Malawian children \textsuperscript{65} (M. Esan personal observation) and may contribute to the high post discharge mortality rates observed in some children \textsuperscript{66, 67}. There are concerns that this policy, and possibly also the recommendations concerning the volume of blood to be transfused, may be too conservative given the high rates of persistent severe anaemia, re-admission and death following treatment of severe anaemia.
Traditionally fluid resuscitation had not been given to children with severe anaemia complicated by shock whilst they were waiting for their blood transfusion. This because of concerns about dilutional anaemia or cardiac decompensation but recently it has been shown that fluid resuscitation is safe for severely anaemic children with circulatory failure.

Management and prevention | Global perspective
Our search strategy focused exclusively on evidence about severe anaemia from Africa. However, many of the issues we have highlighted relate to a lack of resources for fully investigating and treating patients with severe anaemia. Our findings, and in particular, the need to devise standard treatment packages based on knowledge of locally prevalent causes of anaemia, are therefore likely to be applicable to low-income settings beyond Africa. Clinicians in wealthy countries caring for patients from Africa with severe acquired anaemia, will need to consider causes such as malaria or infection with HIV or hookworm (see box) as well as the high likelihood of multiple factors operating in an individual, in addition to their usual list of differential diagnoses.

Over a decade ago it was recognised that a new multi-sectoral approach was needed to generate the evidence needed address the complex aetiology of anaemia and to reduce the burden of anaemia. However, at global and national levels there is still a lack of agreement about strategies to prevent, diagnose and manage severe anaemia. Currently evidence to guide these strategies is fragmented and concentrated in just a few countries. More research is needed to establish whether this evidence is applicable to other low-income countries in Africa and beyond.

Table 1. Summary of recent research evidence concerning causes of severe acquired anaemia in Africa

<table>
<thead>
<tr>
<th>Common causes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malaria, especially <em>P. falciparum</em></td>
</tr>
<tr>
<td>Bacteraemia, especially non-typhoid salmonella</td>
</tr>
<tr>
<td>HIV infection, especially in association with tuberculosis</td>
</tr>
<tr>
<td>Hookworm (<em>Necator americanus</em>, and <em>Ankylostoma duodenale</em>)</td>
</tr>
<tr>
<td>Vitamin A deficiency</td>
</tr>
<tr>
<td>Vitamin B12 deficiency</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Uncommon causes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron deficiency</td>
</tr>
<tr>
<td>Folate deficiency</td>
</tr>
</tbody>
</table>
Conclusions

Prevention and management of severe anaemia must be underpinned by a detailed knowledge of the aetiology but because the aetiology varies geographically, seasonally and between sub-populations, it is difficult to devise strategies that are universally applicable. Over the last decade it has become increasingly apparent that severe anaemia in Africa is a complex multi-factorial syndrome which is unlikely to be amenable to a single intervention. More research and strong partnerships between vertical programmes are needed to identify and address the causes of severe anaemia in a variety of settings and populations. In order to make a significant impact on the huge burden of severe anaemia in Africa this new evidence needs firstly to be replicated in other countries and then used to develop treatment and prevention packages that are tailored to address specific local causes of severe anaemia.

Acknowledgements and contributions

MBvH and FJ conducted the search for research literature concerning severe anaemia and IB conducted the search for policy documents. All authors made substantial contributions to the design of this review, and to the acquisition and synthesis of information. They all contributed to drafting and revising the paper and all approved the final version.
References

27. Sazawal S, Black RE, Ramsan M et al. Effects of routine prophylactic supplementation with iron and folic acid on admission to hospital and mortality in preschool children in a high malaria transmission setting: community-
based, randomised, placebo-controlled trial. Lancet 2006;367(9505):133-143.
63. WHO. Hospital Care for Children: guidelines for the management of common illnesses with limited resources. 2005.
Chapter three

Real-time PCR demonstrates *Ancylostoma duodenale* is a key factor in the aetiology of severe anaemia and iron Deficiency in Malawian pre-school children


PloS Neglected Tropical Diseases 2012; 6(3)
Abstract

Hookworm infections are an important cause of (severe) anaemia and iron deficiency in children in the tropics. The type of hookworm species (Ancylostoma duodenale or Necator americanus) and infection load are considered associated with disease burden, although these parameters are rarely assessed due to limitations of currently used diagnostic methods. Using multiplex real-time PCR assay, we evaluated hookworm species prevalence, infection load and their contribution towards severe anaemia and iron deficiency in pre-school children in Malawi.

A. duodenale and N. americanus DNA loads were determined in 830 faecal samples of pre-school children participating in a case control study investigating severe anaemia. Using multiplex real-time PCR, hookworm infections were found in 34.1% of the severely anaemic cases and in 27.0 % of the non-severely anaemic controls (p<0.05) whereas a 5.6% hookworm prevalence was detected by microscopy. Prevalence of A. duodenale and N. americanus was 26.1 % and 4.9% respectively. Moderate and severe A. duodenale infections were positively associated with severe anaemia (adjusted odds ratio: 2.49 (95% CI 1.16-5.33) and 9.04 (95% CI 2.52-32.47) respectively). Iron deficiency (assessed through bone marrow examination) was positively associated with intensity of A. duodenale infection (adjusted odds ratio: 3.63 (95% CI 1.18-11.20); 16.98 (95% CI 3.88-74.35) and 44.91 (95% CI 5.23-385.77) for low, moderate and high intensity respectively).

This is the first report assessing the association of hookworm intensity and species differentiation with severe anaemia and bone marrow iron deficiency. By revealing a much higher than expected prevalence of A. duodenale and its significant association with severe anaemia and iron deficiency in pre-school children in Malawi, we demonstrated the need for quantitative and species specific screening of hookworm infections; multiplex real-time PCR is a powerful diagnostic tool for public health research to combat (severe) anaemia and iron deficiency in children living in resource poor settings.
Chapter three | *Ankylostoma duodenale*, severe anaemia and iron deficiency

**Introduction**

*Ankylostoma duodenale* and *Neator americanus* are soil transmitted nematodes responsible for an estimated 576-740 million human hookworm infections worldwide\(^1\)\(^-\)\(^3\). Hookworm infection often leads to anaemia and iron deficiency, major causes of sickness and delayed cognitive development, especially in pre-school children\(^4\). Hookworm, therefore, is one of the most important infections in terms of disease burden in developing countries \(^5\)\(^-\)\(^7\) with a major impact on public health \(^8\)\(^-\)\(^11\).

The intensity of the hookworm infection is related to the severity of disease \(^5\)\(^,\)\(^10\). As adult hookworms attach to and feed from the bowel mucosa of the infected host, they are the direct cause of intestinal blood loss, which often gives rise to iron deficiency and anaemia. Crompton and Whitehead proposed a model describing the relationship between the actual number of adult worms present in the intestines, and the host’s iron status; increased infection load was associated with lower iron levels \(^12\)\(^,\)\(^13\). The conventional method for determination of infection load is done with a proxy marker, number of eggs, using Kato-Katz microscopy slides with a fixed amount of faeces \(^14\). However, although useful for estimating prevalence in highly endemic areas, the use of Kato-Katz microscopy for estimating intensity is laborious and frequently omitted.

*N. americanus* and *A. duodenale* are considered to have distinct geographical distribution; *N. americanus* is predominant in tropical environments, whereas *A. duodenale* is adapted to colder and drier circumstances \(^15\)\(^,\)\(^16\). Based on studies of experimental human hookworm infections using labelled erythrocytes, daily blood loss is estimated to range from 0.03 to 0.30 ml per worm per day, with *A. duodenale* causing 2 to 10 times more blood loss per worm than *N. americanus* \(^12\)\(^,\)\(^13\). This is consistent with a few detailed clinical studies which indicated a stronger association of *A. duodenale* infection with anaemia than *N. americanus* \(^10\)\(^,\)\(^15\)\(^,\)\(^17\). Due to the complexity of conventional diagnostic procedures (microscopy by a skilled technician after 7 days of stool culture), differentiation of hookworm species rarely has been done in population-based surveys \(^18\)\(^,\)\(^19\). This has been justified on the basis that prevalence of *A. duodenale* infection was limited and therefore the contribution to global disease burden was low, despite causing higher blood loss \(^15\)\(^,\)\(^20\). Furthermore, as both species respond to the same anthelmintic treatment, differentiation is not considered essential for treatment. Yet post-treatment data is scarce and extremely little is known about the species-specific effects of mass drug administration \(^10\)\(^,\)\(^21\).

An alternative diagnostic procedure is multiplex real-time PCR which allows species-specific identification, as well as semi-quantification of parasite DNA in human stool samples; based on selected targets and tested on a panel of well-defined DNA and stool samples, these assays were found to be 100% specific and substantially more sensitive compared with microscopy \(^22\)\(^-\)\(^25\). Through the use of different fluorescent labels in a closed system, multiple targets can be detected simultaneously within a single reaction tube with a low risk of contamination, which is ideal for high-throughput analysis. These assays offer significant potential for large scale population based surveys.

The objective of this study was, using a species-specific multiplex real-time PCR, to determine prevalence of the two hookworm species, infection load and species association with severe anaemia and bone marrow iron deficiency in pre-school Malawian children.
Methods

Methods | Study design and study population

The study was part of a large case-control study on severe anaemia in pre-school Malawian children. A detailed description of the study has been previously published elsewhere 26, 27. In brief three groups of children were recruited between 2002 and 2004 in an urban and rural setting in Southern Malawi. Cases were children (aged 6-60 months) presenting with severe anaemia (haemoglobin<5.0 gram per decilitre). For each case, two control children were enrolled, a community control living within 100 to 1000 meters of the case, and a hospital control, presenting at the same hospital or outpatient facility as the case. Controls were eligible for recruitment if aged 6-60 months and if their haemoglobin level was at least 5.0 gram per decilitre.

Methods | Ethics Statement

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. Written informed consent was obtained from the parent or guardian.

Methods | Laboratory investigations

Haemoglobin (Hb) concentrations measured by HaemoCue B-Haemoglobin analyser (HaemoCue, Ångelholm, Sweden) were used to assess eligibility for the study; results of full blood count analyses by Coulter counter analyser (Beckman Coulter, Durban, South Africa) were used for statistical analyses. In cases only, a bone marrow sample was collected, and slides were stained with Hematognost Fe (Merck, Darmstadt, Germany) and graded for iron content 28. Bone marrow examination was performed as part of the main study to investigate etiology of severe anaemia. Bone marrow smears were assessed by a histological grading method which classifies iron status into six grades, iron deficiency was defined as a bone marrow smear score of 0 or 129. In all study participants a stool sample was collected for parasitological examination. In order not to delay required treatment in case of hookworm infection, stool samples were examined for ova by microscopy of direct smears and a single 25 mg Kato-slide 30, 31. The slides were read within 30 minutes by well trained microscopists and the number of hookworm eggs and presence of other helminth infections were recorded. An aliquot (approximately 0.5 gram) was stored at -20 °C for later DNA isolation which was performed in a separate room at the research laboratory in Malawi. PCR was performed in Leiden University, the Netherlands. To prevent contamination with PCR products every step of the procedure was performed in separate rooms. DNA was isolated using face suspensions of 200 μl (± 0.5 gram of stool per ml PBS containing 2 % poly-vinyl-poly-pyrolidone (Sigma, Steinheim, Germany)) and heated for 10 minutes at 100°C. After sodium-dodecyl-sulphate-proteinsase K treatment (overnight at 55°C), DNA was isolated with QIAampTissue Kit spin columns (QIAGen, Hilden, Germany) 32. Extracted DNA samples were transported for multiplex real-time PCR assessment in the Netherlands. For detection of inhibition of the amplification process, DNA of Phocin Herpes Virus 1 (PhHV-1) was added to each PCR mixture as an internal control and detected by PhHV-1 specific primers and probe 33. A. duodenale and N. americanus multiplex real-time PCR was performed as described previously; targeting the Internal Transcribed Spacer 2 (ITS-2) sequence of each species and using minor groove binding detection probes 35. ITS-2 is a DNA target part of the whole genome present in all life stages. In brief, amplification reactions were performed in a volume of 25 μl with PCR buffer (HotstarTaq master mix, QIAGen, Germany), 5 mM MgCl₂, 2.5 μgram Bovine Serum Albumin (Roche Diagnostics Nederland B.V., Almere, the Netherlands), 1.5 pmol of each A. duodenale-specific primer, 2.5 pmol of NED-labeled A. duodenale-specific MGB-Taqman probe (Applied Biosystems, Warrington, U.K.), 5 pmol of each N. americanus-specific primer, 2.5 pmol of FAM-labeled N. americanus-specific MGB-Taqman probe (Applied Biosystems, Warrington, U.K.), 3.75 pmol of each PhHV-1-specific primer, and 2.5 pmol of
Chapter three | *Ancylostoma duodenale*, severe anaemia and iron deficiency

PhHV-1-specific Cy5-double-labeled probe (Biolegio, Nijmegen, The Netherlands) and 5 μl of the DNA sample. Amplification consisted of 15 min at 95°C followed by 50 cycles of 15 s at 95°C and 60 s at 60°C. Negative and positive controls were included in each amplification-run. Amplification, detection and data analysis was performed with the AB7500 real-time PCR system (Applied Biosystems, Warrington, U.K.). The cycle threshold (Ct), meaning the amplification cycle in which the level of fluorescent signal exceeds background fluorescence, was used as the PCR output, reflecting parasite species-specific DNA load in the stool samples tested. Hookworm intensity categories were defined as following: low (Ct >35.0 and \(<50\)), moderate (Ct \(>25.0\) and \(\leq50.0\)) or high (Ct \(\leq25.0\)). None of the PCR analyzed samples were excluded due to inhibition as all runs showed a Ct-value for the internal PhHV-1 control within the expected range.

Methods | Statistical methods

Data were analyzed with the use of STATA 9 (Stata Corporation, TX) and PASW Statistics 18 (SPSS, Chicago, Illinois) statistical computer packages. Cross-sectional analyses were completed to assess the correlation of severe anaemia and iron deficiency with PCR detected hookworm load per species. Using Chi-square test hookworm prevalence per species was compared individually across severely anaemic and non-severely anaemic as well across iron deficient and iron-replete study groups. Spearman’s rank correlation (\(\rho\)) was used for calculation of concordance between Ct-values with haemoglobin and number of iron fragments in the bone marrow. The combined association of characteristics related to risk of severe anaemia and iron deficiency was examined by two multivariate logistic-regression models, correcting for potential confounding factors. Potential aetiologies of severe anaemia were entered in the model if they were associated with severe anaemia in univariate analysis (p \(<0.10\)). The model was adjusted for subjects’ baseline characteristics (age, sex) and other potential confounders: recent haematinics or anti-malarial treatment, history of transfusions and death of a parent [22]. With this model we compared all cases with the two control groups combined, stratified for study location. To explore the possibility that different patient characteristics were important in the two control groups, separate secondary analyses were performed with the community and hospital control groups. To specify the association between iron deficiency (dependent variable) and hookworm (independent variable) we adjusted for subjects’ baseline characteristics (age, sex, study location) and other potential confounders (HIV infection and wasting (defined as a Z-score of weight for height \(<-2\)\(^{34}\)). Stepwise backward multiple logistic regression analyses were performed; \(p\) values less than 0.05 were considered as statistically significant. The latter analyses only included case patients with bone marrow aspirate results. For both analyses attributable-risks were calculated using adjusted odds ratios \(^{35}\). When both hookworm species were analyzed together, the lowest Ct-value was counted. Reported \(p\)-values are two-sided.

Results

Stool samples of 830 (72.9%) of the 1138 children enrolled in the severe anaemia study were stored for real-time PCR analysis. Table 1 summarizes mean haemoglobin and the general characteristics per study group. The mean age was lower in the case group; for which we adjusted in the multivariate model. Other baseline characteristics were not statistically different. Bone marrow iron examination was performed in a subgroup of 160 severely anaemic children (cases) (Table 2). Baseline characteristics were not significantly different between children with or without available stool sample or bone marrow sample (data not shown).
Table 1. Baseline characteristics of 830 hookworm-PCR tested children stratified per study group

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Cases Hb ≤5.0 g/dL (N=252)</th>
<th>Hospital Controls Hb &gt;5.0 g/dL (N=291)</th>
<th>Community Controls Hb &gt;5.0g/dL (N=287)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Living in an urban area</td>
<td>134 (53.2 %)</td>
<td>150 (51.5 %)</td>
<td>146 (50.9 %)</td>
</tr>
<tr>
<td>Male</td>
<td>119/252 (47.2 %)</td>
<td>147/291 (50.5 %)</td>
<td>139/287 (48.4 %)</td>
</tr>
<tr>
<td>Age in months (mean ± SD)</td>
<td>19.9 ± 12.6</td>
<td>22.7 ± 12.0</td>
<td>25.6 ± 13.6</td>
</tr>
<tr>
<td>Haemoglobin*in g/dL (mean ± SD)</td>
<td>3.6 ± 0.8</td>
<td>9.6 ± 2.2</td>
<td>9.9 ± 1.9</td>
</tr>
</tbody>
</table>

*n=826

Table 2. Baseline characteristics of severely anaemic cases stratified per iron status

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Iron deficient† (n=68)</th>
<th>Iron replete§ (n=92)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Living in an urban area</td>
<td>45 (66.2 %)</td>
<td>52 (56.5 %)</td>
</tr>
<tr>
<td>Male</td>
<td>25 (36.8 %)</td>
<td>49 (53.2 %)</td>
</tr>
<tr>
<td>Age in months (mean ± SD)</td>
<td>18.1 ± 10.4</td>
<td>21.6 ± 13.8</td>
</tr>
<tr>
<td>Iron fragments (mean ± SD)</td>
<td>0.3 ± 0.5</td>
<td>3.8 ± 1.0</td>
</tr>
</tbody>
</table>

† Iron deficiency was defined as a bone marrow iron grade of none (grade 0) or very slight (grade 1). § Iron replete means sufficient iron (≥ grade 2).

Results

Hookworm

Microscopy was done on 780 (94.0%) of the 830 stool samples. Failure was due to small sample stool volume or constitution (too watery for Kato-slide examination). Hookworm eggs were identified in 44 of 780 (5.6%) samples, of which 18 (2.3%) had a high-load infection, defined by more than 1.000 eggs per gram faeces (epg). In the severely anaemic children (cases), significantly more high-load hookworm infections were detected compared to the controls; 5.9% (14/236) vs. 0.7% (4/544), p < 0.001. Real-time PCR identified 242 (29.2%) hookworm infections in the 830 children. A. duodenale and N. americanus DNA was detected in respectively 217 (26.1%) and 41 (4.9%) children. Six children were infected with both species. Within the healthy study population (community controls) 73 hookworm infections were detected (25.4 %) of which the larger part was caused by A. duodenale (Table 3). In 182 (83.5%) of the 218 PCR positive samples tested parasitologically, microscopy did not identify the hookworm infection, 130 (71.4%) of these samples showed low quantities of hookworm DNA (Ct >35). Furthermore in 8 (18.2%) of the 44 microscopy positive samples the presence of hookworm was not confirmed with real-time PCR. These 8 samples had a median of 220 eggs per gram.
Table 3. PCR-determined hookworm distribution stratified per study group

<table>
<thead>
<tr>
<th></th>
<th>Cases</th>
<th>Hospital Controls</th>
<th>Community Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hb ≤ 5.0 (N=252)</td>
<td>Hb &gt; 5.0 (N=291)</td>
<td>Hb &gt; 5.0 (N=287)</td>
</tr>
<tr>
<td>Any hookworm infection</td>
<td>86 (34.1%) *</td>
<td>83 (28.5%)</td>
<td>73 (25.4%)</td>
</tr>
<tr>
<td>A. duodenale</td>
<td>81 (32.1%) **</td>
<td>72 (24.7%)</td>
<td>64 (22.3%)</td>
</tr>
<tr>
<td>Infection load</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>low</td>
<td>32 (12.7%)</td>
<td>54 (18.6%)</td>
<td>46 (16.0%)</td>
</tr>
<tr>
<td>moderate</td>
<td>27 (10.7%) **</td>
<td>15 (5.2%)</td>
<td>17 (5.9%)</td>
</tr>
<tr>
<td>high</td>
<td>22 (8.7%) ***</td>
<td>3 (1.0%)</td>
<td>1 (0.3%)</td>
</tr>
<tr>
<td>N. americanus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>positive</td>
<td>10 (4.0%)</td>
<td>19 (6.5%)</td>
<td>12 (4.2%)</td>
</tr>
<tr>
<td>Infection load</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>low</td>
<td>6 (2.4%)</td>
<td>14 (4.8%)</td>
<td>7 (2.4%)</td>
</tr>
<tr>
<td>moderate</td>
<td>3 (1.2%)</td>
<td>3 (1.0%)</td>
<td>5 (1.7%)</td>
</tr>
<tr>
<td>high</td>
<td>1 (0.4%)</td>
<td>2 (0.7%)</td>
<td>0 (0 %)</td>
</tr>
</tbody>
</table>

Infection load is defined by the following cycle thresholds (CT): low 35<CT<50; moderate 25<CT≤35; high CT≤25. In case of dual infection the lowest CT-value was counted. Cases are compared with combined control groups using Chi-square * P < 0.05 ** P < 0.01; *** P < 0.001.

Results | Anaemia
Hookworm infections were found in 34.1% of the severely anaemic cases and in 27.0 % of the non-severely anaemic controls (p<0.05). A. duodenale was found in 32.1% of the severely anaemic cases and in 23.5% of the non-severely anaemic controls (p <0.01, Table 3). Also the prevalence of high intensity infections (CT<25) of A. duodenale infections was different; 8.7% among cases and 0.7% among controls ( p <0.001). Additionally, in moderately anaemic children (Hb 5-11g/L) prevalence of A. duodenale was higher, 26.0% (105/404), than in non-anaemic children (Hb ≥11 g/dl), 17.6% (30/170) (p<0.03). There was no difference in prevalence of N. americanus infections between moderately anaemic and non-anaemic children. Within the whole study population (cases and controls combined) hookworm infection load and haemoglobin were negatively correlated (spearman correlation coefficient ρ = 0.176, p < 0.01, n=826), which was mainly due to A. duodenale (ρ = 0.173, p <0.01, n=826) and not to N. americanus (ρ = 0.042, p = 0.2, n=826). After correction for other causal factors of severe anaemia using multivariate analysis, A. duodenale remained a significant risk factor for severe anaemia; odds ratios increased with infection load and are shown in Figure 1 (colour section).

Results | Iron deficiency
Iron deficiency was prevalent in 42.5% (68/160) of severely anaemic children who had available bone marrow samples. Of the iron deficient children 60.3% (41/68) had a hookworm infection, compared to 16.3% (15/92) for the iron-replete group (p < 0.0001). This difference in prevalence was only noted for A. duodenale infections and was greater in children with a high infection load (Table 4). Infection load for A. duodenale, but not for N. americanus, was negatively correlated with the fragmental iron staining score (spearman correlation coefficient ρ = 0.536, p <0.01 and ρ =0.015, p = 0.9 respectively, n=160). The association between hookworm infection load and iron deficiency remained significant after correcting for other factors using multivariate analysis (Figure 2, colour section).
Table 4. PCR-determined hookworm distribution in severely anaemic children stratified per iron status

<table>
<thead>
<tr>
<th></th>
<th>Iron deficient† (n=68)</th>
<th>Iron replete‡ (n=92)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Any hookworm infection</td>
<td>41 (60.3%) **</td>
<td>15 (16.3%)</td>
</tr>
<tr>
<td><em>A. duodenale</em> positive</td>
<td>39 (57.4 %) **</td>
<td>12 (13.0 %)</td>
</tr>
<tr>
<td>Infection load</td>
<td>low</td>
<td>12 (17.6 %)*</td>
</tr>
<tr>
<td></td>
<td>moderate</td>
<td>13 (19.1 %) **</td>
</tr>
<tr>
<td></td>
<td>high</td>
<td>14 (20.6 %) **</td>
</tr>
<tr>
<td><em>N. americanus</em> positive</td>
<td>5 (7.4 %)</td>
<td>4 (4.3 %)</td>
</tr>
<tr>
<td>Infection load</td>
<td>low</td>
<td>0 (0.0 %)</td>
</tr>
<tr>
<td></td>
<td>moderate</td>
<td>1 (1.5 %)</td>
</tr>
<tr>
<td></td>
<td>high</td>
<td>4 (5.9 %)</td>
</tr>
</tbody>
</table>

Infection load is defined by the following cycle thresholds (Cr): low 35<Cr<50; moderate 25<Cr≤35; high Cr≤25. In case of dual infection the lowest Cr-value was counted. Cases are compared with combined control groups using Chi-square. * P < 0.05 ** P < 0.01; *** P < 0.001. † Iron deficiency was defined as a bone marrow iron grade of none (grade 0) or very slight (grade 1). ‡ Iron replete means sufficient iron (≥ grade 2).

