STRATEGIES TO REDUCE ANAEMIA AND IRON DEFICIENCY IN SUB-SAHARAN AFRICAN CHILDREN: TECHNOLOGICAL AND PHYSIOLOGICAL APPROACHES

A dissertation submitted to

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presented by

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ABBREVIATIONS

AGP       α1-acid-glycoprotein
ALAS-E    Aminolevulinic acid synthase
CDC       Center of Disease Control
CRP       C-reactive protein
DCT1      Divalent cation transporter 1
DcytB     Duodenal cytochrome B
DMT1      Divalent metal transporter 1
EGRAC     Erythrocyte glutathione reductase activation coefficient
ELISA     Enzyme-linked immunosorbent assay
EPO       Erythropoietin
FAD       Flavin-adenin mononucleotide
FAO       Food and Agriculture Organization
Fe        Iron
FPN1      Ferroportin 1
FSP       Flame spray pyrolysis
HAMP      Hepcidin gene
Hap       Haptoglobin
Hb        Haemoglobin
HCP1      Haem carrier protein 1
HFE       gene products from a mutation in hereditary haemochromatosis
HH        hereditary haemochromatosis
HIF       hypoxia-inducing factor
HNE       Hydroxy-nonenal
HO1       Haem oxygenase 1
ID        Iron deficiency
IDA       Iron deficiency anaemia
IFN       Interferon
IL        Interleukin
INACG     International Nutritional Anemia Consultancy Group
IPT       Intermittent preventive treatment for malaria
IRE       Iron responsive element
IREG1     Iron-regulated mRNA 1
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRMA</td>
<td>Immunoradiometric assay</td>
</tr>
<tr>
<td>IRP</td>
<td>Iron regulatory protein</td>
</tr>
<tr>
<td>MCV</td>
<td>Mean corpuscular volume</td>
</tr>
<tr>
<td>MTP1</td>
<td>Metal transfer protein 1</td>
</tr>
<tr>
<td>NaFeEDTA</td>
<td>Sodium iron ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>Nramp2</td>
<td>Natural resistance associated macrophage protein 2</td>
</tr>
<tr>
<td>NTBI</td>
<td>Non-transferrin-bound iron</td>
</tr>
<tr>
<td>Pf</td>
<td><em>Plasmodium falciparum</em></td>
</tr>
<tr>
<td>RBP</td>
<td>Retinol-binding protein</td>
</tr>
<tr>
<td>RBV</td>
<td>Relative bioavailability value</td>
</tr>
<tr>
<td>RDT</td>
<td>Rapid diagnostic test</td>
</tr>
<tr>
<td>RR</td>
<td>Risk ratio</td>
</tr>
<tr>
<td>sF</td>
<td>Serum ferritin</td>
</tr>
<tr>
<td>Sh</td>
<td><em>Schistosomas haematobium</em></td>
</tr>
<tr>
<td>Sm</td>
<td><em>Schistosomas mansoni</em></td>
</tr>
<tr>
<td>sR</td>
<td>Serum retinol</td>
</tr>
<tr>
<td>SSA</td>
<td>Sub-Saharan Africa</td>
</tr>
<tr>
<td>sTfR</td>
<td>Soluble transferrin receptor</td>
</tr>
<tr>
<td>STH</td>
<td>Soil-transmitted helminths</td>
</tr>
<tr>
<td>TBI</td>
<td>Transferrin-bound iron</td>
</tr>
<tr>
<td>Tf</td>
<td>Transferrin</td>
</tr>
<tr>
<td>TfR</td>
<td>Transferrin receptor</td>
</tr>
<tr>
<td>TIBC</td>
<td>Total iron-binding capacity</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TTR</td>
<td>Transthyretin (Prealbumin)</td>
</tr>
<tr>
<td>UNICEF</td>
<td>United Nations Children's Fund</td>
</tr>
<tr>
<td>UTR</td>
<td>5'-untranslated region</td>
</tr>
<tr>
<td>VAD</td>
<td>Vitamin A deficiency</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>XB/X1B</td>
<td>Bitot's spots</td>
</tr>
<tr>
<td>XN</td>
<td>Night blindness</td>
</tr>
<tr>
<td>ZPP</td>
<td>Zinc protoporphyrin</td>
</tr>
</tbody>
</table>
SUMMARY

