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

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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\(^1\), conversely treatment of the deficiency with supplementation has been associated with an increased incidence of infection\(^2-7\). 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\(^8\). 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\(^9,10\), 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\(^9\), hookworm\(^11\) and HIV infection\(^9\); drugs (antibiotics\(^10\), tuberculostatics\(^11\) and antiretrovirals\(^12\); genetic disorders (G6PD, alpha-thalassaemia, sickle cell trait and haemoglobin C \(^13\); 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\(^14\). It is defined by a haemoglobin \(<5\) g/dL\(^14\), or \(<6\) g/dL\(^15\)), and is associated with major morbidity and mortality\(^16\). 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\(^16,17\), 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).

![Figure 1. Human cycle of iron metabolism.](image)

Healthy subjects have about 3000-5000 mg of iron in their body. About 2200 mg is located through the blood in the oxygen carrying haemoglobin; most of the rest (approximately 1500 grams) is contained in ferritin complexes that are present in all cells, mostly in the bone marrow, liver, and spleen. The storage of iron tends to be lower in children and women of child-bearing age, than in men. About 500 mg iron is used for cellular processes and cell growth. A relatively small amount (3 mg) circulates through the plasma, bound to transferrin.

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 \textit{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.

\textbf{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\textsuperscript{28, 29}. These measures reflect the haemoglobinization of the early reticulocytes and mature erythrocytes respectively\textsuperscript{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\textsuperscript{11}, 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\textsuperscript{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 \textsuperscript{25, 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\textsuperscript{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\textsuperscript{41}. There are several factors that regulate hepcidin expression including systemic stimuli of iron levels, inflammation, erythropoietic drive and hypoxia\textsuperscript{42} (Figure 3). Hepcidin is down-regulated during iron deficiency, hypoxia and enhanced erythropoietic drive\textsuperscript{43}; conversely it is up-regulated in the process of increased iron levels and during inflammation\textsuperscript{41}.

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

Hepcidin has been shown to be a potential marker to define iron deficiency\textsuperscript{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 supplements\textsuperscript{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 (*Ancylostoma 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 year60). 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° 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 coma71. 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 °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.

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 hepecidin 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 hepecidin, against bone marrow iron assessment in a healthy population of Malawian children
Chapter one | Introduction and outline of the thesis

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