Discussion

Using the multiplex real-time PCR test for hookworm identification we revealed the hidden burden of hookworm in pre-school children in Southern Malawi; hookworm prevalence was much higher than expected. We have shown that hookworm infections, mainly *A. duodenale*, are significantly associated with the development of iron deficiency and severe anaemia in pre-school children in Malawi. One of the strengths of this study is the sample size and comprehensive data set. Including reference standard data on iron deficiency; it is the first report assessing the association of hookworm intensity and species differentiation with iron deficiency measured with the reference standard for iron status.

Severe anaemia is a leading cause of hospital admissions and death in children living in sub-Saharan Africa, where between 12-29% of all children admitted to hospital are severely anaemic and require blood transfusion. This results in high mortality (8-17%) 36-38. The cause of severe anaemia, however, often remains unexplained. Using the same dataset, we have previously reported factors associated with severe anaemia and identified hookworm infection, diagnosed by microscopy, as a significant correlate of infection 36. This applied only to infections with a high load (> 1,000 epg). Using real-time PCR we now demonstrate that high-load infections have the greatest impact on disease burden, however also moderate *A. duodenale* infections were associated with significantly increased risk of severe anaemia. This indicates that the association of microscopy-detected hookworm with severe anaemia is an underestimation of the real impact of hookworm infections 39. The disease burden of *A. duodenale* infections may be a significant contributor to unexplained severe anaemia prevalence in sub-Saharan Africa.

Iron deficiency is often considered the same as anaemia, especially in resource poor settings where both are very common. Estimations of prevalence of iron deficiency in Malawi vary from 20-40% 40. Yet, iron deficiency without anaemia is important to recognize as it may delay cognitive development in pre-school and school-age children 6,7,41. On the other hand, in anaemia without iron deficiency, iron supplementation should
Chapter three | *Ancylostoma duodenale*, severe anaemia and iron deficiency

be avoided, since it has been associated with increased risk of infection \(^7\). \(^{42-46}\) Thus in stead of presumptive treatment of iron deficiency, prevention of iron deficiency would be rather preferred and should include the use of anthelmintics if prevalence of *A. duodenale* is as high as in this study.

Unexpected was the high prevalence of *A. duodenale* whilst we expected *N. americanus* to be the dominant specie in this area \(^{15, 16}\). This is important as we showed that *A. duodenale* was an independent risk factor for both moderate/severe anaemia and iron deficiency. Based on the results from ‘healthy’ community controls, this study suggests that *A. duodenale* was the predominant hookworm species in children below five years of age in this area of southern Malawi. Whether this pattern is similar for all ages needs to be investigated, but PCR based analysis of stool samples from a community-based cross-sectional survey in the nearby Mozambique indicated that the ratio between the two hookworm species changes with age; *A. duodenale* was the predominant species in children, whereas in adults both species were almost equally present (Van Lieshout, unpublished data). Clearly more studies are needed to determine the species specific distribution and their risk factors in different regions, and how they relate to the burden of infection.

A surprising finding was that severe anaemia was less common in children having low-load hookworm infections (35<CT<50) when compared to non-infected children (Figure 1, colour section). We considered two possible explanations. Firstly, low-load hookworm infections cause iron deficiency which protects against bacteraemia, a cause of severe anaemia \(^8\). Alternatively a low hookworm load is seen in children with an effective immune response which are able to control their hookworm infection and other infections that may cause severe anaemia. The proportional benefit of treating low-load hookworm infections requires further study as even these infections may contribute to burden of disease \(^8\).

From a public health perspective the implication of this diagnostic method could be substantial as hookworm prevalence based on conventional microscopy seems largely underestimated, with the consequence that substantial areas may unjustly remain untreated. Screening for hookworm with real-time PCR would lead to more reliable prevalence data which should benefit the efficiency of mass drug administration. In 2007 the World Health Organization stated that in areas with a hookworm prevalence of more than 20%, all pre-school and school-age children should yearly be treated with anthelmintics, and where prevalence is more than 50% they should be treated twice a year \(^{47}\). Although large scale de-worming has been proven to decrease hookworm prevalence and contribute to an improved health and well-being \(^{10, 48}\), the importance of appropriate interventions still remain neglected in most endemic countries \(^{49}\) and there is some debate whether administration of anthelmintic drugs results in substantial improvement of haemoglobin concentration \(^{21}\). Differential effects of treatment might relate to geographic differences in species-specific distribution and infection load. Monitoring de-worming programs using real-time PCR should provide more precise data on the effects of mass treatment. In addition, assessment of differences in treatment effects per species may diminish risk for the development of drug resistance.

Although the value of real-time PCR for clinical diagnostics is limited in resource poor settings, this method brings forward new exciting prospects for epidemiology of intestinal parasites in these settings. The collection of stool samples in ethanol allows storage at room temperature and transportation to central research centres with facilities for real-time PCR. Moreover, using the same DNA isolation method simultaneous monitoring of other parasitic infections can be performed. For example *A. duodenale*, *N. americanus*, *Ascaris lumbricoides* and *Strongyloides stercoralis*-specific DNA can be detected in a single assay \(^{50-52}\). This all simplifies the complex organization of labor-intensive field studies in remote areas. An increasing number of research centres located within low income countries have access to real-time PCR technology. In combination with a trend of
decreasing availability of well trained microscopists, real-time PCR is more recognized as an important diagnostic tool in research.

A concern was that 18.2% (8/44) of the microscopy positives were PCR negative. This finding may indicate genetic variation of the PCR target gene [3]. On the other hand this was not supported by the fact that almost all PCR-missed infections showed low egg counts. Misidentification during microscopy could be another explanation. Nevertheless, most procedures have a certain chance to miss very light infections, and as examination is based on a small test sample volume only, it is probable that both eggs and free DNA are not completely homogeneously distributed within the stool sample. Hookworm was not detected by microscopy in 83.5% (182/218) of the PCR positive samples, since the specificity of real-time PCR was already proved to be close to 100% [2] this demonstrates the limited sensitivity of microscopy to detect hookworm infections even in an ideal laboratory setting. A limitation is that the association between iron deficiency and hookworm was only assessed in severely anaemic children. These children may represent a small group of children with severe disease and interpretation of the associations may be different in the majority of otherwise “healthy children” infested with hookworm.

In conclusion, we have shown the need for quantitative screening of species-specific hookworm infections by demonstrating a much higher than previously expected prevalence of A. duodenale, and identified its significant contribution to severe anaemia and bone marrow iron deficiency in pre-school children in Malawi. Multiplex real-time PCR is a powerful diagnostic tool in public health research to facilitate identification of the causes of (severe) anaemia and iron deficiency in children living in resource poor settings.

Acknowledgments and contributions

We thank the parents and guardians of the children admitted in the study; the SevAna study team; the staffs of the Queen Elizabeth Central Hospital and Chikwawa District Hospital. MBvH and LvL. conceived and designed the study. JJC, KP, MBvH, HK and BJB were responsible for the design and execution of the large severe anaemia (SevAna) research programme in Malawi. EATB, JvV and LvL. were responsible for the design, performance and interpretation of the multiplex real-time PCR analyses. FAMJ and JJC conducted statistical analyses. FAMJ drafted the manuscript. All authors critically revised the manuscript for intellectual content, and read and approved the final version. FAMJ and LvL. are the guarantors of the paper. This study was supported by a grant (064722) from the Wellcome Trust and by independent grants from the Nutricia Research Foundation, the Ter Meulen Fund of the Royal Netherlands Academy of Arts and Sciences, the Dr. P.C. Flu Foundation and JANIVO Foundation. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.
Chapter three | *Ancylostoma duodenale*, severe anaemia and iron deficiency

References


Chapter four
Iron status predicts malaria risk in Malawian preschool children

T. A. M. Marker, J. C. Faiss, M. Boele van Damen, K. S. Phiri, J. D. L. Zeekie, B. J. D. Brabin, T. Leeuw

Plos ONE, accepted for publication
Abstract

Iron deficiency is highly prevalent in pre-school children in developing countries and an important health problem in sub-Saharan Africa. A debate exists on the possible protective effect of iron deficiency against malaria and other infections; yet consensus is lacking due to limited data. Recent studies have focused on the risks of iron supplementation but the effect of an individual’s iron status on malaria risk remains unclear. Studies of iron status in areas with a high burden of infections often are exposed to bias. The aim of this study was to assess the predictive value of baseline iron status for malaria risk explicitly taking potential biases into account.

We prospectively assessed the relationship between baseline iron deficiency (serum ferritin < 30 μg/L) and malaria risk in a cohort of 727 Malawian preschool children during a year of follow-up. Data were analyzed using marginal structural Cox regression models and confounders were selected using causal graph theory. Sensitivity of results to bias resulting from misclassification of iron status by concurrent inflammation and to bias from unmeasured confounding were assessed using modern causal inference methods.

The overall incidence of malaria parasitaemia and clinical malaria was 1.9 (95% CI 1.8-2.0) and 0.7 (95% CI 0.6-0.8) events per person-year, respectively. Children with iron deficiency at baseline had a lower incidence of malaria parasitaemia and clinical malaria during a year of follow-up; adjusted hazard ratio’s 0.55 (95% CI:0.41-0.74) and 0.49 (95% CI:0.33-0.73), respectively. Our results suggest that iron deficiency protects against malaria parasitaemia and clinical malaria in young children. Therefore the clinical importance of treating iron deficiency in a pre-school child should be weighed carefully against potential harms. In malaria endemic areas treatment of iron deficiency in children requires sustained prevention of malaria.
Chapter four | Iron status predicts malaria risk in Malawian preschool children

Introduction

Childhood iron deficiency is prevalent globally with highest estimates in children in developing countries. In sub-Saharan Africa, 33-66% of children are iron deficient, most of whom are under the age of five years 1, 2. The high prevalence of iron deficiency in these populations is a consequence of low nutritional iron intake due to low economic status 2, decreased absorption of dietary iron due to a high prevalence of infections 3 or intestinal blood loss in the case of helminth infections 4. Iron deficiency causes anaemia and developmental dysfunction [2,3] and the current consensus is to treat all iron deficient children 5. Conversely, evidence of a protective effect of iron deficiency against malaria and other infections exists6,12.

In recent years concerns have focused mainly on the possible harmful effects of treating iron deficiency rather than on the effects of iron status itself 13-19. A recent Cochrane meta-analysis reviewing iron supplementation in children in malaria endemic areas concluded that iron supplementation did not adversely effect children in malaria endemic areas when adequate malaria surveillance is provided, although the authors considered their data were limited by various factors including lack of data on baseline iron status 20. There is little conclusive data on the protective effect of iron deficiency against malaria. The reliability of current data on iron status is limited since available iron biomarkers are affected by inflammation which makes interpretation of results from malaria endemic areas problematic 21. In addition, the complexity of factors influencing both malaria susceptibility and iron status are not always taken into account, potentially leading to confounded results. To improve control and management of malaria as well of iron deficiency, reliable data about the influence of iron status on malaria risk are needed. The aim of this study was to assess the predictive value of baseline iron status in children for subsequent malaria risk, explicitly taking misclassification of iron deficiency due to concurrent inflammation and confounding bias into account by utilizing modern causal inference methods in the analyses of a dataset of Malawian pre-school children.

Methods

Methods | Study Design
This is a secondary analysis of follow-up data from a cohort of pre-school children recruited as the control group for the Severe Anaemia Study (SevAna study), a large case-control study investigating the aetiology, pathophysiology and outcome of severe anaemia in southern Malawi 22-24. The analysis is restricted to the non-severely anaemic control population (haemoglobin level ≥5.0 g/dL), because the cases all received a blood transfusion. Follow-up data of the first year after enrolment were used, allowing the observation of a full year of malaria transmission per child.

Methods | Ethical Statement
The SevAna study was approved by the Ethics Committees of the College of Medicine, Malawi (ref. no P.00/01/116, and the Liverpool School of Tropical Medicine, United Kingdom (ref. no 01.29). Prior to enrolment in the study, written informed consent was obtained from the parent or guardian of all study participants.

Methods | Study population
The SevAna study recruited 381 cases and 757 controls between July 2002 and July 2004 (Figure S1, supplements). Every child (6-60 months old) presenting at the hospital with severe anaemia (haemoglobin

49
<5.0 g/dL) was defined as a case. For each case, two controls were enrolled; a community control living within 100-1000 meter of the case, and a hospital control, which was the first child presenting at the outpatient department on the day following presentation of the case patient. Controls were eligible for recruitment if aged 6-60 months and if their haemoglobin level was at least 5.0 g/dL. All children were visited at 1, 3, 6 and 12 months from date of recruitment (active follow-up). If children did not report for follow up, families were visited by the study team. In addition, guardians were asked to present their child to a study-clinic whenever the child was sick (passive follow-up).

Methods | Study setting
The SevAna study took place in Malawi, a land-locked country in the south-eastern part of Africa of which 20% is covered by Lake Malawi. The climate is tropical, but prevalence of malaria and other infectious diseases vary with proximity to the lake and altitude. Rough estimates of the prevalence of iron deficiency vary from 20-40% [2, 25]. Study participants were recruited in two settings; Blantyre district (study site: Queen Elizabeth Central Hospital) and Chikwawa District (study site: Chikwawa District Hospital), both in the southern region of Malawi. Blantyre is the main commercial town of Malawi with a predominantly urban population of half a million. At an altitude of 800m above sea level malaria is mainly seasonal (approximately 1 infectious bite per person per year) [20]. Chikwawa District Hospital, which caters for a predominantly rural population of approximately 400 thousand people, is situated in the lower Sire Valley, 50 km south of Blantyre. With an altitude of 250 m above sea level malaria transmission is year round (approximately 170 infectious bites per person per year) (Milahowa T, personal communication).

Methods | Clinical procedures
At recruitment a detailed medical and socio-economic history was recorded and a physical examination was performed. Samples of blood, stool and urine were collected. Children were treated if indicated using local treatment guidelines. Clinical malaria was defined as a positive blood slide with concurrent fever (axillary temp >37.5°C) or history of fever (caregiver recall of fever in the last week). Severe malaria was defined as a positive blood slide with either severe anaemia (haemoglobin <5.0 g/dl) or coma [27]. Malaria was treated with sulfadoxine-pyrimethamine (SP) and if the child was unable to take oral medication parenteral quinine was administered. As a standard procedure at recruitment all study participants received presumptive malaria treatment (25.0/1.25 mg/kg SP) and according to local guidelines all children received iron supplementation 2 mg/kg/day for 28 days. Follow-up procedures existed of a medical history, physical examination and a blood sample to determine haemoglobin and malaria parasitaemia, and if indicated children were treated using local treatment guidelines. Deaths were recorded and if they occurred outside the study clinics they were investigated as completely as possible using a verbal autopsy form.

Methods | Laboratory procedures
Haemoglobin (Hb) concentrations were measured by HemoCue B-Haemoglobin analyzer (HemoCue, Ängelholm, Sweden) to judge eligibility-criteria. Hb measured by Coulter counter analyzer (Beckman Coulter, Durban, South Africa) was used for statistical analyses. Moderate and severe anaemia were defined as an Hb of < 11.0 g/dL and < 5.0 g/dL, respectively. Plasma levels of C-reactive protein (CRP) and serum ferritin were analyzed on Modular P800 and Monular Analytic E170 systems (Roche Diagnostics, Switzerland). Iron deficiency was defined as serum ferritin < 30 µg/L [25]. Inflammation was defined as CRP > 10 g/L. For malaria diagnoses thin-film blood slides were read by two independent readers; with a third reader being used if results differed by 25% or more. The number of Plasmodium falciparum asexual parasites per 200 white cells was counted and expressed as the number per microliter of blood. Malaria parasitaemia was defined as the presence of one or more Pl. falciparum asexual parasites[28]. Human immunodeficiency virus (HIV)
infection was assessed using two rapid tests (Determine, Abbott Laboratories; and Uni-Gold, Trinity Biotech). Discordant or reactive rapid-test results in children less than 18 months of age were resolved by PCR 29.

Methods | Statistical Analysis
The incidence of clinical malaria and parasitaemia was calculated for a follow-up period of one year. Multiple events per child were registered. A child was considered not to be at risk for incident malaria for the 28 days following malaria treatment (SP), as the prophylactic effect of malaria treatment was assumed 30. This 28 day period was excluded as person-time and malaria events during this period were not counted for analyses. Malaria parasitaemia or clinical malaria occurring during this period were defined as treatment-failure and were excluded from analysis.

The main predictor, iron deficiency, was defined as serum ferritin <30 μg/L. Possible confounders considered for inclusion were: sex, age, area of residence, season, socio economic status, genetic predisposition of malaria, cellular immunity, humoral immunity, cumulative malaria exposure, malaria immunity, history of other infections than malaria, HIV infection, hookworm infection, nutritional status and zinc deficiency (Figure 1). We used DAGitty®, a web-based application based on the Directed Acyclic Graph theory, to model the causal relationship between iron status, malaria risk and any potential (measured and unmeasured) confounders 31, 32. This DAG-program was used to identify sets of confounders that together fully adjust for confounding in multivariable modelling 31, 33, 34. Several of these sets of confounders (minimally sufficient adjustment sets) were identified from figure 1. The set containing HIV-status, socio economic status, age, nutritional status and study site was used for analysis, as these variables were available in our data set. Socio economic status was scored on parents’ education (1-4), job (1-4) and number of assets (0-5). Chronic malnutrition defined as height-for-age < -2 SD was used as the marker for nutritional status 35.

Due to inadequate sample volume in 29% of the children serum ferritin was not available. The missing data were assumed to be missing at random; basic characteristics between children with and without available ferritin assessments were comparable (table S1, supplements). Because complete-cases analysis with a large amount of missing values may lead to loss of efficiency and bias 36, missing data were imputed using multiple imputation by chained equations in the R software version 2.14.0 utilizing the MICE package 37, 38. The imputation model included all baseline covariates in table 1 plus soluble transferrin receptor (sTfR) and mean corpuscular haemoglobin concentration (MCHC) as additional markers of iron status. The outcome variable was included in the imputation model via the event indicator and the Nelson-Aalen estimator of baseline hazard, as studies suggest that these specific components lead to lower bias in estimation 39. Regression estimates and their standard errors from analyses of the 25 separate imputed data sets were summarized using Rubin’s rules 40.

To compare the incidence of malaria during follow-up between iron deficient and iron replete individuals, a marginal structural Cox proportional hazards model was used to adjust for confounding. Marginal structural models are a new class of statistical models to estimate causal effects 41, 42, and are equivalent to non-parametric multivariate standardization methods 43,43,44. Effect estimates can be adjusted for measured confounders using inverse probability of exposure weighting. In addition, marginal structural models allow the quantitative evaluation of unmeasured confounding 42, 45, 46. Stabilized inverse probability weights were estimated using the ‘ipw’ package in R 30, 47. To adjust for the correlation between recurrent malaria events in a single person, robust standard errors were computed. All covariates were treated as time-independent. The proportional hazards assumption was checked by plotting the scaled Schoenfeld residuals against time and
tested using cox.zph procedure in R 48. We assessed whether the effect of iron deficiency on malaria was modified by age or study site by testing their interaction terms with the Wald statistic. Interaction terms were considered significant if p<0.1.

We assessed to which extent the outcomes could have been biased because of possible misclassification of the iron deficiency indicator (serum ferritin), by reanalyzing the data using a more sensitive and a more specific definition of iron deficiency 49; cut-offs for serum ferritin that varied by inflammation status; children without inflammation (CRP <10 g/L) were defined as iron deficient having a serum ferritin of < 12 µg/L; for children with current inflammation (CRP > 10 g/L) serum ferritin cut-offs of 30 and 70 µg/L were explored 25,50.

In addition, to evaluate how large the average difference in susceptibility to malaria at baseline between the iron deficient and iron-replete group would need to have been to partly or fully explain our observed differences in malaria incidence, we used a method for sensitivity analysis for unmeasured confounding developed by Robins et al. 42, 45, 46. The method requires that unmeasured confounding is quantified as a bias parameter u = α (2 x ID - 1), with ID = 1 or 0 as indicator of iron deficiency and α the degree of unmeasured confounding or, in other words, the average difference in malaria risk at baseline on a ratio-scale and conditional on the covariate distribution.

Figure 1. Directed acyclic graph for the relation between iron deficiency and malaria risk.

cell. imm: cellular immunity; cum exp: cumulative malaria exposure; genetic predis: genetic predisposition; humor immun: humoral immunity; hookw: hookworm infection; hist infect: history of other infections than malaria; HIV: human immunodeficiency virus; ID: iron deficiency; Pl.f.immun: immunity for Pl. falciparum; Pl.f: Plasmodium falciparum infection; SE: socio economic score; ZD: zinc deficiency; z.h.a: z-score height for age.
Results

Results | Study population
Follow-up data were available for 96.0% of all study participants (727/757). Baseline characteristics are presented in table 1. Baseline characteristics of children with and without follow up data were comparable (data not shown). Of the remaining 727 children the attendance at active follow-up visits ranged from 86-89%.

Table 1. Baseline characteristics of the study population.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>357/727 (49.1%)</td>
</tr>
<tr>
<td>Age in months: mean (s.d)</td>
<td>23.9 (12.6)</td>
</tr>
<tr>
<td>Age &lt; 2 years</td>
<td>408/727 (56.1%)</td>
</tr>
<tr>
<td>Living in an urban area</td>
<td>376/727 (51.7%)</td>
</tr>
<tr>
<td>SE-score: mean (s.d)</td>
<td>7.2 (2.1)</td>
</tr>
<tr>
<td>Hospital control</td>
<td>357/727 (49.1%)</td>
</tr>
<tr>
<td>Haemoglobin: mean (s.d)</td>
<td>9.7 (2.1)</td>
</tr>
<tr>
<td>Iron deficiency</td>
<td>146/513 (28.5%)</td>
</tr>
<tr>
<td>Malnourished</td>
<td>275/669 (41.1%)</td>
</tr>
<tr>
<td>Fever (&gt; 37.5 °C axillary)</td>
<td>205/727 (28.4%)</td>
</tr>
<tr>
<td>CRP &gt; 10 mg/L</td>
<td>373/630 (59.2%)</td>
</tr>
<tr>
<td>Malaria parasitaemia</td>
<td>308/720 (42.8%)</td>
</tr>
<tr>
<td>Clinical malaria</td>
<td>108/720 (14.9%)</td>
</tr>
<tr>
<td>HIV-infected</td>
<td>39/670 (5.8%)</td>
</tr>
</tbody>
</table>

Haemoglobin in g/dl; CRP: C-Reactive Protein; malnourished is defined as height-for-age < -2 SD. Iron deficiency defined as serum ferritin < 30 µg/L. HIV: Human Immunodeficiency Virus. SE-score: Socio Economic Score, a sum of the following scores: parents’ education (1-4), job parents (1-4) and number of assets (0-6).