Background
Iron deficiency and anaemia are global health problems, with a total of more than half of the world’s population affected. In sub-Saharan Africa, infants, children, women of childbearing age and pregnant women are the most vulnerable groups. The aetiology of anaemia in the tropics is multifactorial, but iron deficiency and chronic infections such as malaria and helminths are thought to be the main causes. Anaemia results in poorer educational achievement, impaired cognition, increased morbidity, increased maternal and perinatal mortality and reduced work capacity and thus, adversely affects economies of entire countries. Proposed strategies to reduce anaemia include iron fortification, treatment against intestinal parasites and malaria control.

Aim
The main aim of this thesis was to investigate potential strategies to reduce anaemia. It included:

1) A comparison of two methods for the assessment of plasma ferritin;
2) A cross-sectional study of the association between riboflavin status and anaemia;
3) A prospective intervention study comparing the efficacy of iron fortification, deworming treatment and/or malaria reduction on anaemia was done;
4) A nano-sized iron phosphate compound was produced for potential use in food fortification and/or supplementation.

Design
1) An automated and a manual method for measuring plasma ferritin were compared by simultaneously measuring a series of samples;
2) In a cross-sectional study conducted in two distinct geographical zones in Côte d’Ivoire, parasitological and nutritional parameters were assessed, including riboflavin and iron status, as well as anaemia;
3) In a 6-month, randomized, double-blind, placebo-controlled trial, school children in rural Côte d’Ivoire (6-14 y-old; n=591) were treated with 1) iron-fortified biscuits containing 20 mg/day as electrolytic iron (4x/week); 2) repeated anthelmintic treatment and/or 3) intermittent preventive treatment of malaria. At baseline and endpoint, haemoglobin and other relevant blood parameters were assessed;
Summary

4) Nanopowders of ferric phosphate were synthesized, characterized, and the relative bioavailability value compared to ferrous sulphate in rats using the haemoglobin repletion method was assessed.

Results

1) The two ferritin methods showed good agreement ($r^2=0.98$), but the automated method showed better performance in terms of intra- and interassay variability;
2) The prevalence of mild riboflavin deficiency was high (65%); in children who were free of malaria, risk for iron deficiency was increased by riboflavin deficiency (odds ratio: 3.07; 95% CI: 1.12, 8.41);
3) In this setting of high anaemia, helminths and malaria prevalence, only the treatment with anthelmintic resulted in a significant reduction in anaemia prevalence and a significant increase in haemoglobin concentration of approximately $\approx 3.0$ g/L; the other interventions had no impact;
4) The commercial and the two in-house synthesized nano-iron phosphate powders had a surface area of 33, 69, and 195 m$^2$/g; their relative bioavailability values were 61, 70, and 96%, respectively; the histology and assessment of oxidative stress found no increased risk of toxicity for smaller powders.

Conclusion

1) Due to the lower variability and operator independency, the automated method for plasma ferritin is the preferred one;
2) A high prevalence of mild riboflavin deficiency was found in Ivorian children, and this may increase the risk for iron deficiency; however, malaria masks this interaction, and riboflavin deficiency has no impact on anaemia;
3) Of the three interventions implemented, only anthelmintic treatment was moderately efficacious in improving haemoglobin concentration or reduce anaemia prevalence; in this setting, the iron fortification was not efficacious due to the low prevalence of iron deficiency or the low availability, likely due to physiological conditions (inflammation, low level of gastric acid); intermittent preventive treatment of malaria was not efficacious in this setting, suggesting the administered dose and interval have to be reassessed.
4) The relative bioavailability of the smallest nano-sized ferric phosphate is similar to a water-soluble iron compound, and preliminary toxicology assessments favour the nano-compounds.
ZUSAMMENFASSUNG

Hintergrund

Zielsetzung
Das Hauptziel dieser Arbeit war es mögliche Strategien zur Verminderung von Anämie zu untersuchen. Der Fokus lag auf:
1) einem Vergleich von zwei Methoden zur Bestimmung von Plasmaferritin;
2) einer Querschnittsstudie zum Einfluss des Riboflavinstatus auf die Anämie;
3) einer Interventionsstudie, welche die Effizienz von Eisenfortifizierung, Entwurmung und/oder Malariakontrolle auf die Anämie verglich;
4) der Entwicklung und Produktion von Nanoeisen (Eisenphosphat) für den möglichen Einsatz in Lebensmittelanreicherungs- und/oder Supplementierungsprogrammen.