Results | Incidence of malaria parasitaemia and clinical malaria
During the follow-up year the overall incidence of malaria parasitaemia and clinical malaria was 1.9 (95% CI 1.8-2.0) and 0.7 (95% CI 0.6-0.8), respectively. This incidence was higher in the rural areas and in the children less than 24 months of age (Table 2). There were four cases of severe malaria, three of which were iron replete. During follow-up 0.7% (1/141) of the iron deficient children and 1.3% (5/372) of iron replete children died.
Table 2. Incidence of malaria parasitaemia and clinical malaria per person year

<table>
<thead>
<tr>
<th></th>
<th>All</th>
<th>Iron deficient group</th>
<th>Iron replete group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Events</td>
<td>Incidence</td>
<td>Events</td>
</tr>
<tr>
<td>Malaria parasitaemia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>896</td>
<td>1,9</td>
<td>180</td>
</tr>
<tr>
<td>Urban</td>
<td>253</td>
<td>1,0</td>
<td>38</td>
</tr>
<tr>
<td>Rural</td>
<td>643</td>
<td>3,1</td>
<td>142</td>
</tr>
<tr>
<td>Age &lt; 24 months</td>
<td>557</td>
<td>2,1</td>
<td>139</td>
</tr>
<tr>
<td>Age ≥ 24 months</td>
<td>339</td>
<td>1,6</td>
<td>41</td>
</tr>
<tr>
<td>Clinical malaria</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>318</td>
<td>0,7</td>
<td>58</td>
</tr>
<tr>
<td>Urban</td>
<td>65</td>
<td>0,2</td>
<td>11</td>
</tr>
<tr>
<td>Rural</td>
<td>253</td>
<td>1,2</td>
<td>47</td>
</tr>
<tr>
<td>Age &lt; 24 months</td>
<td>207</td>
<td>0,8</td>
<td>44</td>
</tr>
<tr>
<td>Age ≥ 24 months</td>
<td>111</td>
<td>0,5</td>
<td>14</td>
</tr>
</tbody>
</table>

Incidence of malaria parasitaemia and clinical malaria per person year stratified per area, age group and iron status. Malaria parasitaemia defined as a positive malaria blood slide; Clinical malaria: positive malaria blood slide with concurrent fever (axillary temp > 37.5°C) or history of fever.

Table 3. Hazard ratios (95% CI) of iron deficiency for the risk on malaria parasitaemia en clinical malaria.

<table>
<thead>
<tr>
<th>Definition iron deficiency</th>
<th>Malaria parasitaemia</th>
<th>Clinical malaria</th>
<th>Malaria parasitaemia</th>
<th>Clinical malaria</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR (95% CI)</td>
<td>HR (95% CI)</td>
<td>HR (95% CI)</td>
<td>HR (95% CI)</td>
<td>HR (95% CI)</td>
</tr>
<tr>
<td>SF &lt; 30</td>
<td>0.59 (0.44-0.78)</td>
<td>0.54 (0.36-0.80)</td>
<td>0.55 (0.41-0.74)</td>
<td>0.49 (0.33-0.73)</td>
</tr>
<tr>
<td>CRP &gt;10: SF &lt; 30</td>
<td>0.57 (0.40-0.82)</td>
<td>0.53 (0.32-0.89)</td>
<td>0.46 (0.31-0.71)</td>
<td>0.43 (0.25-0.73)</td>
</tr>
<tr>
<td>CRP &lt;10: SF &lt; 12</td>
<td>0.66 (0.52-0.85)</td>
<td>0.59 (0.41-0.84)</td>
<td>0.60 (0.46-0.79)</td>
<td>0.52 (0.35-0.75)</td>
</tr>
</tbody>
</table>

Iron deficiency is defined with different cut-offs for serum ferritin (SF) in µg/L, depending on presence of inflammation, defined as C-reactive protein (CRP) > 10 mg/L. The marginal-structural Cox model included HIV-infection, socio economic score, age, nutrition and study site.

Results | Baseline iron status and incidence of malaria
The marginal structural Cox model showed that iron deficiency was an independent predictor of malaria parasitaemia and clinical malaria; HR (95%CI) 0.55 (0.41-0.74) and 0.49 (0.33-0.73) respectively (Table 3). The effect of iron deficiency on malaria parasitaemia and clinical malaria was consistent across different sub-populations; no significant effect measure modification by study site (urban vs. rural) or age (linear term) was seen (all p-values >0.9). In addition, the effects were sustained throughout the study period; the proportional hazards assumption was not refuted and when analysis was done on consecutive periods of 4 months, consistent effect measures were seen (data not shown). Analyses conducted with the complete cases data set (including only children with a ferritin result) showed comparable results (HR (95% CI) 0.61 (0.44-0.81) and HR (95% CI) 0.53 (0.36-0.78) for malaria parasitaemia and clinical malaria respectively.
Chapter four | Iron status predicts malaria risk in Malawian preschool children

Results | Sensitivity analyses
Analyses assessing sensitivity to misclassification of iron status are presented in Table 3 and show consistent results using different definitions of iron deficiency. Hazard ratio estimates for clinical malaria from the marginal structural Cox model including adjustment for varying amounts of potential unmeasured confounding are presented in Figure 2. Two distinct distributions of unmeasured confounding were considered. Figure 2a presents results from analyses in which the net amount of bias per group is specified. The a priori clinical malaria risk in the iron deficient group should have been at least 0.72 times the a priori risk in the iron replete group to remove significance of our findings, and 0.48 times to fully explain our findings (Figure 2a). Figure 2b presents results from analyses in which the amount of unmeasured confounding varied as function of age; increasing log-linearly up to a peak at the age of 3 years and then decreasing to no confounding effect. The ratio of a priori risks would have to have been at least 0.49 at its peak at 3 years-of-age to remove significance and 0.18 to fully explain our finding (Figure 2b). Baseline malaria is often included as a proximate measure of susceptibility to malaria in multivariable analyses. However, since our hypothesis was that baseline iron status influences malaria risk, baseline malaria would technically not be a confounder, i.e. the exposure may not affect the confounder (Causal knowledge as a prerequisite for confounding evaluation\(^{33}\)). We therefore chose to assess the effect of differences in malaria susceptibility at baseline through sensitivity analysis. Notwithstanding, when baseline malaria parasitaemia respectively clinical malaria was included, effect estimates were slightly attenuated though still significant HR (95% CI) 0.66 (0.48-0.91) and HR (95% CI) 0.51 (0.33-0.79) for malaria parasitaemia and clinical malaria respectively.

Figure 2. Sensitivity analysis of the impact of unmeasured confounding on the effect of baseline iron deficiency on risk of clinical malaria. The y-axis represents the hazard ratio for malaria infection comparing iron deficiency to iron replete after adjustment for average differences in prior risk represented by \( x \) on the x-axis. Figure 2a represents analyses in which net amount of confounding is specified for both iron status groups (bias parameter \( u = e^{(z - ID)} \)). Figure 2b represents analyses in which the amount of confounding was varied as a function of age (bias parameter \( u = e^{(2 \times ID \cdot 1) \times (1 - \text{abs(age-36)/36})} \)).
Discussion

In this study, children with iron deficiency at baseline (serum ferritin <30 μg/L) had a lower incidence of malaria parasitaemia and clinical malaria during a follow up period of one year. This finding suggests that iron deficiency protects against malaria parasitaemia and clinical malaria in pre-school children living in malaria endemic areas. The results were consistent across study sites with varying transmission intensity and across age-groups despite changing susceptibility with age. In addition, the difference in risk between baseline iron status groups persisted throughout the follow-up period.

Our findings are in line with results of the few other studies assessing influence of iron status on malaria risk

As with any observational study, the results of this study are subject to bias. Therefore, we explicitly evaluated the potential impact that misclassification and confounding bias could have had on our results. Firstly, because serum ferritin levels are influenced by concurrent inflammation it may not adequately reflect true iron status. As a consequence, misclassification of baseline iron status may have occurred and thus resulted in biased effect measures. We evaluated whether use of a more sensitive and a more specific definition, taking concurrent inflammation into account, would substantially change the results. As expected, use of a more specific definition slightly increased the magnitude of effect and the use of a more sensitive definition led to a slightly weaker effect. Importantly, this did not change our conclusions, suggesting that the findings are robust to misclassification bias.

In the directed acyclic graph (DAG) in Figure 1 all potential confounders of the effect of iron status on malaria risk as conceptualized by the authors, were evaluated. We considered two additional yet unmeasured variables which could theoretically confound our results by both protecting against malaria and causing iron deficiency. We assessed how strong these potential confounding effects should have been to explain the observed difference in malaria risk between iron status groups. The first unmeasured factor was ‘cumulative exposure to malaria’. Recent studies have suggested that chronic infection, including malaria, may reduce iron absorption in the gut by inducing an increase of hepcidin and that malaria infection may lead to iron loss through haemosiderin deposition. In addition, repeated malaria infections (cumulative exposure) result in development of immunity to malaria. The age at which clinical immunity is attained is influenced by local infection pressure represented by the number of infectious bites per year (EIR). If it is true that increased exposure to malaria simultaneously induces iron deficiency and increased malaria immunity, cumulative exposure is a potential confounder in our analyses. In other words, the lower incidence of malaria in the iron deficient group may have been due to higher malaria exposure and subsequently better immunity to malaria, rather than directly due to mechanisms involving iron status. We conducted sensitivity analyses allowing unmeasured confounding bias to vary as a function of age; reflecting the theoretical confounding effect of cumulative exposure, where the difference in immunity (and thus the maximum prior risk difference) gradually changes with increasing age, reaching a peak at the age of 36 months to subsequently decrease after that. The average prior risk of malaria should then have been at least half the risk in the iron replete group at the peak age of 36 months to have led to a spurious significant finding (Figure 2b). Although theoretically a plausible confounder, it is unlikely that in reality ‘cumulative exposure to malaria’ would result in such large differences in prior malaria risk, suggesting that our measured effects are unlikely due to confounding by differences in cumulative exposure. As a second potential confounding factor we considered a (yet undiscovered) genotype simultaneously causing iron deficiency and increasing immunity to malaria, for example a variant in iron metabolism that inhibits maturation of Plasmodium in the red cells.
Chapter four | Iron status predicts malaria risk in Malawian preschool children

Our analysis showed that to be responsible for our statistically significant finding, the average prior risk of malaria in the iron deficient group should have been at least 0.72 times the risk compared to that of the iron replete group (Figure 2a). A genetic predisposition causing a 28% lower prior risk is imaginable (i.e. sickle cell trait) and if such a genotype is discovered this may change the interpretation of our results. In the interpretation of our analyses we assume that iron status measured at baseline remained relatively stable throughout the year of follow-up. Unfortunately, iron status measurements were not repeated after baseline, so we are not able to check this assumption. It is possible that children changed from the iron deficient group to the iron replete group and vice versa. However, because our study population was closely followed and adequately treated for infections it is more likely that iron deficient children became iron replete than the other way around. The resulting misclassification would have resulted in an underestimation of the effect.

For the underlying mechanism of the protective effect of iron deficiency on susceptibility to malaria infection, several hypotheses have been raised. It is suggested that iron deficiency alters the membrane structure of parasitized erythrocytes which facilitates their elimination by phagocytic cells, yet further research is needed to further support this hypothesis.58 Another hypothesis is that the increased zinc protoporphyrin in iron deficient parasitized red blood cells binds to haeme crystals and thus inhibits the formation of haemoglobin, in analogue to antimalarial quinolines.59

We cannot fully exclude that the observed effect was the result of increased susceptibility in the iron replete group resulting from the month of iron supplementation, rather than a protective effect of iron deficiency. In 2006 a large supplementation trial in Zanzibar was stopped prematurely after increased morbidity and mortality was observed in iron replete children receiving iron and folic acid.19 However it remains unclear whether the increased morbidity was due to the effect of the iron, or the folic acid component of the supplements.60, 61. Although the month of iron supplementation in this study complicates the interpretation of our study results, it is a reflection of current practice in such settings in which short courses if iron supplementation are routinely given. In addition we think its influence was minimal for several reasons. Firstly, since the difference in risk was still observable towards the end of follow-up, the influence of the one month of iron supplementation was likely to be negligible. Secondly 59% of the children had an infection at baseline and this was likely to inhibit the absorption of iron in the first two weeks of supplementation.52-54.

In conclusion, iron deficiency seems to protect against malaria parasitaemia and clinical malaria. However, because we lack direct measures of baseline immunity to malaria our results should be confirmed by well conducted randomized controlled trials or observational birth cohort studies. Our findings support the theory of nutritional adaptation to infectious diseases.62, 63. To avoid increased malaria risk due to “neutralizing” the protective effect of iron deficiency, treatment of iron deficiency should occur under simultaneous and sustained control of prevalent infections. We recommend that treatment of iron deficiency occurs within the context of the Integrated Management of Childhood Illness (IMCI) and will benefit from the addition of Integrated Vector Management (IVM)64, 65.

However, because oral iron is often poorly absorbed in individuals with concurrent inflammation62-64, prevention of iron deficiency may be more effective. Treatment and especially prevention of infections, in particular hookworm infections,4, 66, will contribute to prevention of iron deficiency through decreased loss and increased absorption of dietary iron. Whichever strategy is chosen, concurrent and sustained prevention of malaria through Integrated Management of Childhood Illness is imperative in malaria endemic areas.
Supplements

Table S1. Baseline characteristics of the study population.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>All</th>
<th>ferritin available</th>
<th>complete cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>357/727 (49,1%)</td>
<td>239/513 (46,6%)</td>
<td>195/438 (44,5%)</td>
</tr>
<tr>
<td>Age in months: median (IQR)</td>
<td>21,8 (13,4-32,7)</td>
<td>23,3 (13,7-33,3)</td>
<td>23,7 (14,2)</td>
</tr>
<tr>
<td>Age &lt; 2 years</td>
<td>408/727 (56,0%)</td>
<td>268/513 (52,2%)</td>
<td>223/438 (50,9%)</td>
</tr>
<tr>
<td>Living in an urban area</td>
<td>376/727 (51,7%)</td>
<td>264/513 (51,5%)</td>
<td>196/738 (44,7)</td>
</tr>
<tr>
<td>SE-score: mean (s.d)</td>
<td>7.2 (2.1)</td>
<td>7.2 (2.1)</td>
<td>7.2 (2.1)</td>
</tr>
<tr>
<td>Hospital control</td>
<td>357/727 (49,1%)</td>
<td>253/513 (49,3%)</td>
<td>213/438 (48,6%)</td>
</tr>
<tr>
<td>Malnourished</td>
<td>271/669 (40,5%)</td>
<td>19/513 (42,0%)</td>
<td>185/438 (42,2%)</td>
</tr>
<tr>
<td>Fever (&gt; 37.5 °C axillary)</td>
<td>205/727 (28,4%)</td>
<td>142/513 (27,9%)</td>
<td>119/438 (27,4%)</td>
</tr>
<tr>
<td>CRP &gt; 10 mg/L</td>
<td>374/630 (59,4%)</td>
<td>283/513 (55,4%)</td>
<td>238/438 (54,6%)</td>
</tr>
<tr>
<td>Malaria parasitaemia</td>
<td>308/720 (42,8%)</td>
<td>207/513 (40,6%)</td>
<td>180/435 (41,4%)</td>
</tr>
<tr>
<td>Clinical malaria</td>
<td>108/720 (15,0%)</td>
<td>71/513 (13,8)</td>
<td>60/438 (13,7%)</td>
</tr>
<tr>
<td>HIV-infected</td>
<td>39/670 (6,8%)</td>
<td>30/513 (6,3%)</td>
<td>38/438 (6,6%)</td>
</tr>
<tr>
<td>Haemoglobin: mean (s.d)</td>
<td>9.7 (2.1)</td>
<td>9.8 (2.0)</td>
<td>9.8 (1.9)</td>
</tr>
</tbody>
</table>

Haemoglobin in g/dl; CRP: C-Reactive Protein; malnourished: height-for-age < -2 SD; HIV: Human Immunodeficiency Virus. Iron deficient denoted as serum ferritin < 30 μg/L. SE-score: Socio Economic Score, a sum of the following scores: parents’ education (1 - 4), job parents (1 - 4) and number of assets (0-6).

Figure S1. Flow chart study population. This flowchart presents number of children enrolled in the main study, a case control study investigating aetiology of severe anaemia (I); number of children enrolled in the cohort study (II) and the number of children attending the follow-up visits (III).
Acknowledgments and contributions

We thank the parents and guardians of the children admitted in the study; the SevAna study team; the staffs of the Queen Elizabeth Central Hospital and Chikwawa District Hospital. MBvH, JJC, KP and BJB conceived and designed the experiments; JJC, KP, MBvH and KP performed the experiments; FAMJ, TL and RG analyzed the data; TL and RG contributed reagents/materials/analysis tools; FAMJ, TL, RG, JJC, BJB and MBvH wrote the manuscript; FAMJ and TL were guarantors of the paper. This study was supported by a grant (064722) from the Wellcome Trust and by independent grants from the Nutricia Research Foundation, the Ter Meulen Fund of the Royal Netherlands Academy of Arts and Sciences, the Dr. P.C. Flu Foundation and JANIVO Foundation. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.
Chapter four | Iron status predicts malaria risk in Malawian preschool children


49. Lash TL, Silliman RA (2000) A sensitivity analysis to separate bias due to confounding from bias due to predicting misclassification by a variable that does both. Epidemiology 11: 544-549.


Chapter five

Iron deficiency anaemia in children with HIV-associated anaemia: a systematic review and meta-analysis

M.O. Esan, F.A.M. Jonker, M. Boele van Hensbroek, J.C.J. Calis, K.S. Phiri

Abstract

We conducted a systematic review and meta-analysis to determine the prevalence of iron deficiency in HIV-infected children from high and low-income settings and compared it with that of HIV-uninfected controls.

We searched five major databases for primary studies reporting on anaemia and iron markers in HIV-infected children. A pooled analysis was done using random-effects models, with Forest plots and heterogeneity test estimates provided. Fifteen articles (2778 children) met the inclusion criteria. In the pooled analysis, mean overall prevalence of iron deficiency in HIV-infected children was 34% (95% CI 19-50%). Prevalence rates were similar in high-income (31%; 95% CI 2-61%) and low-income settings (36%; 95% CI 17-54%) (p=0.14). Studies that included a HIV-uninfected control population (n=4) were only available from low-income settings and showed less iron deficiency in HIV-infected children (28%) compared with HIV-uninfected controls (43%); OR 0.50 (0.27-0.94); p=0.03.

The findings suggest that HIV-infected children are less likely to be iron deficient when compared with HIV-uninfected children. Possible explanations for this include HIV-induced haematosuppression and associated hypoferraemia, with adequate iron stores. Nevertheless iron deficiency is a common co-morbidity in HIV. Studies are needed to determine the role of iron deficiency in HIV-associated anaemia and the effects of iron supplementation in this population.
Chapter five | Iron deficiency anaemia in children with HIV-associated anaemia

Introduction

Anaemia is a common haematological complication of HIV infection and has been consistently found to be independently associated with HIV-disease progression and mortality. Mild to moderate anaemia is the most common presentation in HIV-infected children from both high and low-income settings, with a prevalence of 3-82% and 22-94% respectively. The aetiology of HIV-associated anaemia is thought to be multi-factorial with HIV-associated infections, neoplasms, drug-related side effects, and micronutrient deficiencies being the most important aetiological (sub) groups involved. Red cell production failure is often the most important underlying pathogenic mechanism.

Iron deficiency is considered to be the most important micronutrient deficiency causing anaemia globally. In low-income settings, iron deficiency is estimated to be responsible for up to 50% of cases of anaemia seen in pregnant women and children. However, the contribution of iron deficiency to anaemia in HIV-infection is unclear. Poor dietary iron intake and bioavailability, as well as reduced intestinal absorption due to repeated infections could result in a diminished iron status. On the other hand, there is evidence that iron metabolism is altered in HIV-infection, resulting in an immune-mediated relative increase in iron stores. Increased iron levels have been associated with advanced stages of HIV disease and mortality and iron supplementation in this context may be detrimental. The purpose of this review is to determine the prevalence of iron deficiency in HIV-infected children from high and low-income settings and compare it with that of HIV-uninfected controls. The results are then used to discuss the role of iron deficiency, the use of iron markers and inflammation in HIV-associated anaemia.

Methods

Methods | Search Strategy
Primary studies reporting on haemoglobin (Hb) and markers of iron status in HIV-infected children were searched for in the following databases (excluding Web of Science) in November 2009, with an updated search done in January 2012: PubMed (1950-2012), Embase (1980-2012), Africa Index Medicus (1960-2009), Africa Journals On-line (1998-2009) and Web of Science (1975-2012). Conference abstracts were searched via conference proceedings citation index database available on Web of Science. A standardized search protocol was developed based on the Cochrane Collaboration guidelines using the following key words: ‘HIV’, ‘children’, ‘iron status’ and ‘anaemia’. The search strategy aimed to identify all relevant papers and conference abstracts regardless of language or publication status (published, unpublished, in press or in progress). Relevant papers were translated where necessary. Finally, references lists of all selected articles were reviewed for relevant articles. Selection of papers and data extraction was done independently by two of the reviewers (MOE and FAMJ); discrepancies were resolved by discussion.

Methods | Selection Criteria
All studies that met the following criteria were included: presented data in HIV-infected children (<18 years) on mean haemoglobin/haematocrit levels and one or more of the following markers of iron status: serum ferritin, serum transferrin receptor (STfR), serum transferrin receptor-log ferritin index (STfR-F Index), zinc protoporphyrin (ZPP), serum iron, serum transferrin (Trf), total iron binding capacity (TIBC), mean cell haemoglobin concentration (MCHC), mean corpuscular volume (MCV), haemosiderin or bone marrow iron. If both haemoglobin and haematocrit values were provided, the former was used. Where more than one marker of iron status was used, bone marrow iron was considered first, but where this was not available,
peripheral blood iron markers (ferritin, Trf, STfR, TIBC, TrR-F Index) were preferred over indices of the full blood count (MCV, MCHC). All observational and interventional studies that met the inclusion criteria were included. The following studies were excluded: individual case reports and case reviews, studies assessing restricted populations such as children with haemoglobinopathies; or studies done in children with specific HIV-related morbidities only. All other relevant non-primary articles on anaemia and iron deficiency in HIV-infected children were used for the discussion section.

Methods | Definitions
For the purpose of this review, anaemia was defined as haemoglobin of less than 11.5 g/dl or haematocrit of less than 33%. This value was derived from the cut-off values for anaemia used by the selected studies, and is the WHO cut-off for anaemia for children >5 years. Different markers and cut-offs for iron deficiency were used in the included studies and included ferritin (6 -40 µg/dl), serum transferrin receptor-log ferritin >5.6 and >0.75, with a CRP <1.0 mg/L, serum iron <40 µmol/l; mean corpuscular volume <70 fl and mean cell haemoglobin concentration <32.4g/dl.

After careful consideration, studies were classified into 2 broad groups: low-income and high-income settings, taking into account several factors which included socio-economic factors, accessibility/availability of health care and infection pressure. Studies done in Africa, Asia, and South America were classified as from low-income settings. Studies done in Europe and North America were classified as from high-income settings.

Methods | Statistical analysis
Comprehensive meta-analysis software version 2 (Biostat Inc., Englewood NJ, USA) and STATA version 10 (StataCorp, College Station TX, USA) were used for data analysis. Descriptive data of all identified studies are presented in Table 1. Pooled mean prevalence estimates (95% Confidence Intervals) using a random-effects model were generated for studies done in HIV-infected children using Forest plots stratified by setting (high versus low-income settings) and presented in Figure 2. A pooled analysis of available data from controlled studies (Figures 3 & 4) and of iron deficiency prevalence stratified by highly active anti-retroviral therapy (HAART) use (Figure 5) was done, with Forest plots and heterogeneity test estimates provided. A two-sample z-test was used to compare the prevalence of iron deficiency in HIV-infected children in high-income versus low-income settings, and the prevalence of iron deficiency in HIV-infected children on HAART with those not on HAART. We visually assessed for publication bias using funnel plots. Two-sided p-values of <0.05 were considered statistically significant.

Results
We followed the preferred reporting items for systematic reviews and meta-analyses (PRISMA) guidelines in presenting the findings of this review.

Results | Selection of articles
A combined search of PubMed, Web of Science and Embase databases retrieved 507 hits, which included 13 conference abstracts. Thirty-eight articles qualified using the selection criteria described above (Figure 1). Africa Index Medicus and Africa Journals On-line were excluded from the updated search as the initial search of these databases gave a low return of articles and did not provide any additional literature to that which was available on PubMed or Embase. From the 38 articles left, 23 were excluded either because they did not present relevant findings exclusively in the study target population (children <18 years) or they did not present data on iron deficiency or anaemia.
Chapter five | Iron deficiency anaemia in children with HIV-associated anaemia

Figure 1. Systematic review flow diagram. Study results were either not reported exclusively for children <18 years or exclusively for the paediatric HIV-infected sub-population; Did not present results of iron markers for determination of iron status or haemoglobin cut-off levels for definition of anaemia in their respective study populations.

Results | Description of selected studies
Fifteen articles representing 2778 children met the inclusion criteria. Eleven studies were from low-income settings: Brazil,9, 18, 24 Thailand,19 South Africa,2 Uganda,3, 21-23 Malawi25 and India;26 and four were from high-income settings: USA.3, 28, 29 and Italy.12 Twelve of the studies were cross-sectional studies1, 2, 9, 12, 18, 19, 21, 22, 25, 26, 28, 29 and three were cohort studies.3, 23, 24 Four of the fifteen studies presented data on iron deficiency in an HIV-uninfected control group, all from low-income settings (Figure 4).22-25

Three of the four controlled studies were cohorts of children born to HIV-infected and uninfected mothers followed from birth with blood parameters assessed periodically; HIV PCR testing was used determine which HIV-exposed children made up the HIV-infected cohort, all other children (HIV-exposed and unexposed) were used as controls.22-24 The fourth study was a case-control study in which healthy, non-severely anaemic (haemoglobin >5g/dl) community and hospital controls were recruited for HIV-infected children admitted for severe anaemia.25

All HIV-infected children from two of the studies presented were on HAART;18, 29 four studies had some of their HIV-infected cohorts on HAART24, 9, 26, 28 seven studies were done before HAART use was adopted as part of the standard of care in the countries where they were carried out 1-3, 21-23, 25 and two studies did not provide any information on HAART use.12, 19 Four of the five studies which provided prevalence estimates for iron deficient HIV-infected children on HAART were made up of cohorts that were either followed from birth or started on HAART at an early age; 9, 18, 24, 29. The fifth study cohort was made up of HIV-infected children initiated on HAART using national guidelines, adapted from WHO criteria.26 Only children on daily maintenance medications with HAART were considered for inclusion the HAART subgroup, children who received HAART only as part of peri-natal prophylaxis protocols were excluded.

Results | Anaemia
The prevalence of anaemia in HIV-infected children from high and low-income settings ranged from 15-94% and 63-100% respectively (Table 1). Prevalence of anaemia in uninfected controls from high and low-income settings ranged from 5-31% and 36-100% respectively (Table 1).
Results | Iron deficiency
The iron markers and their cut-off values used to define iron deficiency for each study are presented in Table 1. In the pooled analysis, the mean overall prevalence of iron deficiency in HIV-infected children was 35% (95% CI 18-51%). Prevalence rates were similar in high-income settings (31%; 95% CI 2-61%) and low-income settings (36%; 95% CI 17-56%) (p=0.14; Figure 2). Studies that included a HIV-uninfected control population (n=4) were only available in low-income settings and showed that HIV-infected children were less likely to be iron deficient when compared with HIV-uninfected controls [28% vs. 43%; OR 0.50 (0.27-0.94); p=0.03]; (Figures 3 and 4).

Pooled estimates of the prevalence of iron deficiency in HIV-infected children stratified by HAART use are provided in Figure 5. The prevalence of iron deficiency was significantly lower in HIV-infected children on HAART (24%; 95% CI 7.41%) when compared with those not on HAART (37%; 95% CI 17-58%) (p=0.005). Visual assessment of funnel plots for the prevalence of iron deficiency in HIV-infected children (not shown) revealed some asymmetry, with 3 outlying studies detected 3,18,23.

![Figure 2](image_url). Pooled analyses of prevalence of iron deficiency in HIV-infected children. 1) n=257, test for heterogeneity (random): Q=1.52; d.f.=3 (p=0.02); I² =0. 2) n=964, test for heterogeneity (random): Q=4.75; d.f.=10 (p=0.01); I² =0. 3) Test for overall effect (random): z=4.28 (p<0.001).
<table>
<thead>
<tr>
<th>Country</th>
<th>Authors</th>
<th>Period</th>
<th>Age in months</th>
<th>Description</th>
<th>HIV+ N</th>
<th>HIV- N</th>
<th>Definition of Iron deficiency $a$</th>
<th>Prevalence of Anemia $b$ (%)</th>
<th>Prevalence of Iron deficiency $c$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thailand</td>
<td>Tishkoff, et al.</td>
<td>1994</td>
<td>2-46</td>
<td>Review looking at nutritional problems in hospitalized children in Thailand</td>
<td>48</td>
<td>-</td>
<td>&lt;10 serum iron &lt;40</td>
<td>88.0</td>
<td>-</td>
</tr>
<tr>
<td>Uganda</td>
<td>Kay, et al.</td>
<td>1995-1996</td>
<td>6</td>
<td>Cross-sectional study of usefulness of stunted growth in predicting iron deficiency in HIV</td>
<td>134</td>
<td>-</td>
<td>&lt;11 ferritin &lt;12</td>
<td>90.7</td>
<td>-</td>
</tr>
<tr>
<td>Uganda</td>
<td>Tonin, et al.</td>
<td>1997-1998</td>
<td>9</td>
<td>Study on contribution of iron deficiency &amp; HIV disease to anemia</td>
<td>100</td>
<td>25</td>
<td>&lt;11 ferritin &lt;12</td>
<td>92.6</td>
<td>78.9</td>
</tr>
<tr>
<td>Uganda</td>
<td>Clark, et al.</td>
<td>2000-2001</td>
<td>6-60</td>
<td>Longitudinal study characterizing risk factors for anemia in HIV</td>
<td>228</td>
<td>-</td>
<td>&lt;11 MCV &lt;12.4</td>
<td>81.7</td>
<td>-</td>
</tr>
<tr>
<td>Brazil</td>
<td>Silva, et al.</td>
<td>1995-1997</td>
<td>3-18</td>
<td>Longitudinal study of hematological parameters in HIV-infected children</td>
<td>87</td>
<td>-</td>
<td>&lt;11.5 ferritin &lt;12</td>
<td>100.0</td>
<td>-</td>
</tr>
<tr>
<td>Brazil</td>
<td>Silva, et al.</td>
<td>1998-1999</td>
<td>1-48</td>
<td>Longitudinal study of hematological aspects of vertical exposure to HIV</td>
<td>27</td>
<td>18</td>
<td>&lt;11.5 ferritin &lt;12</td>
<td>72.1</td>
<td>42.6</td>
</tr>
<tr>
<td>Zambia</td>
<td>Miller, et al.</td>
<td>1997-1999</td>
<td>0-24</td>
<td>Nested cohort study of hematological changes in HIV-infected children</td>
<td>89</td>
<td>490</td>
<td>&lt;11.3 ferritin &lt;12</td>
<td>77.6</td>
<td>26.6</td>
</tr>
<tr>
<td>Brazil</td>
<td>Silva, et al.</td>
<td>2000-2001</td>
<td>6-48</td>
<td>Descriptive study of hematological alterations in HIV</td>
<td>8</td>
<td>-</td>
<td>&lt;11.0 Haemoglobin decreased</td>
<td>82.6</td>
<td>-</td>
</tr>
<tr>
<td>South Africa</td>
<td>EHW, et al.</td>
<td>Prior to 2002</td>
<td>13-39</td>
<td>Descriptive study on iron status in children stable on HIV</td>
<td>80</td>
<td>-</td>
<td>&lt;11 ferritin &lt;12</td>
<td>72.0</td>
<td>-</td>
</tr>
<tr>
<td>Malawi</td>
<td>Cells, et al.</td>
<td>2002-2004</td>
<td>6-60</td>
<td>Cross-sectional (case-control) study on risk factors for severe anemia</td>
<td>81</td>
<td>458</td>
<td>&lt;11 TIBC Index &lt;35</td>
<td>80.0</td>
<td>42.2</td>
</tr>
<tr>
<td>India</td>
<td>Patel, et al.</td>
<td>2007-2008</td>
<td>24-344</td>
<td>Cross-sectional study on prevalence &amp; etiology of anemia in HIV + children</td>
<td>80</td>
<td>-</td>
<td>&lt;11.5 TIBC Index &lt;27.5</td>
<td>82.8</td>
<td>-</td>
</tr>
<tr>
<td>USA</td>
<td>Mueller, et al.</td>
<td>1987-1994</td>
<td>4-159</td>
<td>Retrospective review of bone marrow aspirations</td>
<td>60</td>
<td>-</td>
<td>&lt;11.0 Haemoglobin decreased</td>
<td>84.0</td>
<td>-</td>
</tr>
<tr>
<td>USA</td>
<td>Blamir, et al.</td>
<td>Prior to 1995</td>
<td>2-99</td>
<td>Hematological profile of symptomatic HIV- patients</td>
<td>100</td>
<td>47</td>
<td>&lt;11 ferritin &lt;12</td>
<td>54.0</td>
<td>32.0</td>
</tr>
<tr>
<td>Italy</td>
<td>Castaldo, et al.</td>
<td>Prior to 1995</td>
<td>1-150</td>
<td>Study assessing contribution of CMV in HIV</td>
<td>71</td>
<td>-</td>
<td>&lt;11.5 TIBC &lt;10.5</td>
<td>88.0</td>
<td>-</td>
</tr>
<tr>
<td>USA</td>
<td>Buhler, et al.</td>
<td>Prior to 2000</td>
<td>&lt;110</td>
<td>Observational study classifying anemia as either due to ID or chronic illness</td>
<td>76</td>
<td>-</td>
<td>&lt;11.0 HDV + HBV</td>
<td>18.0</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 1. Prevalence of anaemia and iron deficiency in HIV-positive children and controls. Values for serum ferritin presented in ug/L; serum iron in umol/L; MCHC in g/dl & MCV in IL $a$: Estimate based on mean (SD) ferritin values for HIV-infected cohort at 6 months; $b$: Age specific cut-offs for anaemia and serum iron are presented. Anaemia defined as Hb <11.5g/dl for children >24months, and < 10.5g/dl for children 6-24months. ID defined as serum iron <30 for children <12months; serum iron <53 for children >12months. $c$: Laboratory cut-off value for Hb not referenced by the authors.
Figure 3. Prevalence of iron deficiency in: A) HIV-uninfected controls, B) HIV uninfected controls
1) n= 291 (HIV-infected cohort), 1510; data obtained from studies on low-income settings, no data available for controlled studies from high-income settings. 2) Pooled estimates; Test of heterogeneity (random effects model): Q=1.75, d.f.=3 (p=0.43) I²=0. 3) Pooled estimates; Test of heterogeneity (random effects model): Q=2.94, d.f.=3 (p<0.001), I²=0.

Figure 4. Pooled analyses of iron deficiency prevalence in. 1) n=1801 (HIV positive: 291); compares prevalence rates of iron deficiency in HIV-infected children with controls in the four controlled studies, only studies done in low-income settings had a control group included. 2) Test of overall effect: Z=-2.17 (p=0.03).
Chapter five | Iron deficiency anaemia in children with HIV-associated anaemia

Results | Intervention studies

Only one of the studies reviewed was an intervention study, done in a high-income setting looking at the effect of oral iron therapy on iron deficiency and intestinal malabsorption in HIV-infected children.\textsuperscript{12} Iron deficiency was present in 48% of the study population, and was significantly associated with intestinal malabsorption.

Discussion

In this first review on iron deficiency in paediatric HIV-infection, we observed that there is limited data on iron status in HIV-infected children. However, we identified that HIV-infected children are less likely to be iron deficient when compared with HIV-uninfected children. This comparison could only be made for children from low-income settings, since there were no studies from high-income settings reporting on the prevalence of iron deficiency in HIV-uninfected controls. We also observed that prevalence rates of iron deficiency in HIV-infected children are significantly modified by HAART use.

True versus functional iron deficiency in HIV-associated anaemia

Our results suggest that iron deficiency in HIV-infected children is a problem in both high and low-income settings, despite the increasing availability of HAART- with studies from both areas reporting similar mean prevalence rates. Our results also suggest that iron deficiency may be less common in HIV-infected children than in uninfected children. HIV-infected children are commonly malnourished due to macronutrient and multi-micronutrient deficient diets, as well as increased metabolic demands during infections.\textsuperscript{30} Iron deficiency however does not appear to be more common.

One of the studies presented in this review which looked at bone marrow aspirates in a subset of their HIV cohort found normal iron content in all 20 specimens examined- although other bone marrow studies done in children reported higher iron deficiency prevalence rates.\textsuperscript{3,9,28} Pathophysiologic mechanisms that may explain the lower prevalence of iron deficiency seen in HIV-infected children compared with controls include a
cytokine-mediated inhibition of erythroid progenitor cells and an inflammation-induced hypoferremia, with haemoglobin levels closely correlated with low serum iron levels. These mechanisms may result in functional iron deficiency, where indices of body iron stores are relatively normal while indices of peripheral body iron are reduced. This is in contrast with true iron deficiency where iron stores (notably ferritin levels) are depleted. Functional iron deficiency, which may be seen in HIV and other chronic inflammatory disorders, may render iron unavailable for erythropoiesis resulting in anaemia but also makes it unavailable for infective organisms that require iron for growth and proliferation - thus may be protective against severe bacterial infections and malaria in regions with a high pressure of infection.

The use of iron markers in infection: the role of inflammation
The interpretation of serological markers of body iron status is unclear in areas with a high pressure of infection. Ferritin, which is commonly used to estimate body iron is also a mediator of the acute phase response and is raised in HIV-infection while serum iron and blood transferrin levels are decreased. Some of the studies identified in our search did not adequately adjust their ferritin cut-off values for inflammation-thus true iron deficiency may be present even when the indices of iron status exceed the recommended normal cut-off value. This could have contributed to the lower prevalence of iron deficiency seen in the HIV-infected cohorts of studies that used ferritin alone to assess iron status. However, two of the three controlled studies (both done in regions of high infection pressure) that used ferritin alone to assess iron status did not report a significant difference in mean ferritin values or the prevalence of iron deficiency between their HIV-infected and control populations. More importantly, the only controlled study which used serum transferrin receptor-log ferritin index to determine iron status- a marker which is less influenced by infection showed that iron deficiency was less prevalent in HIV-infected children compared with controls, further adding weight to our findings. In the absence of bone marrow iron values, ideally studies using a combination of two or more serological iron markers, preferably including a marker of inflammation in regions with a high pressure of infections should have been used but this was not possible as the number of eligible studies presenting this data was too small.

Iron states in HIV-associated anaemia: is intervention needed?
The results of this review casts some doubt as to the role of iron deficiency in the aetiology of anaemia of HIV-infection, and raises important questions on how HIV-associated anaemia should be managed. It is known that anaemia in HIV disease is associated with an increased risk of death and anaemia treatment is associated with an improved survival and quality of life in HIV-infected persons. However, it is unknown if iron deficiency plays a major role in the aetiology of anaemia in HIV-infected children, with the results suggesting that it is less common when compared with uninfected children. An important question remains: if the WHO policy of iron supplementation for the prevention and treatment of anaemia is favourable in HIV-infected children, especially in the era of increased availability of HAART. Castaldo et al demonstrated that iron supplementation is beneficial in HIV-infected children; however the six patients that received iron in this study were neither randomised nor blinded, and there was no control group with which to compare their findings- thus their results were subject to selection and measurement bias. They also did not report if there were any untoward effects seen as a result of iron supplementation. The detrimental effects of iron supplementation for the treatment of anaemia in iron-replete children have been clearly demonstrated in previous studies, with the results showing an increased risk of severe infections and death in populations with a high prevalence of malaria. Given the increased risk of susceptibility to infections in HIV-infected children, this evidence is needed to inform management, especially in regions with a high prevalence of infections, including malaria.
Chapter five | Iron deficiency anaemia in children with HIV-associated anaemia

In trying to assess the impact of important factors that could have affected our results, we examined the effect of HAART on our prevalence estimates. We observed that routine HAART use in HIV-infected children was associated with a lower prevalence of iron deficiency when compared with HIV-infected children not on HAART. The explanation for this observation is unclear, but it may be related to the beneficial effect of HAART on anaemia and haematopoiesis, where a reduction in viral load results in reversal of cytokine-mediated haematosuppression. A recent study carried out in India by Shet et al in which a cohort of HIV-infected children managed according to national guidelines were followed for 6 months reported that HAART when given with iron supplementation was associated with a better haemoglobin response than either HAART or iron given alone, without any serious adverse effects seen. These observations make a strong case for the conduct of studies examining the effects of HAART on iron status in HIV-infected children.

Other factors that could potentially have had an impact on our findings include poor dietary intake and bioavailability of iron, inadequate HIV disease monitoring and management in resource-limited settings, prevention and treatment of opportunistic infections, and management of co-morbidities such as malnutrition and parasitic infections—especially in regions with a high pressure of infection. We have tried to limit the impact of these factors by classification of the studies included in this review; however their importance cannot be understated, especially in low-income settings.

Limitations of this review

The number of studies identified by our search was small and heterogenous, restricting the differential analytic power of the meta-analysis. We partly corrected for this by doing sub-group analyses using high and low income settings. However, as we found some asymmetry on visual assessment of funnel plots (not presented), we cannot rule out publication bias which is not uncommon with diagnostic studies.

None of the studies from high-income settings provided data on iron deficiency in HIV-uninfected controls, thus it was not possible to compare findings in controlled studies from different geographic (socio-economic) settings. There were no randomized clinical trials on iron supplementation in HIV-infected children with anaemia identified in our search.

Clinical impact and areas for future research

Our findings suggest that iron deficiency may be less common in HIV infected children compared with HIV-uninfected children. Studies examining the role of iron deficiency in the aetiology of HIV-associated anaemia are needed. There is a need for more studies in which the iron status of HIV-infected children is correctly defined, ideally using bone marrow iron estimates, and also for studies examining the effect of HAART on iron status. There is a need for randomized clinical trials evaluating the safety and benefit of iron supplementation in the treatment of HIV-associated anaemia, especially in low-income settings.
Conclusions

Though the data available was limited, the findings suggest that HIV-infected children are less likely to be iron deficient when compared with HIV-uninfected children. Iron deficiency however remains a common co-morbidity. Studies are needed to determine the role of iron deficiency in HIV-associated anaemia and to assess the safety and benefit of iron supplementation. This information is required to guide management, especially in low-income settings with a high prevalence of infections, including malaria.

Acknowledgements and contributions

The manuscript was prepared by MOE and FAMJ. JCJC, MBVH and KSP provided editorial support. Statistical analysis was done by MOE and JCJC. The authors would like to thank Caroline Veraart and Mw. Dyserineck of Emma Children’s Hospital AMC for logistics and help with the database search strategies respectively. Funding: Netherlands-African partnership for capacity development and clinical interventions against poverty-related diseases (NACCAP). Competing interests: None declared. Ethical approval: None required.
Chapter five | Iron deficiency anaemia in children with HIV-associated anaemia

References


16. WHO. Assessing the iron status of populations. 2007.


Chapter six

Unexpected low hepcidin levels in severely anaemic Malawian children with high incidence of infectious diseases and bone marrow iron deficiency


Submitted for publication
Abstract

Iron supplementation targeting only iron deficient children is preferred in infection endemic areas as supplementing iron replete children may increase their infection risk. A reliable iron biomarker for individual assessment is lacking in these populations. The iron-regulator, hepcidin, controlled by host iron status, anaemia and inflammation, is a potential candidate marker for identifying iron deficiency and guiding supplementation programs. However, circulating hepcidin levels have never been evaluated against bone marrow iron status in African children.

In this cross-sectional study, bone marrow iron status was evaluated in 237 severely anaemic (haemoglobin <5.0 g/dL) Malawian children (6-60 months) in which hepcidin levels were assessed. It was found that hepcidin levels were unexpectedly low, and compared to other peripheral iron markers, poorly predicted bone marrow iron status. Structural-equation-modelling indicated a strong down-regulating stimulus of erythropoietin, which counterbalanced up-regulation by CRP and IL-6. Furthermore CRP, not hepcidin, was negatively associated with insufficient erythroblast iron incorporation.

In these severely anaemic children hepcidin was a poor marker for bone marrow iron status. Low serum hepcidin would be an unreliable indicator for iron supplementation in these children, and may expose children to an increased infection risk.
Chapter six | Hepcidin levels in severely anaemic children

Introduction

Iron deficiency is a nutritional disorder which is common world-wide. Pre-school children in developing countries are one of the most important risk groups, as iron deficiency not only causes anaemia but also developmental dysfunction and is a major contributor to child morbidity and mortality in these settings. Iron supplementation is considered a cost-effective strategy to prevent and treat anaemia, although there is concern that it may increase risk of infection in iron replete children living in malaria endemic areas. The World Health Organization therefore recommends restricting iron supplementation to children with proven iron deficiency or signs of severe anaemia. In these circumstances a reliable iron biomarker is required in order to identify those iron deficient children who may benefit from iron supplementation without experiencing an increased infection risk. Such a biomarker is currently lacking.

The recently discovered key iron regulator ‘hepcidin’ plays a central role in the interaction between iron deficiency, anaemia and inflammation. In iron loaded conditions hepcidin levels increase during inflammation/infection through degradation of the cellular iron exporter ferroportin. This reduces intestinal absorption of iron and down regulates release of iron from iron-stores. Conversely, iron deficiency, anaemia/hypoxia and enhanced erythropoiesis, decrease hepcidin levels and will subsequently increase iron levels. In view of this central role, it has been suggested that serum hepcidin could be an ideal marker to guide iron supplementation requirements. Nevertheless in subjects living in areas of Africa with a high infection pressure, hepcidin has never been validated against bone marrow iron, the “gold standard” of iron status. We therefore evaluated serum hepcidin as a predictor of deficient bone marrow iron stores, as well of erythroblast iron incorporation in severely anaemic Malawian children. We further evaluated the impact of the different stimuli, iron levels, inflammation, hypoxia and erythropoiesis, on serum hepcidin levels in this population.

Methods

Methods | Study design and population

This study formed part of a large research programme investigating the etiology, pathophysiology and outcome of severe anaemia in Malawian children. A detailed description of the study methodology has been given elsewhere. In brief, 381 children (6–60 months old) presenting with severe anaemia (haemoglobin <5.0 g per deciliter) were enrolled as ‘cases’ if they had not been transfused in the previous 4 weeks. For each case, two controls were enrolled, a community-control living within 100 to 1000 meters of the case, and a hospital-control, presenting at the same hospital or outpatient facility as the case. Controls were eligible for recruitment if they were aged between 6-60 months and if their haemoglobin level was 5.0 gram per decilitre or more. At recruitment a venous blood sample was obtained and, (in cases) if the clinical condition allowed, a bone marrow aspiration was performed.

For the hepcidin study, all cases with available bone marrow aspirates were included. In addition, in order to compare hepcidin levels between severely anaemic and non-severely anaemic children, hepcidin was also assessed in a small sub-sample of controls (without available bone marrow aspirates). Written informed consent was obtained from a parent or guardian of each child. The study was approved by the ethics committees of the College of Medicine, Malawi, and the Liverpool School of Tropical Medicine, United Kingdom.
Methods | **Laboratory investigations**

Haemoglobin, mean cellular volume (MCV) and mean corpuscular haemoglobin concentration haemocrit (MCHC) were measured by a Coulter counter analyzer ( Beckman Coulter, Durban, South Africa). Bone marrow slides were stained with Hematognost Fe (Merck, Darmstadt, Germany) and graded for intracellular iron content using a histological grading method which classifies iron status into six grades (0-6). In addition, all marrow smears were also assessed using an alternative grading method where utilizable iron was specifically assessed through determination of iron in the erythroblasts. At high power magnification (*1000) 20 fields surrounding the marrow fragments were examined whereby hundred erythroblasts were examined to enumerate the percentage containing iron granules in their cytoplasm.

Within the first hour after collection 200µl plasma and serum aliquots were stored at -80 °C. As outcome of assessments of plasma and serum hepcidin is comparable we will refer as *serum* hepcidin. These samples were used for later assessment of hepcidin-25 (the mature, active form of the peptide) by a combination of weak cation exchange chromatography and time-of-flight mass spectrometry (WCX-TOF-MS). An internal standard (synthetic hepcidin-24; Peptide International Inc., Louisville, KY) was used for quantification. Peptide spectra were generated on a Microflex LT matrix-enhanced laser desorption/ionization (MALDI) TOF-MS platform (Bruker Daltonics, Bremen, Germany). Serum hepcidin-25 concentrations are expressed as nanomoles per liter (nmol/L). The lower limit of detection of this method was 0.5 nmol/L. Samples found to have a hepcidin concentration < 0.5 nmol/L were imputed with a random value out of a uniform distribution with a minimum of 0.01 nmol/L and a maximum of 0.5 nmol/L. Ferritin was determined using electro-chemiluminescence immunoassay (Modular Analytics E170, Roche Diagnostics, Switzerland). Erythropoietin was determined using Immulite 2000 (maximum detection limit <200 and after dilution >1000 IU/L) (Siemens DPC, new Jersey, USA). (Immunoturbidimetric assay (Modular P800, Roche Diagnostics, Switzerland) was used to determine serum transferrin and C-reactive protein (CRP). Soluble transferrin receptor (sTfR) was measured using an enzyme immunoassay (Ramco Laboratories, TX, USA, detection limit 1.0µg/l). Interleukin-6 (IL-6) was measured by Cytometric Bead Array on a FACS- Calibur flow- cytometer (Becton-Dickson, South Africa). Clinical malaria was defined as a positive blood slide with concurrent fever (axillary temp >37.5° C), or history of fever within the previous 48 hours.

Methods | **Definition and cut-off values**

Deficiency of bone marrow iron stores was defined as grade 0 or 1. Erythroblast iron incorporation was used as a proxy for utilizable iron in the bone marrow; insufficient erythroblast iron incorporation (functional bone marrow iron deficiency) was defined as less than 30% of the erythroblasts having visible iron granules while having replete iron stores. Cut-off values for the peripheral iron markers were as follows: hepcidin <0.5 nmol/L; ferritin <30 µg/L; sTfR >8.3 µg/mL; MCHC <32 g/L; MCV <67 fl (<2 years old) and <73 fl (2-5 years old). Soluble transferrin log-ferritin index (sTfR-F index) was defined as $sTfR + \log ferritin$ in which log refers to ‘base-10 log’. Using the cut-offs sTfR >8.3 µg/L and ferritin <30 µg/L, a cut-off for sTfR-F index was calculated (>5.6).

Methods | **Statistical Methods**

Data were analyzed with use of SPSS 20.0 and AMOS 20.0 statistical computer packages (SPSS, Chicago, Illinois). Correlations with bone marrow iron grading were assessed with the Kendall rank correlation test for ordinal data. Median hepcidin levels between the different sub groups were compared using the Mann-Whitney U test. Using the dichotomous outcome of bone marrow iron status and erythroblast iron incorporation, receiver operating characteristics (ROC) curves and corresponding areas under the curve (AUCROC) were created for hepcidin and conventional iron markers. The ROC curves were used to assess
a cut-off for hepcidin providing optimal sensitivity and specificity in predicting bone marrow iron deficiency, and for all iron markers in predicting insufficient erythroblast iron incorporation. To evaluate different signalling pathways of hepcidin, all relevant available covariates relating to hepcidin were analyzed using linear regression models with bone marrow iron deficiency as outcome. These predictors of hepcidin included erythropoietin, reticulocytosis, haemoglobin, bone marrow iron grading, CRP, IL-6, malaria and bacteraemia. To evaluate the association between hepcidin and insufficient erythroblast iron incorporation, all relevant available covariates relating to erythroblast iron incorporation were analyzed using logistic regression models, these predictors of erythroblast iron incorporation included hepcidin, use of haematinics in the previous 4 weeks and CRP. Missing observations were included in analyses by creating missing-value categories. Reported p-values are two-sided; statistical significance was set at the conventional 5% level.

Structural equation model
More complex multivariate analyses allowing interaction of covariates were performed using structural equation modelling.34 This model was created containing all possible associations between all relevant available variables relating to hepcidin, after which all non-significant arrows or variables (p ≥0.05) were removed, unless pathophysiologically deemed relevant (dashed arrows). Skewed variables were log-transformed. Missing observations were considered as missing at random and were imputed by single imputation.34

Results
From the 381 severely anaemic children, in children 237 a bone marrow aspirate was performed. In 139 of these children a serum sample with sufficient volume for hepcidin assessment was available. Haematological status of children with or without hepcidin and/or bone marrow assessment was comparable (data not shown). From the 757 controls, 43 children were randomly selected for hepcidin assessment. Haematological status of this sub-group was comparable to all controls (data not shown). In Table 1 baseline characteristics of the severely anaemic study group and the non-severely anaemic control group are presented. Bone marrow iron deficiency (30.2%), inflammation (81.2% CRP >40.0 mg/L) and high levels of erythropoietin (92.9% epo >1000 u/L), were common in the severely anaemic children.

Results | Hepcidin values in severely and non-severely anaemic children
The median value of hepcidin in the severely anaemic children was 0.40 nmol/L (IQR 0.19-2.10 nmol/L) and 1.40 nmol/L (0.31-5.60 nmol/L) in the non-severely anaemic control group (p =0.04).

Results | Hepcidin and conventional iron markers predicting bone marrow iron stores
Hepcidin levels poorly correlated with bone marrow iron grading (Kendall rank correlation coefficient 0.05, p=0.5). The ROC-curve for hepcidin identifying bone marrow iron deficiency showed an area under curve (AUCROC) of 0.598, not quite significantly higher than 0.500 (p=0.07), the cut-off to indicate an effective test 35. The AUCROC of ferritin, sTfR, sTfR-F index and MCHC were all greater than 0.500 (p<0.05) (Table 2). The optimal cut-off for hepcidin to predict bone marrow iron deficiency, derived from the ROC-curve, was 0.5 nmol/L. Using this cut-off for hepcidin and standard cut-offs for the conventional iron markers, sensitivity and specificity to detect bone marrow iron deficiency were calculated (Table 2). Sensitivity and specificity of hepcidin to predict bone marrow iron deficiency were 66.7 % and 49.5%, respectively.
Table 1. Baseline characteristics of the study population

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>severely anaemic cases</th>
<th>non-severely anaemic controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Demographics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>48.8% (101/207)</td>
<td>69.8% (30/43)</td>
</tr>
<tr>
<td>Age (months)</td>
<td>14.0 (9.7-26.3)</td>
<td>20.1 (10.6-28.3)</td>
</tr>
<tr>
<td>Haematological status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Haemoglobin (g/dl)</td>
<td>3.6 (0.8)</td>
<td>9.7 (1.9)</td>
</tr>
<tr>
<td>MCHC (g/dl)</td>
<td>32.60 (6.8)</td>
<td>33.2 (5.4)</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>82.7 (15.4)</td>
<td>71.2 (8.0)</td>
</tr>
<tr>
<td>Reticulocytes %</td>
<td>3.6 (2.1-6.7)</td>
<td>2.3 (1.7-4.3)</td>
</tr>
<tr>
<td>Erythropoietin &gt;1000 U/L</td>
<td>95.6% (108/113)</td>
<td>0% (0/39)</td>
</tr>
<tr>
<td>Iron status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deficiency of bone marrow iron stores</td>
<td>30.2% (42/139)</td>
<td>n/a</td>
</tr>
<tr>
<td>Insufficient erythroblast iron incorporation</td>
<td>41.4% (48/116)</td>
<td>n/a</td>
</tr>
<tr>
<td>Hepcidin (nmol/L)</td>
<td>0.40 (0.19-2.10)</td>
<td>1.40 (0.31-5.60)</td>
</tr>
<tr>
<td>Ferritin (µg/l)</td>
<td>404 (157-905)</td>
<td>50 (12-171)</td>
</tr>
<tr>
<td>sTfR (µg/ml)</td>
<td>13.4 (8.1-22.4)</td>
<td>12.5 (7.2-17.5)</td>
</tr>
<tr>
<td>sTfR-F index</td>
<td>5.1 (3.1-9.6)</td>
<td>5.9 (3.1-12.0)</td>
</tr>
<tr>
<td>Inflammatory status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacteraemia</td>
<td>12.6% (25/198)</td>
<td>0% (0/20)</td>
</tr>
<tr>
<td>Malaria parasitaemia</td>
<td>60.9% (126/207)</td>
<td>37.2% (16/43)</td>
</tr>
<tr>
<td>HIV infected</td>
<td>7.7% (15/195)</td>
<td>3.7% (2/43)</td>
</tr>
<tr>
<td>CRP &gt; 40 mg/L</td>
<td>81.2% (159/196)</td>
<td>37.7% (12/31)</td>
</tr>
<tr>
<td>Interleukin-6 pg/ml</td>
<td>41.6 (20.9-142.3)</td>
<td>25.6 (10.0-67.3)</td>
</tr>
</tbody>
</table>

Deficiency of bone marrow iron stores was defined as a bone marrow iron score of 0 or 1; Deficiency of functional bone marrow iron was defined as < 30% iron deficient erythroblasts while having replete iron stores 25,26; CRP: C-Reactive Protein; HIV: Human Immunodeficiency Virus; MCHC: mean cell haemoglobin concentration; MCV mean corpus volume; sTfR: soluble transferrin receptor; sTfR-F index: sTfR-log ferritin index 25. Normally distributed variables are presented with their mean value (s.d.); skewed variable are presented with their median value (inter quartile range)

Table 2. Performance of biochemical iron markers to identify children with deficiency of bone marrow iron stores or insufficient erythroblast iron incorporation

<table>
<thead>
<tr>
<th>Iron marker</th>
<th>bone marrow iron deficiency</th>
<th>insufficient erythroblast iron incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AUCROC</td>
<td>p value</td>
</tr>
<tr>
<td>Hepcidin (nmol/L)</td>
<td>0.598</td>
<td>0.07</td>
</tr>
<tr>
<td>Ferritin (µg/l)</td>
<td>0.855</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>sTfR (µg/ml)</td>
<td>0.823</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>sTfR-F index</td>
<td>0.815</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>MCHC (g/dl)</td>
<td>0.727</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>0.489</td>
<td>0.9</td>
</tr>
</tbody>
</table>

Deficiency of bone marrow iron stores was defined as a bone marrow iron score of 0 or 1; CRP: C-Reactive Protein; HIV: Human Immunodeficiency Virus; MCHC: mean cell haemoglobin concentration; MCV mean corpus volume; sTfR: soluble transferrin receptor; sTfR-F index: sTfR-log ferritin index 25. Insufficient erythroblast iron incorporation: defined as < 30% iron deficient erythroblasts. AUC area under the curve; ROC: receiver operating characteristics. sens: sensitivity; spec: specificity.
Chapter six | Hepcidin levels in severely anaemic children

Results | **Hepcidin, iron status, inflammation, hypoxia and erythropoiesis**
To further explore the hepcidin levels in our population we evaluated potential predictors of hepcidin. The signalling pathways for hepcidin are theoretically classified in four main categories: iron status, inflammation, hypoxia and erythropoiesis. Associations with available variables belonging to these pathways were assessed using univariate linear regression analyses (Figure 1, colour section). The multivariate linear regression model predicting log-hepcidin included a proxy variable \( \beta \) for each pathway (bone marrow iron status, CRP, haemoglobin and erythropoietin). To assess more complex interactions between hepcidin and the hepcidin pathways, a structural equation modelling was created (Figure 3, colour section). The iron signalling pathway, represented by bone marrow iron stores had a weak association with hepcidin (regression coefficient 0.03, \( p=0.6 \)). The inflammation signalling pathway, represented by CRP and IL-6, was strongly positively associated with hepcidin (regression coefficients of 0.23, \( p<0.0001 \) and 0.33, \( p<0.0001 \) respectively). CRP was adjusted for malaria and bacteraemia; IL-6 was adjusted for bacteraemia. The hypoxia signalling pathway reflected by haemoglobin concentration did not significantly associate with hepcidin (regression coefficient 0.02, \( p=0.7 \)). Regarding the erythropoietic signalling pathway, erythropoietin had a strong negative association with hepcidin (regression coefficient -0.56, \( p<0.0001 \)). The overall root mean square area of approximation, an indicator for model fit, was 0.288 (95% CI 0.272-0.304).

Results | **Hepcidin and conventional iron markers predicting insufficient erythroblast iron incorporation**
From the 139 available bone marrow smears, 23 were too poorly stained for erythroblast reading and were excluded from analyses concerning erythroblast iron incorporation. From the remaining samples, 49 (41.4\%) showed insufficient erythroblast iron incorporation. The value of hepcidin and the conventional iron markers to predict erythroblast iron incorporation were evaluated (Table 2). The AUC\(^{ROC} \) for hepcidin was 0.591 (\( p=0.1 \)). Using the optimal cut-off of 0.5 nmol/L, sensitivity and specificity of hepcidin to predict insufficient erythroblast iron incorporation were 45.2\% and 61.8\%, respectively (Table 2).

Results | **Hepcidin, inflammation and erythroblast iron incorporation**
To explore the association between hepcidin levels and erythroblast iron incorporation we evaluated potential predictors of erythroblast iron incorporation, which were assessed using univariate and multivariate logistic regression analyses (Figure 2, colour section). Applying these variables into the structural equation model (Figure 3, colour section), hepcidin levels were weakly associated with insufficient erythroblast iron incorporation (regression coefficient -0.04, \( p=0.6 \)). CRP was associated with insufficient erythroblast iron incorporation (regression coefficient 0.15, \( p=0.02 \)).

Discussion
This is the first study evaluating the possible role of serum hepcidin in identifying iron deficiency in children living in areas with a high infection pressure, and comparing hepcidin with bone marrow iron and other hepcidin signalling pathways. Hepcidin levels in these anaemic children were unexpectedly low in view of their high infection burden. This may result from a dominance of the erythropoietin signal for hepcidin in these severely anaemic children. As a consequence, hepcidin was found to be a poor marker for bone marrow iron stores. Furthermore hepcidin was not associated with insufficient erythroblast iron incorporation.
Bone marrow iron stores and hepcidin

Previous studies showed a positive association between hepcidin and serum and liver iron levels \(^{13,14,37}\). With the high prevalence of bone marrow iron deficiency in our population we would have expected low hepcidin values. However, although the hepcidin values were low in this study, the structural equation model showed they were only weakly associated with bone marrow iron stores. This can be a result of the stronger effect on hepcidin production of other signalling pathways, erythropoietic drive and inflammation (Figure 3, colour section), both of which were strongly positively associated with hepcidin. As a consequence hepcidin was not a better iron marker than the previous evaluated conventional iron markers in this population from which sTIR-F index best performed. \(^{23}\)

Inflammation and hepcidin

In view of the strong association of hepcidin with IL-6 and CRP in our population, one would expect elevated hepcidin levels in such a population with high levels of IL-6 and CRP. \(^{9,10,12,38}\) Yet the hepcidin levels were low. As described below, this is explained by the strong down regulating factor of erythropoietin.

Erythropoiesis and hepcidin

Levels of erythropoietin were very high in this severely anaemic population, and strongly associated with low serum hepcidin. The regression coefficients determined in the structural equation model, and the occurrence of low hepcidin levels despite a high incidence of infection, suggested that the down-regulating stimulus due to erythropoietin exceeded that of the up-regulation due to inflammation. This could be confirmed by the higher hepcidin values in our control group with lower erythropoietin and higher CRP and IL-6 levels. Erythropoietin dominating inflammation has been reported in an infectious murine model in which low hepcidin values were observed following administration of erythropoietin. \(^{18,39,40}\) Similar findings have been reported in humans although these subjects had less inflammation compared to children in the present study. \(^{41,42}\) It is unclear whether erythropoietin is directly down regulating hepatic hepcidin synthesis\(^{43}\), or indirectly through induced production of bone marrow erythropoietic factors, i.e. growth differentiation factor-15 (GDF15) and twisted gastrulation protein homolog-1 (TWSG1). \(^{44,45}\)

Hypoxia/anaemia and hepcidin

Hypoxia/anaemia may directly induce a reduction in hepcidin release \(^{38,43,46,47}\) and as a consequence haemoglobin concentration was expected to positively correlate with hepcidin concentration, although this was not observed in this selected group of severely anaemic children. The degree of severity of anaemia in all children may have reduced the heterogeneity of the hepcidin response.

Hepcidin and erythroblast iron incorporation

As regulator of available utilizable iron, hepcidin was expected to be positively associated with erythroblast iron incorporation. This association was not observed. This finding differs from results in Gambian children that showed hepcidin strongly predicted red blood cell iron incorporation measured after 14 days of oral iron supplementation. \(^{19}\) Conversely Cercamondi et al observed that although hepcidin (assessed after 25 days) was associated with intestinal iron absorption, there was no association with red blood cell iron incorporation. \(^{48}\) These studies suggest that intermediate factors may interfere with iron incorporation and may explain the lack of correlation between hepcidin and erythroblast iron incorporation in our study. Furthermore, iron availability (through absorption and release from storage), and iron incorporation is a dynamic process which may be difficult to profile with cross-sectional data alone. This is further underlined by the fact that also all iron markers in this study poorly predicted erythroblast iron incorporation.
Chapter six | Hepcidin levels in severely anaemic children

Alternatively inflammatory factors other than hepcidin may inhibit erythroblast iron incorporation as erythrocyte iron incorporation did not correlate with hepcidin, malaria, or IL-6, but significantly correlated with CRP.

It may be argued that our study population may have been an extreme population with severe anaemia, high erythropoietin levels, and a high infection exposure, and the hypothetic model may not be applicable to populations with less severe morbidity. However, these children are very common in resource limited settings in Africa. Furthermore, the model presented in Figure 3 is the most complete model available to date. Ideally the fit of this structural equation model, represented by the root mean square area of approximation (RMSEA), would be smaller then 0.050. Our RMSEA was 0.288 which may partly be explained by missing values. Application of this model to a similar but complete data set should decrease the RMSEA and provide a stronger hypothetical model. An alternative explanation for the increased RMSEA could be a missing intermediate variable in the model, such as an unmeasured erythropoietic or inflammatory factor.

It is important to note that variation in bone marrow findings can result \(^4\) from an uneven distribution of iron in the bone marrow. \(^5\) Therefore a minimum of 10 bone marrow particles per aspirate was examined to optimise sensitivity of the bone marrow examination.\(^6\) Bone marrow assessment still has generally been considered the most reliable diagnostic test for iron status. \(^5\)-\(^6\)

Clinical implications
Translated into practice, our observations raise critical questions concerning the use of iron supplements in severely anaemic children. In these children hepcidin concentrations were low, despite their highly infectious status. Normally a rise in hepcidin would be expected, which would diminish iron absorption and its availability for pathogens, such as malaria.\(^3\) Iron supplementation in such severely anaemic children with concurrent infections could therefore enhance their infection risk, as their hepcidin response is stunted (Figure 4). Currently iron supplementation is recommended for severely anaemic children.\(^6\) There is an urgent need for studies investigating iron uptake in severely anaemic children with malaria, or other infectious diseases, and to assess infection incidence in children with low hepcidin levels receiving iron supplementation.
Figure 4. Hypothetic model of the iron metabolism in severely anaemic children.
Severe anaemia is associated with severe infections; the associated high levels of IL-6 induce hepcidin. However in severe anaemia hypoxia stimulates the kidneys to produce erythropoietin; the erythropoietin induced erythropoietic drive will down-regulate hepatic hepcidin expression and may overrule the hepcidin stimulation of IL-6. The resulting low hepcidin values are associated with a decreased degradation of the cellular iron exporter ferroportin; in iron replete subjects or during iron supplementation this will lead to a rise in plasma iron levels. In infection endemic areas this may increase infection risk. In addition inflammatory related factors possibly inhibit erythroblast iron incorporation. CRP: C-reactive protein; ery: erythroblast; epo: erythropoietin; Fe: plasma iron; FPN: ferroportin; hep: hepcidin-25; IL-6; interleukin 6.

Acknowledgments and contributions
The authors thank the parents and guardians of the children admitted in the study; the SevAna study team; the staffs of the Queen Elizabeth Central Hospital and Chikwawa District Hospital. This study was supported by a grant (064722) from the Wellcome Trust and by independent grants from the Nutricia Research Foundation, Stichting Emma Steun, and JANIVO Foundation. F.A.M.J analyzed the data, interpreted the results, and wrote the manuscript; J.C.J.C., K.P., B.J.B and M. BvH designed the research, coordinated data collection, interpreted study results; B.J.F. performed statistical analyses. D.W.S., E.T.G. and H.T. were responsible for the design, performance and interpretation of study results. All authors critically revised the manuscript for intellectual content, and read and approved the final version. Conflict-of-interest disclosure: D.W.S and H.T are medical director and managing director, respectively, of the www.hepcidinanalysis.com initiative that serves the scientific and medical community with hepcidin measurements at a fee-for-service basis. The remaining authors declare no competing financial interests.
Chapter six | Hepcidin levels in severely anemic children

References

4. WHO. Guideline for the use of iron supplements to prevent and treat iron deficiency anemia. 1998.
6. WHO. Conclusions and recommendations of the WHO Consultation on prevention and control of iron deficiency in infants and young children in malariacendemic areas. 2007.
Chapter seven

Conventional and novel peripheral blood iron markers compared against bone marrow in Malawian children


Submitted for publication
Abstract

Iron deficiency is an important global child health problem. Diagnosis of iron deficiency is complicated as peripheral blood markers have not been validated against bone marrow iron, the reference standard and may have a reduced diagnostic efficiency in infection endemic areas.

In 87 healthy Malawian children (6-66 months) scheduled for elective surgery, ferritin, soluble transferrin receptor (sTfR), soluble transferrin log-ferritin index (sTfR-F), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration and two novel iron markers, hepcidin and Red blood cell Size Factor (RSf), were compared to assessment of bone marrow iron. Prevalence of bone marrow iron deficiency was 44.8%. Ferritin, sTfR, sTfR-F, hepcidin and MCV showed AUCROC >0.500 (0.797;0.691;0.801;0.673 and 0.674 respectively). Using new cut-offs, ferritin (<18ug/L, sensitivity:73.7%/specificity:77.1%) and sTfR-F (>0.8, sensitivity:72.5%/specificity:75.0%) best predicted bone marrow iron deficiency. Hepcidin (<1.4 nmol/L) was a sensitive marker (73.0%) although specificity was 54.2%. RSf poorly predicted bone marrow iron deficiency.

In this first study evaluating peripheral iron markers against bone marrow iron deficiency in healthy African children, ferritin and sTfR-F best predicted bone marrow iron status and would be preferred for surveillance and research. For clinical diagnosis of iron deficiency there is an urgent need for a more reliable bio-marker in African children.


**Introduction**

Iron deficiency is thought to have an important impact on global child health, especially in resource limited settings \(^1\)\(^-\)\(^2\). In sub-Saharan Africa prevalence of iron deficiency in children ranges from 33 to 63% \(^3\). This variation partly relates to demographic differences, but also to variation in choice of biomarkers and definitions of iron deficiency. Diagnosing iron deficiency using peripheral biomarkers is complicated as bone marrow iron status is considered the accepted standard even though it is invasive and limited for widespread use. Available peripheral iron markers are considered unreliable in populations with high infection pressure, \(^4\) although they have not been validated against bone marrow iron status in these settings. As a consequence different biomarkers and cut-offs, are used in epidemiological and clinical studies assessing iron status and as a result these are difficult to compare and interpret.

There is an urgent need for reliable data on the accuracy of peripheral iron markers in infection endemic populations. This study evaluates the diagnostic accuracy of commonly iron markers against bone marrow iron deficiency in healthy Malawian children. In addition, two new markers were tested; hepeidin, a recently discovered key iron regulating hormone \(^5\)\(^-\)\(^7\), and the Red cell Size Factor (RSf) an iron marker calculated from the mean cell volume (MCV) and mean reticulocyte volume (MRV) reflecting the haemoglobin content of both reticulocytes and red blood cells \(^8\)\(^,\)\(^9\). The diagnostic accuracy of these markers was compared to a reference bone marrow standard for iron status \(^10\)\(^-\)\(^12\), in order to identify the most reliable marker for iron deficiency in this high risk group of children.

**Methods**

**Methods | Study participants and study design**

Study participants were prospectively identified from children aged 6–66 months, scheduled for elective surgery at Queen Elizabeth Central Hospital and Beit Cure Orthopedic Hospital, in Blantyre, Malawi. Exclusion criteria were: blood transfusion within the previous 4 weeks, signs of infection (axillary temperature >37.5 °C or current infectious diagnosis, (suspected) neoplasm, known haemoglobinopathy, or a haemoglobin level below 8.0 g/dL. (local guidelines for elective surgery). The recruitment procedure included a detailed history and physical examination, a venous blood sample and a bone marrow aspiration. Both samples were collected during generalized anaesthesia and prior to surgical intervention. Written informed consent was obtained from the parent or guardian of each child. The study was approved by the Ethical Committees of the College of Medicine, Malawi and of the Academic Medical Centre of Amsterdam, the Netherlands.

Ferritin, soluble transferrin receptor (sTfR), soluble transferrin log-ferritin index (sTfR-F), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC) hepeidin and Red blood cell Size Factor (RSf) were assessed in peripheral blood and compared against the reference standard for iron status, bone marrow iron.

**Methods | Bone marrow iron**

On the same day a bone marrow aspiration was performed. Bone marrow smears were prepared, fixed with methanol and stored in a dry place at room temperature. To minimize diagnostic variation, fixed smears were all stained in one batch at the end of the study period (Hematognost Fe, Merck, Darmstadt, Germany) and graded for iron content. Histological grading classified iron status into six grades \(^13\)\(^,\)\(^14\). To optimize sensitivity a minimum of 10 bone marrow particles per aspirate \(^12\) were identified by two readers using a double reading
system. Readers were unaware of patient characteristics or each others results.

Methods | Haematology
Peripheral blood samples were collected in EDTA-anticoagulant (Becton-Dickinson Vacutainer, Plymouth, UK) and assessed on the same day for haemoglobin and red blood cell indices using a Beckman Coulter AcT 5 diff AL Hematology Analyzer (Villepinte, France). MCV and mean reticulocyte volume (MRV) were assessed using a Beckman HmX Coulter counter analyzer (Durban, South Africa) for calculation of RSf. Basic characteristics of these children (n=43) were comparable to the first study population (data not shown).

Methods | Iron biomarkers
Heparin-plasma samples (Becton-Dickinson Vacutainer, Plymouth, UK) were stored in -80 °C and assessed at the end of the study period. Ferritin and sTfR were assessed using a Beckman Coulter Chemistry System Dxl880i with reagent kits from Beckman Coulter, and followed the manufacturers’ procedure. Serum hepcidin-25 measurements (the mature, active form of the peptide) were performed by a combination of weak cation exchange chromatography and time-of-flight mass spectrometry (WCX-TOF-MS)\textsuperscript{15}. An internal standard (synthetic hepcidin-24; Peptide International Inc., Louisville, KY) was used for quantification\textsuperscript{16}. Peptide spectra were generated on a Microflex LT matrix-enhanced laser desorption/ionization (MALDI) TOF-MS platform (Bruker Daltonics, Bremen, Germany). Serum hepcidin-25 concentrations are expressed as nanomoles per liter (nmol/L). The lower limit of detection of this method was 0.5 nmol/L\textsuperscript{15}. Samples found to have a hepcidin concentration <0.5 nmol/L were imputed with a random value out of a uniform distribution with a minimum of 0.01 nmol/L and a maximum of 0.5 nmol/L.

Methods | Infection markers
C-reactive protein (CRP) was assessed using a Beckman Coulter Chemistry System Dxc880i; HIV testing was performed using two rapid tests (Determine, Abbott-Laboratories, Japan; Unigold, Trinity-Biotech, Ireland). Inflammation was defined as CRP >10 g/L (method specific reference value). Discordant results and reactive results in children less than 18 months were resolved by HIV-PCR\textsuperscript{17}. Malaria was excluded by a negative blood slide and a negative rapid test (Standard Diagnostics, Korea).

Methods | Definitions
Bone marrow iron stores deficiency was defined as grade 0 or 1 {Finch, 1982 2397 /idGale, 1963 22 /id}. Iron deficiency using peripheral iron markers was defined as: hepcidin <0.5 nmol/L\textsuperscript{15}; ferritin: two definitions were tested a) <12 μg/L and <30 μg/dL, the latter being recommended in populations were infections are common\textsuperscript{20, 21}; sTfR >3.3 μg/mL; and MRV <100 fl (method-specific reference values); MCHC <32 g/L; MCV <67 fl(<2 years old) and <73 fl (age 2-5 years)\textsuperscript{2}. sTfR-F was defined as [sTfR + log ferritin] in which log refers to ‘base-10 log’\textsuperscript{22, 23}. Using these cut-off values for sTfR and ferritin, a sTfR-F cut-off of >2.2 was calculated to define deficient iron stores. RSf was calculated as the square root from [MCV * MRV]\textsuperscript{24}; cut-off values of MCV and MRV were used for calculation of the cut-offs for RSf (81.9 (age<2 years ) and 84.5 (age 2-5 years )). Anaemia was defined as a haemoglobin level of less than 11.0 g/dL\textsuperscript{3}.

Methods | Statistical Methods
We compared all peripheral iron markers by calculation of Receiver Operating Characteristic (ROC) curves and corresponding areas under the curve (AUC\textsuperscript{ROC})\textsuperscript{25}, and by calculation of the sensitivity, specificity, positive and negative predictive values. ROC-curves demonstrate the trade-off between
Chapter seven | Peripheral blood iron markers compared against bone marrow
sensitivity and specificity (any increase in sensitivity will be accompanied by a decrease in specificity). The
closer the curve to the upper left corner of the plot, the more accurate the test. The AUC is a measure to test
accuracy, with higher values indicating greater accuracy. ROC-curves were created using the dichotomous
outcome of bone marrow iron status {Finch, 1982 2397 /id;Gale, 1963 22 /id} and the corresponding
AUCROC was compared against the AUCROC cut-off for an effective test (0.500)20. Differences between
AUCROC were tested using a method described by Hanley and McNeil 20. New cut-off values providing
optimal sensitivity and specificity were derived from AUCROC. Correlations between peripheral iron markers
and bone marrow iron scores were assessed using the Kendall rank correlation test for ordinal data. Data
were double entered in Access (Microsoft Office 2007, USA) and analyzed using SPSS 20 statistical computer
packages (IBM statistics, USA). All p values reported are two-sided.

Results
Between March and October 2011 a total of 93 children were recruited. In four children the bone marrow
aspiration resulted in a “dry tap”, in two children bone marrow sample quality was insufficient (no marrow
fragments found). These children were excluded from analysis. From the remaining 87 children the mean age
was 37 months, 64% of children were anaemic and bone marrow iron deficiency was found in 39 children
(44.8 %) (Table 1). No adverse events after bone marrow aspiration or blood sampling occurred.
Apart from RSf all iron makers were significantly correlated with bone marrow iron grading (Table 2). Hepcidin, sTfR, ferritin, sTfR-F and MCV had a greater AUCROC than 0.500 (p >0.05) (Table 2, Figure 1,
colour section). The AUCROC of sTfR-F (0.801) was greater than that for hepcidin (0.673), sTfR (0.691) or
MCV (0.674)
(all p <0.05), although did not differ from that for ferritin (0.797, p=0.2) (Table 2). The AUCROC of ferritin
was greater than that for hepcidin (p <0.05), but did not significantly differ from that for sTfR-F (p=0.9),
sTfR (p=0.2) or MCV (p=0.09) (Table 2).
Sensitivity, specificity, positive predictive values (PPV) and negative predictive values (NPV) for detection of
bone marrow iron deficiency were calculated using published, as well as newly derived cut-offs  based on
ROC values (Table 3). Using the standard cut-offs for ferritin (<12 µg/ml and <30 µg/ml) the sensitivity and
specificity of ferritin were respectively 44.7% and 89.6% (<12), and 81.6% and 37.5% (<30). Applying the
new cut-off (<18 µg/L) the sensitivity and specificity of were 73.7% and 77.1% respectively. The
standard cut-off for sTfR-F (>2.2 µg/ml) had a sensitivity of 51.4% and specificity of 89.6% for bone
marrow iron deficiency; using the new cut-off sensitivity increased to 72.5% and specificity decreased to
75.0%. All other markers had either a sensitivity or specificity below 70% (Table 3).
Table 1. Baseline characteristics

<table>
<thead>
<tr>
<th>Demographic information</th>
<th>n</th>
<th>87</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td></td>
<td>42.5% (37/87)</td>
</tr>
<tr>
<td>Age in months</td>
<td></td>
<td>36.5 (17.1)</td>
</tr>
<tr>
<td>Iron status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bone marrow iron deficiency</td>
<td>44.8% (39/87)</td>
<td></td>
</tr>
<tr>
<td>Hepcidin (nmol/L)</td>
<td></td>
<td>1.10 (0.37-3.1)</td>
</tr>
<tr>
<td>RSf (fL)</td>
<td></td>
<td>83.3 (9.8)</td>
</tr>
<tr>
<td>Ferritin (μg/L)</td>
<td></td>
<td>19 (12-36)</td>
</tr>
<tr>
<td>sTfR (μg/L)</td>
<td></td>
<td>2.2 (1.8-2.6)</td>
</tr>
<tr>
<td>sTfR-F index</td>
<td></td>
<td>1.8 (1.2-2.4)</td>
</tr>
<tr>
<td>Haematology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anaemia (Hb &lt;11.0 g/dL)</td>
<td></td>
<td>71.3% (62/87)</td>
</tr>
<tr>
<td>MCHC (g/dL)</td>
<td></td>
<td>33.6 (1.4)</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td></td>
<td>76.1 (10.0)</td>
</tr>
<tr>
<td>Inflammation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRP &gt;10 g/L</td>
<td></td>
<td>5.7% (5/87)</td>
</tr>
<tr>
<td>Malaria parasitaemia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIV-infected</td>
<td></td>
<td>6.0% (5/87)</td>
</tr>
</tbody>
</table>

Bone marrow iron deficiency was defined as a bone marrow smear score of 0 or 1 (GALE et al., 1963); RSf: Red cell Size Factor; sTfR: soluble transferrin receptor; sTfR-F index: soluble transferrin-log ferritin index (Punnonen et al., 1997b); MCHC: mean cell haemoglobin concentration; MCV: mean corpus volume; CRP: C-Reactive Protein; HIV: Human Immunodeficiency Virus; Normally distributed variables are presented with means and their standard deviation; skewed variables with median values and their inter quartile range.

Table 2. Iron markers and their correlation with bone marrow iron stores and the diagnostic to identify bone marrow iron deficiency represented by their AUROC

<table>
<thead>
<tr>
<th>Iron marker</th>
<th>correlation</th>
<th>AUROC (95% CI)</th>
<th>AUROC &gt; 0.500</th>
<th>AUROC compared with (p-value) :</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>(p-value)</td>
<td>hepcidin</td>
<td>ferritin</td>
</tr>
<tr>
<td>sTfR-F index</td>
<td>-0.46***</td>
<td>0.801 0.706 0.895 &lt; 0.0001</td>
<td>0.05 0.9 0.0002</td>
<td>-</td>
</tr>
<tr>
<td>Ferritin (μg/mL)</td>
<td>0.39***</td>
<td>0.797 0.696 0.897 &lt; 0.0001</td>
<td>&lt;0.05</td>
<td>- 0.02</td>
</tr>
<tr>
<td>sTfR (μg/mL)</td>
<td>-0.29***</td>
<td>0.691 0.578 0.804 0.005</td>
<td>0.8 0.2 -</td>
<td>0.0002</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>0.28***</td>
<td>0.673 0.553 0.792 0.004</td>
<td>0.9 0.09</td>
<td>0.8</td>
</tr>
<tr>
<td>Hepcidin (nmol/L)</td>
<td>0.24**</td>
<td>0.673 0.553 0.792 0.005</td>
<td>- &lt;0.05</td>
<td>0.8 0.05</td>
</tr>
<tr>
<td>MCHC (g/dL)</td>
<td>0.20*</td>
<td>0.602 0.470 0.846 0.06</td>
<td>- - - -</td>
<td>- - -</td>
</tr>
<tr>
<td>RSf (fL)</td>
<td>0.13</td>
<td>0.589 0.377 0.776 0.3</td>
<td>- - - -</td>
<td>- - -</td>
</tr>
</tbody>
</table>

This table presents the Kendall rank correlation with bone marrow iron grading (*<0.05 **<0.01 ***<0.001) and the area under the curve (AUROC) of all iron makers in detecting iron deficiency. The difference of all markers with a AUROC >0.500 was tested. Iron deficiency was defined as a bone marrow smear score of 0 or 1. MCHC: mean cell haemoglobin concentration; MCV: mean corpus volume; sTfR: soluble transferrin receptor; RSf: Red cell Size Factor; sTfR-F index: soluble transferrin-log ferritin index.
Table 3. Peripheral iron markers and their performance to identify children with iron deficiency

<table>
<thead>
<tr>
<th>Iron marker</th>
<th>cut-off*</th>
<th>Iron deficient (%)</th>
<th>Sensitivity (%)</th>
<th>95% CI</th>
<th>Specificity (%)</th>
<th>95% CI</th>
<th>PPV</th>
<th>95% CI</th>
<th>NPV</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>sTfR-F index</td>
<td>&gt; 2.2</td>
<td>31.9 (29/91)</td>
<td>51.4</td>
<td>34.7-67.8</td>
<td>89.6</td>
<td>76.6-96.1</td>
<td>79.2</td>
<td>57.3-92.1</td>
<td>70.5</td>
<td>57.3-81.1</td>
</tr>
<tr>
<td></td>
<td>&gt; 1.85</td>
<td>4.8 (44/91)</td>
<td>70.2</td>
<td>52.8-83.6</td>
<td>75.9</td>
<td>60.1-83.9</td>
<td>68.4</td>
<td>51.2-82.0</td>
<td>76.6</td>
<td>61.6-87.2</td>
</tr>
<tr>
<td>Ferritin (µg/L)</td>
<td>&lt; 30</td>
<td>71.7 (66/92)</td>
<td>81.6</td>
<td>65.1-91.7</td>
<td>57.3</td>
<td>24.3-32.7</td>
<td>30.8</td>
<td>27.8-63.7</td>
<td>72.0</td>
<td>50.4-87.1</td>
</tr>
<tr>
<td></td>
<td>&lt; 12</td>
<td>27.2 (25/92)</td>
<td>44.7</td>
<td>30.0-61.5</td>
<td>89.6</td>
<td>76.1-96.1</td>
<td>77.3</td>
<td>54.2-91.3</td>
<td>67.2</td>
<td>54.2-78.1</td>
</tr>
<tr>
<td></td>
<td>&lt; 16</td>
<td>46.7 (43/92)</td>
<td>73.7</td>
<td>56.6-86.0</td>
<td>77.1</td>
<td>62.3-87.5</td>
<td>71.8</td>
<td>59.4-92.4</td>
<td>78.7</td>
<td>63.9-88.0</td>
</tr>
<tr>
<td>sTfR (µg/mL)</td>
<td>&gt; 3.3</td>
<td>16.5 (15/91)</td>
<td>24.3</td>
<td>12.4-51.2</td>
<td>91.7</td>
<td>79.1-97.3</td>
<td>69.2</td>
<td>38.9-89.6</td>
<td>68.1</td>
<td>48.9-72.2</td>
</tr>
<tr>
<td></td>
<td>&gt; 2.2</td>
<td>34.9 (30/91)</td>
<td>64.9</td>
<td>47.4-79.3</td>
<td>58.3</td>
<td>43.3-72.1</td>
<td>54.5</td>
<td>39.0-69.3</td>
<td>68.3</td>
<td>51.8-81.4</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>&lt; 77.0 (&lt;2 years)</td>
<td>29.0 (27/93)</td>
<td>35.9</td>
<td>21.7-52.8</td>
<td>79.2</td>
<td>64.6-89.9</td>
<td>58.3</td>
<td>37.0-77.2</td>
<td>60.3</td>
<td>47.2-72.2</td>
</tr>
<tr>
<td></td>
<td>≥ 73.0 (≥2 years)</td>
<td></td>
<td>74.4</td>
<td>57.7-86.4</td>
<td>43.8</td>
<td>29.8-58.7</td>
<td>31.8</td>
<td>38.2-65.2</td>
<td>67.7</td>
<td>48.5-82.3</td>
</tr>
<tr>
<td>Hepcidin (nmol/L)</td>
<td>&lt; 0.5</td>
<td>28.9 (26/90)</td>
<td>-35.2</td>
<td>27.5-60.4</td>
<td>83.3</td>
<td>69.2-92.0</td>
<td>66.6</td>
<td>44.7-83.6</td>
<td>65.6</td>
<td>32.2-77.0</td>
</tr>
<tr>
<td></td>
<td>&lt; 1.4</td>
<td>59.9 (53/90)</td>
<td>75.0</td>
<td>55.6-85.6</td>
<td>54.2</td>
<td>39.3-68.4</td>
<td>55.1</td>
<td>40.3-69.1</td>
<td>72.2</td>
<td>54.6-85.2</td>
</tr>
<tr>
<td>MCHC (g/dL)</td>
<td>&lt; 32.0</td>
<td>18.6 (8/93)</td>
<td>7.7</td>
<td>2.0-22.0</td>
<td>94.7</td>
<td>79.0-97.9</td>
<td>42.9</td>
<td>11.5-79.2</td>
<td>55.0</td>
<td>43.5-66.0</td>
</tr>
<tr>
<td></td>
<td>&lt; 33.9</td>
<td>53.8 (50/93)</td>
<td>66.7</td>
<td>49.7-80.4</td>
<td>60.4</td>
<td>46.3-73.9</td>
<td>57.8</td>
<td>42.2-72.0</td>
<td>69.0</td>
<td>52.8-81.9</td>
</tr>
<tr>
<td>Rsf (fL)</td>
<td>81.9 (≤2 years)</td>
<td>26.9 (8/43)</td>
<td>57.9</td>
<td>34.0-78.9</td>
<td>40.9</td>
<td>21.5-63.3</td>
<td>58.8</td>
<td>26.2-66.8</td>
<td>45.9</td>
<td>28.3-76.1</td>
</tr>
<tr>
<td></td>
<td>78.8 (≥2 years)</td>
<td>39.5 (17/43)</td>
<td>45.5</td>
<td>25.1-67.3</td>
<td>72.7</td>
<td>49.6-88.4</td>
<td>62.5</td>
<td>35.9-83.7</td>
<td>57.1</td>
<td>37.4-75.0</td>
</tr>
</tbody>
</table>

This table shows the performance of the iron markers to indentify iron deficiency. The cut-offs used are internationally accepted or, if not available, method-specific cut-offs (grey boxes) as well as cut-offs newly derived form the ROC-curves (white boxes). Iron deficiency was defined as a bone marrow smear score of 0 or 1 (FINCH and Huebers, 1982; GALE et al., 1963). MCHC: mean cell haemoglobin concentration; MCV: mean corpus volume; sTfR: soluble transferrin receptor; Rsf: Red cell Size Factor; sTfR-F index: soluble transferrin-log ferritin index. The iron markers are presented in order of size of AUCROC (Table 2).
Discussion

This is the first study to evaluate the diagnostic accuracy of the conventional peripheral iron markers and two new potential iron markers, hepcidin and RSf, against bone marrow iron levels in healthy African children. None of the markers had both a high sensitivity and specificity. Ferritin and sTfR-F were the most accurate markers in distinguishing children with bone marrow iron deficiency from children with iron repletion. Hepcidin, sTfR and MCV performed moderately, whereas RSf and MCHC poorly performed.

Ferritin

Ferritin is widely used and considered the most specific peripheral iron biomarker correlating with iron stores. The generally accepted cut-off for children under five years of age, is <12 µg/L. \textsuperscript{27} In this study this cut-off had poor sensitivity. Ferritin is also an acute phase protein that is elevated in response to inflammation. For this reason a raised cut-off of 30 µg/L is recommended for use in populations with high infection exposure \textsuperscript{27}. Applying this cut-off in the present study increased sensitivity (81.6 %), but specificity was low (37.5 %). These results contrast with earlier findings in severely anaemic Malawian children in which the cut-off <30 µg/L showed higher specificity than sensitivity \textsuperscript{28}. This difference may relate to the low prevalence of infections in the current study population (5.7 %), and suggests that a cut-off 30 µg/L is too high for use in an otherwise healthy African population.

For ferritin the cut-off with maximal accuracy in predicting iron deficient stores was <18 µg/L, which gave a sensitivity of 73.7% and specificity of 77.1%. In the two other paediatric African studies evaluating ferritin against bone marrow iron deficiency, higher cut-offs were derived (<50 µg/L) \textsuperscript{29} and <273 µg/L. \textsuperscript{28} This may be explained by the high prevalence of infection in the these studies. Notably the ferritin assay used by Fleming \textit{et al} may not have been standardized \textsuperscript{30}, limiting comparability. In a western adult study comparing ferritin with bone marrow iron in healthy subjects, a cut-off of 12 µg/L was derived \textsuperscript{31}, whereas in hospitalized subjects a higher cut-off was required (30-70 µg/L) \textsuperscript{20,32,33}.

The data from the present study suggests that ferritin is to one of the best iron biomarkers for detecting deficiency in healthy African children. Yet even in this healthy population the cut-off may need to be raised, although not to as high a value as 30 µg/L, as previously suggested.

Soluble transferrin receptor

sTfR measured in serum has been shown to be proportional to the mass of membrane-bound transferrin receptor (mTfR) which represents the marrow erythropoietic activity and iron need of the red cell precursor \textsuperscript{34}. Tissue in need of iron will induce a rise in mTfR and thus sTfR \textsuperscript{35}.

In this study the performance of sTfR to detect bone marrow iron deficiency was inferior to ferritin and sTfR-F. Using the method-specific reference value, sTfR appeared to be very specific but lacked sensitivity. This is in contrast to findings in severely anaemic children with multiple infections \textsuperscript{28}, suggesting that performance of sTfR may depend on the presence of anaemia or inflammation. This contrasts with previous studies promoting sTfR as the preferred iron biomarker in populations experiencing inflammation \textsuperscript{36-38}, but is consistent with other studies reporting interference of inflammation with sTfR \textsuperscript{39-42}.

Applying a newly derived cut-off (>2.2 µg/ml) did not improve diagnostic accuracy in the present study population. Other paediatric and adult studies comparing sTfR with bone marrow iron showed cut-offs for maximal accuracy varying from 2.0-2.8 µg/ml \textsuperscript{32,33,43} to 8.0-8.3 µg/ml. \textsuperscript{28,44} Apart from the differences in inflammatory status, different methodologies and lack of standardization of immunoassays for sTfR \textsuperscript{45} seriously limit comparability.
Chapter seven | Peripheral blood iron markers compared against bone marrow

**sTfR-log ferritin index**

As serum ferritin reflects iron stores, and sTfR is assumed to vary with the degree of tissue iron, the sTfR-log ferritin index (sTfR-F) has been suggested to be a complete estimate of the iron level of the individual. In this population the sTfR-F was one of the best predictors of bone marrow iron deficiency, although it was not better than ferritin alone. This differs from results from populations with a high prevalence of infections, in which the sTfR-F index was a more accurate marker than ferritin, but is comparable to findings in African hospitalized adults whereby the AUCROC of ferritin (0.94) was larger than the AUC of sTfR-F (0.84) (significance of difference not reported). The preferred cut-off for maximal accuracy is difficult to establish across studies due to the variable interference of inflammation as well as the lack of standardization of the sTfR assays are.

In the present study the determination of sTfR, either alone or using the sTfR ratio, does not appear to have added value over the single marker ferritin for the diagnosis of iron deficiency in healthy African children, although the combination have added value in populations with high infection exposure.

**Hepcidin**

Hepcidin inhibits iron absorption in iron loaded conditions and enhances absorption in iron deficiency. In the present study hepcidin was a less accurate predictor of bone marrow iron deficiency compared to other iron biomarkers. This is the first study to compare serum hepcidin levels against bone marrow iron assessment in African children. Other studies evaluating hepcidin as a marker of iron deficiency showed better results, although comparison was with other peripheral biomarkers. Van Santen et al described hepcidin as a potential marker for differentiation of iron deficiency anaemia from anaemia of chronic disease in rheumatoid arthritic patients, using a reference combination of peripheral iron markers. Pasricha et al also proposed serum hepcidin as a useful marker for iron deficiency using ferritin and sTfR-index as their reference standards.

The sub-optimal performance of hepcidin as a marker of bone marrow iron deficiency in our study could result from other factors than iron concentration influencing hepcidin release. These factors may be stimulatory (inflammation) or suppressive (hypoxia and erythropoietic drive), both of which probably reduce sensitivity or specificity. Anaemia, a suppressor of hepcidin, was highly prevalent in these children and although acute infection (stimulating hepcidin) was infrequent, nevertheless low grade or chronic inflammation may have influenced hepcidin production. Its poor performance may be also explained by the large number of the children with hepcidin values below the minimal detection limit (0.5 nmol/L). A more sensitive test for hepcidin may show an improvement of the ROC-curve characteristics. In this setting hepcidin was not an optimal peripheral marker to diagnose bone marrow iron deficiency.

However as a key regulator of iron, hepcidin may provide additional value to ferritin for evaluation of the effects and safety of iron supplementation, as shown in Gambian children in whom hepcidin strongly predicted red blood cell iron incorporation after oral iron supplementation. Although contrasting results have been reported.

**Red cell Size factor**

The reticulocyte haemoglobin concentration has been shown to be a useful screening tool for iron deficiency in young children, and it can adequately detected bone marrow iron deficiency in adults. The reticulocyte haemoglobin concentration assessed on the ADVIA analyzer of Siemens (named CHr), on a Sysmex analyzer (named Ret-He) or Beckman analyzer (named RSt) showed equivalent results.
In this study RSf poorly predicted bone marrow iron deficiency. As the reticulocyte haemoglobin content is a sensitive marker for recent iron restricted erythropoiesis \textsuperscript{65}, then its performance may be limited in the present study in which children may be chronically iron deficient. Nutritional vitamin B12 deficiency, which is common in Malawi \textsuperscript{66} or folate deficiency, both influence the volume of the red blood cell and reticulocyte, and may interfere with RSf values. Although age could be a confounding factor, as MCV is generally higher in young children, adjustment in a multivariate logistic regression model did not change the correlation between RSf and bone marrow iron deficiency (data not shown).

The reticulocyte haemoglobin content does not to appear a reliable marker to detect iron deficiency in this population of (chronically) iron deficient children.

**Other iron markers**

The red cell size indices MCV and MCHC, both often used when biochemical markers are not accessible, were inferior to ferritin in detecting bone marrow iron deficiency. This is consistent with current knowledge \textsuperscript{21}, and may relate to interfering factors such as other nutritional deficiencies (vitamin B12 and folate deficiency) and/or inflammation.

**Considerations and limitations**

**Bone marrow iron examination**

Bone marrow iron smear was used as the reference for the diagnosis of deficiency of iron stores. It must be noted that variation in results can appear \textsuperscript{67} due to the uneven distribution of iron in the bone marrow \textsuperscript{10}. Nevertheless bone marrow examination has generally been considered the most reliable diagnostic test for iron status\textsuperscript{10-12}.

**Methodology**

For clinical purposes, the major emphasis in laboratory measurements of iron status is detecting iron deficiency rather than in estimating its severity. The cut-off for maximal accuracy to detect iron deficient stores is therefore important. However, some iron biomarkers lack standardization due to the absence of a suitable international calibrator \textsuperscript{68, 69}. As a consequence results differ between methodologies, and cut-offs may be difficult to compare.

**Implications**

Even in this 'healthy' population of African children no single marker showed both high sensitivity and specificity. The frequent reason for poor performance of iron markers: infection \textsuperscript{4} cannot fully explain the limited performance of iron biomarkers in populations under high infection pressure.

Other factors including anaemia, previous infections, age, co-morbidities and the chronicity of the deficiency could influence different iron markers and this underlines the complexity of iron metabolism in children living in these settings. Depending on the purpose of diagnosing iron deficiency our data can be used to select diagnostic tests.

To ‘estimate prevalence’ of iron deficiency a sensitive marker such as ferritin (cut-off <30 \textmu g/L) could be used. Since one would like to avoid underestimation rather than overestimation, and the NPV is more important than the PPV. Yet for ‘research purposes’ a high accuracy (optimal sensitivity and specificity) would be preferred to avoid classification bias (ferritin cut-off <18 \textmu g/L).
Chapter seven | Peripheral blood iron markers compared against bone marrow

For ‘interventional purposes’ other aspects appear to be more relevant than identifying the iron deficient population, for which this study cross-sectional was not designed. For example iron supplementation programmes would be aided by a marker to predict ‘efficacy of iron supplementation’ (intestinal and erythrocyte iron uptake) and ‘safety of the intervention’, as iron supplementation may increase infection risk in certain populations. Hepcidin has been suggested to serve these purposes although further research is needed.

Conclusion

In this first study evaluating peripheral iron markers against bone marrow iron deficiency in healthy African children ferritin and sTfR-F best predicted bone marrow iron status and should be used in epidemiologic studies and for research purposes. Since none of the markers had both a high sensitivity and specificity, there is an urgent need for a more reliable peripheral iron marker in African children.

Acknowledgments and contributions

The authors thank the parents and guardians of the children admitted in the study; the study team; the staffs of the Queen Elizabeth Central Hospital and Cure Beit Hospital. This study was supported by a grant from the Stichting Emma Steun and JANIVO Foundation. Contributions: F.A.M.J designed the research, coordinated data collection, analyzed the data, interpreted the results, and wrote the manuscript; J.C.J.C., designed the research, interpreted study results; M.B.V.H supervised interpretation of study results; T.L. supervised interpretation of study results; R.J.W.M.V supervised bone marrow iron readings; N.M, M.B.G, and M.E were responsible for data collection; R.K. was responsible for the performance and interpretation of ferritin and sTfR assessments; D.W.S. and H.T. were responsible for the design, performance and interpretation of hepcidin assessments. All authors critically revised the manuscript for intellectual content, and read and approved the final version. Conflict-of-interest disclosure: D.W.S. and H.T are medical director and managing director, respectively, of the www.hepcidinanalysis.com initiative that serves the scientific and medical community with hepcidin measurements at a fee-for service basis. The remaining authors declare no competing financial interests.
References

60. Ceramondi CI, Egli IM, Ahoandjinou E et al. Afebrile Plasmodium falciparum parasitemia decreases


73. WHO. Conclusions and recommendations of the WHO Consultation on prevention and control of iron deficiency in infants and young children in malarial endemic areas. 2007.
Chapter eight

Discussion and Recommendations
Anaemia, iron deficiency and infections

Iron deficiency is an important child health problem in sub-Saharan Africa. Although iron supplementation may increase haemoglobin level\(^1\), it has also been associated with an increased malaria risk in certain populations\(^2\), and as a result its use is controversial. Due to the limited quality of the available data on host iron status and infection risk, a consensus on anaemia and iron deficiency management has not yet been reached. The aim of this thesis is to provide reliable data on prevalence, causes and diagnostic tests of iron deficiency in African children and to examine their relation to susceptibility to malaria infection. Hepecin, a recently discovered key iron regulator, may provide new insights into iron homeostasis; this thesis reports research on its measurement in severely and non-severely anaemic African children. Our data may contribute to improvements in management of iron deficiency, and (severe) anaemia.

Severe anaemia management

To generate novel approaches for treatment and prevention of severe anaemia in Africa, the available literature was reviewed to highlight recent research findings (Chapter 2). New insights concerning the pathophysiological mechanisms and aetiologies of severe anaemia contrast with previously accepted understanding; bacterial infections and hookworm infections, and furthermore deficiencies of vitamin B12 and vitamin A, but not of iron or folic acid, were associated with severe anaemia. In view of these findings, policies and practices concerning treatment and prevention of severe anaemia in this area need to be substantially revised in order to make a significant impact on the huge burden of severe anaemia in Africa.

Severe anaemia management | iron supplementation

Iron supplementation is currently recommended in children with severe anaemia\(^3\); although new insights may question this policy. Firstly in Malawian children a negative association between iron deficiency and severe anaemia has been reported\(^4\), suggesting that iron deficiency may even protect against severe anaemia. This is partly explained by a protective effect against bacterial infections\(^4\).

Secondly in Chapter 6 hepecin values in severely anaemic children were found to be surprisingly low despite concurrent infections. Low hepecin values are thought to reflect an ideal and safe condition for iron supplementation\(^5\), since it is associated with increased intestinal iron absorption and a probable absence of inflammation. Normally inflammation will increase hepecin levels which subsequently will inhibit iron absorption\(^6,7\), a mechanism which is considered to withhold iron from pathogens during infection (functional iron deficiency). Despite the fact that infection prevalence in our population was high, the elevated erythropoietin levels due to severe anaemia, inhibited hepecin production, and this influence of erythropoietin exceeded the inflammation stimulus on hepecin production. Theoretically this may mean that during severe anaemia iron is absorbed, despite the presence of infections which may benefit from the increased iron availability. There is an urgent need for studies investigating iron uptake in severely anaemic children with concurrent infectious diseases, and to assess infection incidence and mortality in children with low hepecin levels who are receiving iron supplementation. Possibly labelled iron studies may facilitate these future investigations.

Severe anaemia management | Ancylostoma duodenale

Hookworm infestation (Ancylostoma duodenale and Necator americanus) causes intestinal blood loss and through
this mechanism leads to iron deficiency and anaemia\textsuperscript{8,10}. In Chapter 3 hookworm infection with A. duodenale, was substantially associated with severe anaemia in Malawian pre-school children. This association was assessed using a novel and more sensitive test for detection of hookworm, real-time PCR. Hookworm prevalence was much higher than previously had been determined by microscopy, the conventional method (29.2\% vs. 5.9\%). Since real-time PCR for hookworm allows quantitative measurement we were able to show that even less severe A. duodenale infections contributed to disease burden and thus were of clinical importance. Another surprising finding was the high prevalence of the sub-species, A. duodenale, whereas N. americanus was expected to be the dominant specie in this area\textsuperscript{11,12}. A. duodenale causes 2 to 10 times more blood loss per worm than N. americanus\textsuperscript{13,14} and is therefore more likely to contribute to anaemia and iron deficiency.

This novel highly sensitive test for detection of hookworm enabling differentiation of sub-species as well as quantification of infection is a useful tool to estimate prevalence of hookworm species and their contribution to disease burden in different populations. Data from Mozambique indicated that the ratio between the two hookworm species changed with age (Van Lieshout, unpublished data), suggesting that the contribution of hookworm (A. duodenale) to severe anaemia and iron deficiency may differ per area and/or age group. And since post-treatment data are scarce and extremely little is known about the species-specific effects of mass drug administration\textsuperscript{15,16} further research on treatment effects of the different hookworm species may increase efficacy of hookworm control and thereby contribute to a decrease in prevalence of severe anaemia and iron deficiency.

Iron deficiency and infection

Iron deficiency and infection | Malaria
The relation between host iron status and risk of malaria infections has been the subject of a long standing debate. Studies have described a possible protective effect of iron deficiency against malaria risk\textsuperscript{17,18}. However the available data are inconclusive due to limitations in study designs. In Chapter 4 we have applied modern causal inference methods to analyze the relation between iron deficiency and malaria risk. We found that children who were iron deficient at baseline had a lower incidence of malaria parasitaemia (0.55, 95\%CI 0.41-0.74), and clinical malaria (0.49, 95\%CI 0.33-0.73), during a year of follow-up. These findings suggest that iron deficiency protects against malaria parasitaemia and clinical malaria. However, because we lacked direct measurements of baseline immunity to malaria our results should be confirmed by well conducted randomized controlled trials, or observational birth cohort studies. Until recently these type of studies were lacking, although a well designed birth cohort study has shown a decreased risk of malaria in young children during iron deficient periods\textsuperscript{19}.

Our findings support the theory of nutritional adaptation to infectious diseases\textsuperscript{20,21}: Iron deficiency, highly prevalent in African children, seems to be associated with an increased chance of survival as it may protect against malaria, a major morbidity and mortality factor in these settings. Treatment of iron deficiency should occur concurrently with sustained control and surveillance of prevalent infections to avoid “neutralisation” of the protective effect of iron deficiency andincreasing malaria risk. We recommend that, prevention and treatment of iron deficiency occurs within the context of the Integrated Management of Childhood Illness\textsuperscript{22}. Prevention and treatment of infections such as malaria and hookworm may at the same time prevent iron deficiency and decrease the need for iron supplementation.
Iron deficiency and infection

Iron supplementation is often provided to children with HIV as iron deficiency is considered the most important micronutrient deficiency causing HIV-associated anaemia. The prevalence of iron deficiency in HIV-infection is little studied, and iron supplementation may even be detrimental in these children who are particularly vulnerable to infectious diseases. In Chapter 5 the literature on studies investigating iron status in HIV-infected children was systematically reviewed to assess prevalence of iron deficiency. Pooled analyses of studies that included an HIV-uninfected control population suggested that HIV-infected children were less likely to be iron deficient compared with HIV-uninfected children (OR 0.50; 95%CI 0.27-0.94). Nevertheless iron deficiency was still common in HIV-infected children with a prevalence of 34%, and like other iron deficient children, may require intervention. Our systematic review further showed that iron deficiency prevalence was lower in HIV-infected children on HAART compared with HIV-infected children not on HAART (24% vs. 37%). Although confounding factors such as disease stage may partly explain this difference, this observation makes a case for research examining the effects of HAART on iron status, and of its role as a potential intervention for iron deficiency in HIV-infected children.

Especially in HIV-infected children, studies are needed to determine the safety (and benefit) of iron supplementation. Furthermore, as for other African children, alternative strategies to control iron deficiency should be considered, such de-worming, malaria control and HAART.

Assessment of iron deficiency in African children

Determination of iron status is difficult in sub-Saharan Africa as prevalent infections and other nutritional deficiencies interfere with the accuracy and validity of iron biomarkers. The extent to which this affects our current knowledge on iron status assessments, is unknown as these iron biomarkers have not been validated against iron staining of bone marrow aspirate, the reference standard of iron status, in African children.

In Chapter 7 we therefore tested the diagnostic accuracy of the currently used iron biomarkers against bone marrow iron assessment in Malawian children. Two new potential iron markers, hepcidin and Red cell Size Factor (RSF) (haemoglobin content) were included. No iron marker in this study showed both a high sensitivity and specificity; which confirms the difficulty of assessing status in these settings. Ferritin, the widely criticized marker seemed to be one of the most accurate markers in discriminating children with bone marrow iron deficiency from children with iron replete stores; a newly defined cut-off for ferritin (<18 μg/L) resulted in a maximal accuracy of 73.7% sensitivity and 77.1% specificity. STfR-F, the ratio from sTfR over log-ferritin, performed in an equivalent way to serum ferritin; a new cut-off (>1.85) showed 72.5% sensitivity and 75.0% specificity. It must be noted these assays for sTfR are not uniformly standardized and the latter cut-off should not be universally applied. Hepcidin, sTfR and MCV moderately; and RSF and MCHC poorly performed as iron predictive biomarkers.

Unlike previous data in African populations, sTfR-F had no added value over ferritin alone. However those studies included hospitalized populations whereas our study population consisted of “healthy” preschool children and may better represent the average population of African children.

Our data can be used to guide diagnostic testing, depending on the purpose of the determination. For ‘estimating prevalence’ of iron deficiency, a sensitive marker such as ferritin (cut-off <30 μg/L) could be used. For ‘research purposes’ a high accuracy test (optimal sensitivity and specificity) would be preferred to avoid classification bias (ferritin cut-off <18 μg/L). For ‘interventional purposes’ other aspects appear to be more...
relevant than identifying the iron deficient population, for which this cross-sectional study was not designed. The effect of iron supplementation is a dynamic process which requires a longitudinal study design. As hepcidin regulates intestinal absorption of oral iron, it is considered to play an important role in this process and may function as guide for iron supplementation \(^2\) which is shortly discussed below.

**Hepcidin**

Hepcidin, a recently discovered key iron regulator \(^{25-27}\), inhibits iron absorption in iron loaded conditions whilst decreased levels stimulate iron absorption during iron deficiency \(^7,\ 28,\ 29\). In Chapter 7 compared to other iron markers, hepcidin was a not an accurate predictor of bone marrow iron deficiency in healthy children. Also in severely anaemic children hepcidin poorly predicted bone marrow iron deficiency (Chapter 6). This was probably explained by concurrent conditions influencing the other signalling pathways for hepcidin, such as anaemia and (even low grade) inflammation.

As a key regulator of iron, hepcidin also already been proposed as a predictor of the effect and safety of iron supplementation; in Gambian children hepcidin strongly predicted red blood cell iron incorporation after oral iron supplementation \(^3\). Yet since this was not fully confirmed in another study that demonstrated that regulation of iron absorption, but not the actual incorporation of iron into the erythrocyte, associated with hepcidin vlues \(^{20}\). In addition we described low hepcidin levels in severely anaemic children (Chapter 6) suggesting that iron absorption may even occur in children with severe infections. Further longitudinal research is needed to determine the potential role of serum hepcidin for guiding iron therapy.
Chapter eight | Discussion and Recommendations

References

3. WHO. Conclusions and recommendations of the WHO Consultation on prevention and control of iron deficiency in infants and young children in malnourished areas. 2007.


Samenvatting
Anemie, ijzerdeficiëntie en infecties

Ijzerdeficiëntie (ijzergebrek) kan leiden tot anemie (bloedarmoede) en een vertraagde cognitieve ontwikkeling. Het komt veel voor bij kinderen in Afrika en is daarmee een groot gezondheidsprobleem in dit gebied. Hoewel ijzersupplementatie (toediening van ijzersupplementen) aan de ene kant het risico op anemie kan verkleinen, wordt het eveneens in verband gebracht met een verhoogd risico op malaria-infecties. Het toedienen van ijzer is daarom omstreden.

Door de geringe kwaliteit van de beschikbare informatie over het ijzergehalte bij kinderen en het daaraan gerelateerde infectierisico, ontbreekt een consensus over de manier waarop anemie en ijzerdeficiëntie zouden moeten worden behandeld.

Eén van de factoren die de kwaliteit van de huidige data beperken, is het meten van de ijzerstatus. Infecties en voedingsdeficiënties (anders dan ijzerdeficiëntie) interfereren met de resultaten van de beschikbare methoden om het ijzergehalte te meten in het bloed. Om dit probleem te omzeilen wordt anemie ten onrechte vaak gebruikt om de diagnose ijzerdeficiëntie te stellen. Want hoewel anemie en ijzerdeficiëntie vaak overlappen, komt anemie zonder ijzerdeficiëntie ook voor, en vice versa; de twee ziektebeelden hebben daarom aparte behandelingssstrategieën nodig.

Het doel van dit proefschrift is om enerzijds betrouwbare gegevens te verstrekken over de prevalentie (het vóórkomen), de oorzaken en de diagnose van ijzerdeficiëntie bij Afrikaanse kinderen, en anderzijds om de relatie tussen ijzerdeficiëntie en de vatbaarheid voor malaria-infecties te onderzoeken. De populatie die voor dit proefschrift is onderzocht betreft jonge kinderen in Malawi (Afrika), zowel gezonde kinderen als ernstige anemische en/of zieke kinderen. De recente ontdekking van hepcidin, een belangrijke regulator van onze ijzerstofwisseling, schept de mogelijkheid nieuwe inzichten in de complexiteit van de ijzerstofwisseling te verschaffen en is tevens bij deze kinderen bepaald. Hieronder volgt een korte samenvatting van de in dit proefschrift gerapporteerde bevindingen welke een bijdrage zouden kunnen leveren aan de verbetering van de richtlijnen voor de bestrijding van ijzerdeficiëntie en anemie.

Ernstige anemie

Onder ernstige anemie bij kinderen in Afrika verstaan we niet alleen een extreme vorm van anemie, maar ook een op zich zelf standaard ziektebeeld dat een andere etiologie (oorzakenpatroon) dan matige anemie blijkt te hebben en een hoge morbiditeit (ziektelast) en mortaliteit (sterfte) met zich mee brengt. Omdat er nog weinig bekend is over de etiologie van ernstige anemie, zijn in Hoofdstuk 2 recente onderzoeksresultaten uit de beschikbare literatuur betreffende de etiologie en diens relatie tot de behandeling en preventie (voorkomen) samengevat. De nieuwe inzichten in de toestandkoming van ernstige anemie bleken in verrassend contrast te staan met wat men tot op heden als belangrijkste oorzaken van ernstige anemie beschouwt en toepast in huidige behandelingssstrategieën. Niet ijzer- en foliumzuurdeficiëntie, maar bacteriële infecties, mijnworminfecties en een gebrek aan de vitamines B12 en A zijn belangrijke factoren bij het ontstaan van ernstige anemie. De huidige richtlijnen voor de behandeling en preventie van ernstige anemie zullen daarom moeten worden herzien.
Ernstige anemie | IJzersuppletie

Volgens de huidige richtlijnen in Afrika voor ernstige anemie bij kinderen is ijzersuppletie standaard therapie, ondanks het feit dat nieuwe inzichten dit beleid in twijfel trekken. Één van de studies beschreven in Hoofdstuk 2 deed onderzoek naar voorzaken van ernstige anemie bij kinderen in Malawi en stelde vast dat ijzerdeficiëntie minder vaak voorkwam bij kinderen met ernstige anemie dan bij kinderen zonder ernstige anemie. Omdat ijzerdeficiëntie mogelijk bescherming biedt tegen bacteriële infecties, een belangrijke risicofactor voor het ontstaan van ernstige anemie, zou ijzerdeficiëntie zelfs tegen ernstige anemie kunnen beschermen.

Een andere factor die eveneens de universele ijzersuppletie bij kinderen met ernstige anemie in twijfel trekt wordt beschreven in Hoofdstuk 6 waarin we bij kinderen met ernstige anemie hepcidin waarden hebben bepaald. Kinderen met ernstige anemie bleken verrassend lage hepcidinwaarden te vertonen, ondanks de gelijktijdig voorkomende infecties. Dit was verrassend omdat voorsnog lage hepcidinwaarden met afwezigheid van infecties en ijzerdeficiëntie in verband wordt gebracht, met een verhoogde ijzerabsorptie tot gevolg; lage hepcidinwaarden komen voor wanneer er voldoende ijzervoorraad is en/of er een infectie aan de gang is, gesuppleerd ijzer in deze situatie verminderd opgenomen. Deze hoge hepcidinwaarden en de daarmee gepaard gaande verminderde absorptie wordt beschouwd als een beschermingsmechanisme: er wordt ijzer onthouden aan pathogenen (ziekteverwekkers) ten tijde van een infectie. Eerdere studies suggereerden daarom lage hepcidinwaarden als voorwaarde voor het veilig toedienen van ijzersupplementen. Bij onze populatie zagen we echter dat de ernstige anemie en de daarbij behorende verhoogde erythropoëtine gehalte (een hormoon dat de bloedaanmaak stimuleert maar ook hepcidine remt), de hepcidine-verhogenende werking van de infecties onderdrukte, met lage hepcidinwaarden tot gevolg. Theoretisch kan dit betekenen dat tijdens ernstige anemie, zelfs bij aanwezigheid van infecties, gesuppleerd ijzer wél wordt geabsorbeerd en dat aanwezige pathogenen vervolgens kunnen profiteren van de toegenomen beschikbaarheid van ijzer. Om deze nieuwe theorie nader te onderzoeken zal er moeten worden onderzocht of de ijzeropname daadwerkelijk toeneemt bij kinderen met ernstige anemie en infecties, en of vervolgens de morbiditeit (ziektelast) en mortaliteit (sterfte) bij deze kinderen toeneemt.

Ernstige anemie | Mijnworm infecties

Mijnworminfecties (Anisakis duodenale en Necator americanus) veroorzaken intestinaal (in de darm) bloedverlies en lijden daardoor vaak tot ijzerdeficiëntie en anemie. Een infectie met A. duodenale veroorzaakt 2 tot 10 keer meer bloedverlies per worm dan de N. americanus en vormt dan ook een groter risico voor anemie en ijzerdeficiëntie. De gangbare methode om mijnworminfecties vast te stellen is de microscopische bestudering van feces (ontlasting). Om met deze methode de subtypes van mijnworm te onderscheiden is echter zeer tijdrovend en wordt daarom zelden gedaan, ook het meten infectiegraad is gecompliceerd en wordt vaak niet bepaald. Klinische relevantie van deze twee parameters is echter niet uitgesloten. Real-time PCR is een nieuwe methode om mijnworm te diagnostieceren en is gebaseerd op de detectie van het DNA van de mijnworm in de feces. Met deze methode is subtype-differentiatie en kwantificering van de infectie zeer eenvoudig, bovendien is in voorgaande studies de test vele malen gevoeliger gebleken. In Hoofdstuk 3 wordt de klinische relevantie van deze nieuwe methode geëvalueerd bij kinderen in Malawi. Het bleek dat bij veel meer kinderen mijnworminfecties werden vastgesteld dan eerder was bepaald met microscopie (29.2% versus 5.9%). Verrassend genoeg werd het merendeel van de infecties veroorzaakt door de A. duodenale, terwijl tot nu toe werd gedacht dat de N. americanus de dominante soort in dit gebied was. Verder zagen we dat zelfs minder ernstige A. duodenale infecties tot anemie en/of ijzerdeficiëntie leidden en daarmee dus tevens klinisch relevant bleken. Gegevens van een andere studie in Mozambique die deze methode gebruikte, lieten zien dat de verhouding tussen de twee mijnwormsoorten kan veranderen met de leeftijd, wat suggereert dat
Samenvatting

de bijdrage van de mijnworm aan ernstige anemie en ijzerdeficiëntie kan verschillen per gebied en/of leeftijdsgroep. Real-time PCR voor mijnworm zou zeer nuttig kunnen zijn bij het vaststellen van de prevalentie van mijnworm en diens bijdrage aan de morbiditeit in verschillende populaties. Omdat er nog weinig bekend is over de soortspecifieke effecten van de behandeling van mijnworm is daar tevens verder onderzoek nodig.

Ijzerdeficiëntie en infecties

Ijzerdeficiëntie en infecties | Malaria
De relatie tussen ijzerdeficiëntie en het risico op malaria-infecties vormt reeds lange tijd bron van discussie. Verschillende studies hebben een mogelijk beschermend effect van ijzerdeficiëntie tegen malaria aangetoond, deze studies zijn echter van beperkte methodologische kwaliteit, dientengevolge is een consensus nog niet bereikt. In Hoofdstuk 4 zijn daarom vernieuwende statistische methodes toegepast om de relatie tussen ijzerdeficiëntie en malariarisico te analyseren. Het bleek dat bij kinderen met ijzerdeficiëntie bij aanvang van de studie, gedurende de volgperiode van een jaar, minder vaak malaria werd aangetoond. Dit suggereert dat een tekort aan ijzer inderdaad zou kunnen beschermen tegen malaria infecties. Een bevinding die de theorie van evolutionaire adaptatie ondersteunt: ijzerdeficiëntie, vaak voorkomend bij Afrikaanse kinderen, lijkt samen te hangen met een verhoogde kans op overleving in gebieden waar veel malaria voorkomt; malaria vormt immers een groot sterft eerisico. In deze gebieden zal de behandeling van ijzerdeficiëntie daarom altijd gelijktijdig moeten plaatsvinden met preventie en behandeling van malaria.

Ijzerdeficiëntie en infecties | HIV
Anemie is een veelvoorkomend probleem bij kinderen met HIV. Omdat ijzerdeficiëntie als de belangrijkste oorzaak van HIV-gesociaeerde anemie wordt beschouwd, worden deze kinderen op grote schaal gesuppleerd met ijzer, hoewel over de daadwerkelijke prevalentie van ijzerdeficiëntie bij HIV-geïnfecteerde kinderen weinig bekend is. Omdat ijzersuppeltie zeker bij deze kinderen die bijzonder kwetsbaar zijn voor infectieziekten, schadelijk zou kunnen zijn i.v.m. een verhoogd infectierisico, is een beter beeld van de noodzaak van ijzersuppeltie bij HIV-geïnfecteerde kinderen gewenst. Om een schatting te maken van de prevalentie van ijzerdeficiëntie bij kinderen met HIV is in Hoofdstuk 5 de literatuur van studies over de ijzerstatus van deze populatie systematisch samengevat. De analyses van studies met een controlepopulatie (kinderen zonder HIV), lieten zien dat kinderen met HIV minder vaak een tekort aan ijzer hadden dan niet geïnfecteerde kinderen (OR 0.50; 95%BI 0.27-0.94). Toch kwam ijzerdeficiëntie nog steeds vaak voor bij kinderen met HIV (34%) en is behandeling gewenst. In verband met een mogelijk verhoogd infectie risico, dienen er alternatieve strategieën om ijzerdeficiëntie te behandelen in overweging te worden genomen, zoals ontwormen, behandeling van malaria en toepassen van anti retro virale middelen (HAART); onze analyses lieten zien dat kinderen met HIV die behandeld werden met HAART, minder vaak ijzerdeficiënt waren dan kinderen zonder behandeling (24% versus 37%). Dit verschil kan behalve door een mogelijk positief effect van de behandeling mogelijk ook worden verklaard door andere factoren zoals de ziekteprogressie, die veelal gerelateerd is aan het wel of niet starten van HAART.
De diagnose van ijzerdeficiëntie bij kinderen in Afrika

Het bepalen van de ijzerstatus wordt problematisch geacht in gebieden waar veel infectieziekten en veel verschillende voedingsdeficiënties voorkomen omdat deze factoren interfereren met de meetresultaten van beschikbare methoden om de ijzergehalte te meten. De mate waarin de meetresultaten worden verstoord is echter onbekend. Dat komt doordat deze ijzermarkers (stof in het bloed die ijzergelde weergeeft) bij kinderen in Afrika nog nooit zijn gevalideerd ten opzichte van de gouden standaard voor het meten van de ijzerstatus (beenmerguizerkleuring).

In Hoofdstuk 7 zijn daarom de meest gebruikte ijzermarkers vergeleken met de beenmerguizerkstatus van kinderen in Malawi, om zo hun sensitiviteit (gevoeligheid) en specificiteit (de mate waarin een test specifiek is) te bepalen, twee eigenschappen die samen de nauwkeurigheid van een test bepalen. Tevens werden twee nieuwe potentiële ijzermarkers, hepcidine en de Red cell Size Factor (RSF) getest. Uit onze analyses bleek dat geen van de ijzermarkers in deze studie zowel sensitief als specifiek was, wat de eerdere twijfels over het gebruik van de ijzermarkers in deze gebieden bevestigde.

Ferritine, één van de meest bekritiseerde markers, bleek echter één van de meest accurate markers te zijn. Met een nieuw bepaalde afkapwaarde van 18 ug/L had deze marker een sensitiviteit van 73.7% en specificiteit van 77.1%. sTfR-log Ferritin, de ratio van de transferrine receptor (sTfR) over de log van ferritine, presteerde in gelijke mate als ferritine, met een nieuwe afkapwaarde van 1.85 vertoonde deze marker een sensitiviteit van 72.5% en specificiteit van 75.0%. Belangrijk is om te melden dat de manier van testen van sTfR nog niet gestandaardiseerd is en dat daarom de hiergenoemde afkapwaarde niet universeel kan worden toegepast. Hepcidine, sTfR en MCV presteerden matig, en RSF en MCHC waren zelfs slechte markers voor de ijzerstatus.

In tegenstelling tot voorgaande studies was in onze studie sTfR-log ferritin geen betere marker dan ferritine alleen. Deze eerdere studies betroffen echter ziekenhuispopulaties, terwijl onze onderzoekspopulatie bestond uit gezonde kinderen en daardoor beter de gemiddelde populatie van Afrikaanse kinderen vertegenwoordigt. Omdat de waarde van een test tevens afhangt van het doel waartoe de test dient, kunnen de gegevens in hoofdstuk 7 worden gebruikt om de verschillende ijzermarkers hiervoor te selecteren. Voor een schatting van de prevalentie van ijzerdeficiëntie is de sensitiviteit belangrijker dan de specificiteit, en kan bijvoorbeeld ferritine met een afkapwaarde van <30 ug/L worden gebruikt. Voor wetenschappelijke doeleinden daarentegen is zowel een optimale sensitiviteit als specificiteit gewenst om misclassificatie van ijzerstatus te voorkomen en zou ferritine met een afkapwaarde van 18 ug/L een betere optie zijn om ijzerdeficiëntie vast te stellen. Voor behandelingsdoeleinden zijn weer andere aspecten meer relevant, waarvoor deze cross-sectionele studie niet is ontworpen; het effect van ijzersuppletie is een dynamisch proces dat een longitudinale studie vereist. Omdat hepcidine de intestinale absorptie van ijzer regelt, zou deze marker als leidraad kunnen fungeren voor het geven van ijzersuppletie. Dit zal hieronder kort worden besproken.
Samenvatting

Hepcidine

Hepcidine, een recent ontdekte belangrijke ijzerregulator, remt de absorptie van ijzer wanneer er voldoende ijzer aanwezig is, en stimuleert de ijzerabsorptie ten tijde van ijzerdeficiëntie. Als ijzermarker bleek hepcidine niet optimaal te functioneren, zowel bij gezonde Afrikaanse kinderen niet (Hoofdstuk 7) als wel bij kinderen met ernstige anemie en infecties niet (Hoofdstuk 6). Dit is te verklaren door het feit dat niet alleen ijzerstatus maar ook andere factoren de hepcidinewaarden beïnvloeden, zoals anemie en (zelfs laaggradige) infecties.

Omdat een infectie de ijzerabsorptie kan reguleren via hepcidine, suggereerden eerdere studies om hepcidine te gebruiken als voorspeller van de effectiviteit en veiligheid van ijzersuppletie. In één van die studies bleek hepcidine echter vooral geassocieerd met de absorptie en niet met de daadwerkelijke inbouw van ijzer in de rode bloedcellen. Daarnaast beschreven wij in Hoofdstuk 6 lage hepcidine-waardes bij kinderen met ernstige anemie en gelijktijdige infecties, waardoor ijzer wel degelijk zou kunnen worden opgenomen door kinderen met ernstige infecties en kan worden “misbruikt” door de aanwezige pathogenen. Nader longitudinaal onderzoek is nodig om de mogelijke rol van hepcidine bij de behandeling van ijzerdeficiëntie bij verschillende populaties vast te stellen.
Acknowledgements
This thesis was made possible by the efforts and support of many people. It is impossible to name each and every one of them, but I want to express my gratitude to all of them.

However, I wish to single out a few people that have played a special role in the completion of this thesis.

First of all, I want to thank all the children and their parents or guardians that participated in both the Iron Study and the SevAna Study.

I am indebted to all who made the Iron Study possible: all the staff of the surgical wards of Cure Beit Hospital and Queen Elizabeth Hospital. Special thanks go out to Doctor John Cashman, Prof. Eric Borgstein, Prof. Geert Tom Heijkens and Prof. Elizabeth Molynex who allowed me to conduct my research on their wards, to Mr Banda for teaching us how to perform a bone marrow aspiration, to everybody who prayed for the Iron Study, to Alice Bandawe Malili, Margreet Schilt and Tim Hall for their logistic support on the surgical ward, to Nelson Maseko, Montfort Bernard and Mercy Emana for recruiting study participants, to David Kachala for performing the laboratory tests and keeping me stocked with supplies, to Mike Moore, Bridget Denise, Danielle De Clercq, Wezi Gondwe and Mavis Menyere and the rest of the Welcome Trust Laboratory and to Beckman Coulter South Africa for enabling me to perform the reticulocyte measurements, to Kamija Phiri, Mary Makoti, Herbert Longwe for providing logistic support in the College of Medicine in Blantyre, to Harold Tjalsma, Erwin Wiegertink, Siem Klaver and Anita van Schijndel from Hepcidinanalysis.com for performing the hepcidin measurements, to Rob Kraaijenhagen and Annet de Bats from the Clinical Laboratory of the Meander Medical Centre for assisting in the iron marker assasements. Furthermore, I wish to thank Job Calis and all the other staff of the SevAna study team for supplying the extensive and excellent data set, which among others was extended by the support and effort of the Parasitology Department of the LUMC.

I express my thanks to my supervisor Prof. dr. Bernard Brabin and to co-supervisors Dr. Job Calis and Dr. Michael Boele van Hensbroek.

Dear Bernard, you are the one who has passed the final judgment on my work. With your international experience in the field of science and health and your inexhaustible knowledge of the literature on tropical paediatrics you have steered our research group around the intricacies and pitfalls of scientific research. Also, being a native speaker of English, you have made sure that we did not all too blatantly abuse your mother tongue in the texts we produced.

Dear Michael, as my second co-supervisor you took more than a casual glance at the progression of my PhD. Over the years I have gotten to know you as an enthusiastic, highly creative, nicely chaotic though very disciplined researcher; you have made me feel at home in the Global Child Health Group. I hope to continue to work with you in future.

Dear Job, at a symposium on tropical paediatrics in 2007, you held a talk about your PhD project in Malawi. My enthusiasm for research in the tropics was ignited there and then and we kept in touch. Being colleagues in the St. Lucas Andreas Hospital, we wrote a grant proposal together. I was delighted to be able to start my research project in 2009. You boosted my confidence by trusting me to find my own way forward along the path of scientific research. You have been a great mentor. I have greatly benefited from your ability to provide clear answers to difficult questions, from your enthusiasm, your perceptive comments and, of course, your subtle humour. When we met to discuss the progress of my work you always encouraged me and you even had an open ear for any private concerns. These meetings allowed me to recharge my batteries and gave
me the energy to get on with the job.

Special thanks go out to Tjalling Leenstra for his crucial role in the prolonged but successful search for reliable statistics. I have appreciated his critical insight, his enthusiasm and his persistence during this process. I also want to thank Brian Faragher and Ronald Geskus for sharing with us the benefits of their innovative statistical approach.

I am grateful to Heleen Dyserinck for the great assistance with the literature searches for Chapter 2 and 5.

I want to thank all co-authors for their critical and useful comments on my work, especially Lisette van Lieshout, Imelda Bates and Dorine Swinkels.

I wish to express my gratitude to the members of the committee, Prof. dr. F.G.J. Cobelens, Prof. dr. H. S. A. Heymans and Prof. dr. V. Mwapasa, Dr. M. Peters and Prof. A. J.A.M. van der Ven, for reviewing this thesis and for agreeing to serve on the thesis committee. I am indebted to Prof. Heymans in particular for his moral support during the process of researching and writing my PhD.

I want to thank the Global Child Health Group for supporting my PhD project. Special thanks go out for Caroline Verraart for offering me a warm welcome at the start of my PhD, and for providing logistic support in the Netherlands as well as in Malawi; and, of course, for being such pleasant company over the past few years.

I am indebted to all the sponsors who made this thesis possible (see colophon), Numico, Janivo Foundation, Stichting Emma Steun and AIGHD, special thanks to Hans Sybrandy, Bert Veldhuizen Ella van den Brink, Esther Keijser, Wouter Keuning Wytske de Man Hanold van de Pol, Jan Bosch, Marco van der Spek, Peter Buitema en Ronald Kranendonk for cycling with the 250 kilometer to raise funding for my project.

I want to express my thanks to the Emma’s Children Hospital, to Diederik Bosman, to prof. Heymans for supporting and believing in the projects of the GHCG. Thanks to the St Lucas Andreas Hospital for the experience as clinical doctor which were useful during my research. I also want to thank the Liverpool School of Tropical Medicine for their statistic support.

Fellow researchers Amber, Machtelt, Marlies and Quirine, thank you for welcoming me into your metabolic group despite my hematologic background. Dear Quirine, how we would have wished that you had been able to defend your thesis! Unfortunately, fate had other plans. You continued to support us till the end. I am indebted to Michael Esan for asking me to be his co-author and for being such agreeable company in Malawi as well in Amsterdam. I also thank Jessica, Veronica, Imke, Evelien, Servet and all the other colleagues in the Rode Luifel for their moral support. Thanks to Carlene Tacke for providing diversity during my PhD.

I am grateful to Annemiek Tuinhofdemoed for her excellent work on the lay-out of this thesis and to Karst Janneke Rogaar, for her artistic embellishments of my work.

I am indebted to all my friends in Malawi for giving me the experience of living and working in Malawi and enjoying Malawian life. Special thanks go out for Lucia, Clementine and Karen for the warm home from first till the last day.
I thank all my friends in the Netherlands for their patience, their understanding and their invaluable support. Esther, Welmoed en Wytske also thanks for the distant support while I was abroad. Esther and Rianne, thank you for co-reading. Gwen van Lammeren, my saving neighbour, thanks for helping me out when needed.

I am grateful to my parents Leendert en Maaike, for believing in me and supporting me throughout my project, for listening to me in good times and in bad times, for reading my work, for their unconditional love and care. I thank my sisters Roos and Noor for their patience and their encouragement and for keeping me grounded during the whole process of preparing my thesis.

I gratefully remember and thank my beloved grandfather Prof. dr. Lambertus de Rijk. His passion for knowledge has motivated and inspired me and will continue to do so.

Finally, I would like to thank Manook, for being the special person that he is.
Resume

Femkje A.M. Jonker was born on January 4th, 1979 in Leiden, The Netherlands. She completed her secondary school (Athenaeum) at the Agnes College in Leiden in 1997 and started medical training at the University of Amsterdam in 1999. As a part of the masters program she performed a research internship at the University of Bahia, Salvador da Bahia in Brazil in 2004 evaluating the protective value of breastfeeding against obesity in childhood. After obtaining her medical degree in 2007 she worked as a house officer in the paediatric department of the St. Lucas Andreas Hospital in Amsterdam. In 2008 she successfully obtained a two year grant from the Numico Research foundation to start a PhD project at the Global Child Health Group of the Emma Children’s Hospital at the Academic Medical Centre in Amsterdam under supervision of prof. dr. Bernard J. Brabin, Dr. Michael Boele van Hensbroek and Dr. Job C. J. Calis. In 2010 she received a second grant from the Janivo Foundation and the Stichting Emma Steun. In September 2012 she will continue her clinical career as a resident in paediatrics clinician at the Emma Children’s Hospital.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIDS</td>
<td>Acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>AOR</td>
<td>Adjusted odds ratio</td>
</tr>
<tr>
<td>AUC</td>
<td>Area Under the Curve</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>CC</td>
<td>Community control</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>Ct</td>
<td>Cycle threshold</td>
</tr>
<tr>
<td>DAG</td>
<td>Directed acyclic graph</td>
</tr>
<tr>
<td>EIR</td>
<td>Entomological inoculation rate (number of infectious bites per year per individual)</td>
</tr>
<tr>
<td>G6PD</td>
<td>Glucose-6-phosphate dehydrogenase deficiency</td>
</tr>
<tr>
<td>HAART</td>
<td>Highly active anti-retroviral therapy</td>
</tr>
<tr>
<td>HC</td>
<td>Hospital control</td>
</tr>
<tr>
<td>Hb</td>
<td>Haemoglobin concentration</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HR</td>
<td>Hazard Ratio</td>
</tr>
<tr>
<td>ID</td>
<td>Iron deficiency</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IMCI</td>
<td>Integrated Management of Childhood Illness</td>
</tr>
<tr>
<td>IQR</td>
<td>Interquartile range</td>
</tr>
<tr>
<td>IVM</td>
<td>Integrated Vector Management</td>
</tr>
<tr>
<td>MCH</td>
<td>Mean cellular hematocrit</td>
</tr>
<tr>
<td>MCHC</td>
<td>Mean cellular concentration</td>
</tr>
<tr>
<td>MCV</td>
<td>Mean cellular volume</td>
</tr>
<tr>
<td>MRV</td>
<td>Mean reticulocyte volume</td>
</tr>
<tr>
<td>NPV</td>
<td>Negative predictive value</td>
</tr>
<tr>
<td>OR</td>
<td>Odds Ratio</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>Pf</td>
<td>Plasmodium falciparum</td>
</tr>
<tr>
<td>PPV</td>
<td>Positive predictive value</td>
</tr>
<tr>
<td>RCPF</td>
<td>Red cell production failure</td>
</tr>
<tr>
<td>RMSEA</td>
<td>Root Mean Square Error of Approximation</td>
</tr>
<tr>
<td>ROC</td>
<td>Receiver operating characteristics</td>
</tr>
<tr>
<td>RSF</td>
<td>Serum ferritin</td>
</tr>
<tr>
<td>SP</td>
<td>Soluble transferrin receptor</td>
</tr>
<tr>
<td>SF</td>
<td>Red cell Size Factor</td>
</tr>
<tr>
<td>sTrF</td>
<td>Serum transferrin</td>
</tr>
<tr>
<td>TIBC</td>
<td>Total iron-binding capacity</td>
</tr>
<tr>
<td>WBC</td>
<td>White blood cell count</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>ZPP</td>
<td>Zinc protoporphyrin</td>
</tr>
</tbody>
</table>
Colour section
Figure 1. PCR-detected hookworm infection and its association with severe anaemia

Displayed are the adjusted Odds Ratios and 95% confidence intervals for hookworm infection and its association with severe anaemia. Hookworm infection is defined as an *A. duodenale* and/or *a. americanus* infection; infection load is defined by the following cycle thresholds (Ct): low 35<Ct<50; moderate 25<Ct≤35; high Ct≤25. In case of dual infection the lowest Ct-value was counted. Severe anaemia is defined as haemoglobin <5.0 g per decilitre. The multivariate model was adjusted for age, sex, recent use of haematinics or anti-malarial treatment, history of transfusions, death of a parent, limited maternal education (mother did not attend secondary school), wasting (defined as a Z-score of weight for height < -2), vitamin B12 deficiency (< 20 ng /dL), vitamin A deficiency (< 20 ng /dL), HIV, Epstein-Barr virus, bacteraemia, malaria parasitaemia, G6PD deficiency and IL-10-23 mutations.
Figure 2. PCR-detected hookworm infection and its association with iron deficiency
Displayed are the adjusted Odds Ratios and 95% confidence intervals for hookworm infection and its association with iron deficiency. Hookworm infection is defined as an *A. duodenale* and/or *N. americanus* infection; infection load is defined by the following cycle thresholds (Ct); low 35<Ct<50; moderate 25<Ct≤35; high Ct≤25. In case of dual infection the lowest Ct-value was counted. Iron deficiency is defined as a bone marrow smear score of 0 or 1 iron containing particles 29. The multivariate model was adjusted for age, sex, study location, HIV (human immunodeficiency virus) infection and wasting (defined as a Z-score of weight for height < -2). These analyses include only children with severe anaemia.
Figure 1. Univariate and multivariate linear regression analyses with all relevant covariates predicting log hepcidin. Regression coefficients are presented with 95% confidence interval. Bone marrow iron stores was scores in a range from 0 to 6\(^4\). CRP: c-reactive protein; IL-6: interleukin 6. Bacteraemia was analyzed as dichotomous variable. Malaria was defined as a positive blood slide with concurrent fever (axillary temp $>37.5^\circ$ C), or history of fever within the previous 48 hours.

![Linear regression correlation coefficients (CI 95%) for loghepcidin](image1)

Figure 2. Univariate and multivariate logistic regression analyses with all relevant covariates predicting erythroblast iron incorporation. Regression coefficients are presented with 95% confidence interval. Insufficient erythroblast iron incorporation was defined as less than 30% erythroblasts having visible iron granules while having replete iron stores\(^25\). CRP: c-reactive protein. Previous use of haematinics is defined as use of iron supplements in the previous four weeks.

![Log-hepcidin](image2)

![Log-CRP](image3)
Chapter six | Hepcidin levels in severely anaemic children

![Graphical representation of structural equation model for hepaticin, hypoxia/erythropoiesis, iron status and inflammation.](image)

**Figure 3.** Structural equation model for hepaticin, hypoxia/erythropoiesis, iron status and inflammation. In this exploratory model of the factors associated with serum hepaticin, the sizes of the associations are indicated by the standardized regression coefficients. An inverse association is indicated by a red line. This model was created containing all possible associations between the displayed variables, after which all non-significant arrows ($p \geq 0.05$) were removed. A few non-significant correlations, deemed relevant by the authors, were retained in the figure, displayed as dashed arrows. C-reactive protein (CRP) was adjusted for malaria and bacteremia; interleukin 6 (IL-6) was adjusted for bacteremia (omitted for clarity). Iron in stores was defined as bone marrow iron score (0-6) [21]. Insufficient erythrocyte iron incorporation was defined as less than 30% erythroblasts having visible iron granules while having replete iron stores. Erythroblast iron was defined as >30% of the erythroblasts having visible iron granules while having replete bone marrow iron stores [25]. Alternative analyses without any non-significant arrow resulted in a virtually identical mode. The overall root mean square area of approximation, an indicator for model fit, was 0.288 (95% CI 0.272-0.304).

Chapter seven | Peripheral blood iron markers compared against bone marrow

![Graphical representation of receiver operating characteristic curves.](image)

**Figure 1.** Receiver operating characteristic curves of all iron markers with an AUCROC >0.500 (sTfR, ferritin, sTfR-F, hepaticin and MCV) in the identification of bone marrow iron stores deficiency.