Design
1) In einem Methodenvergleich wurden eine automatisierte gegen manuelle Methode zur Messung von Plasmaferritin verglichen und evaluiert.
2) Eine Querschnittsstudie wurde in zwei verschiedenen geografischen Zonen der Elfenbeinküste durchgeführt. Schwerpunkt war die Untersuchung möglicher
Zusammenfassung

Zusammenhänge von parasitologischer und ernährungs-relevanter Indikatoren (Riboflavin- und Eisenstatus) auf Anämie.

3) In einer 6-monatigen Studie in der Elfenbeinküste (Studiendesign doppeltblind, randomisiert und placebo-kontrolliert) wurden Schulkinder (Alter 6-14 Jahre; n=591) folgender Behandlung unterzogen: 1) Eine Behandlung mit eisenfortifizierten Keksen mit 20 mg Eisen/Tag während 4 Tagen/Woche; 2) wiederholter Helminthen-Behandlung und/oder 3) intermittierender präventiver Malaria-Behandlung. Am Anfang und Ende der Studie wurden Hämoglobin und andere relevante Blutindikatoren erhoben.


Ergebnisse

1) Die beiden Ferritinmethoden zeigten eine gute Übereinstimmung ($r^2=0.98$), die automatisierte Methode wies jedoch eine bessere Robustheit in Bezug auf intra- und interexperimentelles Variabilität auf.

2) Die Prävalenzrate von mildem Riboflavinmangel lag bei 65%. In Kindern, die keine Malaria-Parasiten aufwiesen, war bei Riboflavinmangel das Risiko dreimal höher Eisenmangel zu entwickeln (95% Vertrauensintervall: 1.12, 8.41).

3) Bei einer hohen Anämie-, Helminthen- und Malaria-Praevalenz hatte ausschließlich die Helminthen-Behandlung einen signifikanten Einfluss auf eine Anämiereduktion oder eine Verbesserung der Hämoglobin-Konzentrationen um etwa 3.0 g/L; die beiden anderen Interventionen (Eisengabe und Malaria-Behandlung) hatten keinen Einfluss.

4) Ein kommerzielles und zwei im Labor synthetisierte Eisenphosphat-Nanopartikel wiesen Oberflächen von 33, 69, und 195 $m^2/g$ Pulver auf; die dazugehörigen relativen Bioverfügbarkeiten waren 61, 70 und 96%. Die histologischen Untersuchungen, sowie Messungen des oxidativen Stresses wiesen auf kein erhöhtes toxikologisches Risiko der Nanopartikel hin.
Zusammenfassung

**Schlussfolgerungen**

1) Aufgrund der niedrigeren Variabilität sowie der Anwenderunabhängigkeit ist die automatisierte Methode zu bevorzugen.

2) Es wurde eine hohe Prävalenz von mildem Riboflavinmangel gefunden, was zu einer Erhöhung des Risikos an Eisenmangel zu erkranken führen kann. Die Erkrankung an Malaria verdeckt diesen Einfluss und es konnte gezeigt werden, dass Riboflavinmangel keinen Einfluss auf Anämie hat.


INTRODUCTION

The reduction of iron deficiency and anaemia remains a health challenge worldwide, but in particular in the developing world. It is estimated that around 2 billion people are anaemic and one billion suffer from IDA and a further billion from ID without anaemia, with high proportions of these diseases among children and women of childbearing age from the developing world (WHO, 2002). Anaemia may result in poorer educational achievement, impaired cognition, increased mortality and overall morbidity in children, reduced work capacity in adults and poor pregnancy outcomes (Gilgen & Mascie-Taylor, 2001; Boccio & Iyengar, 2003; Horton & Ross, 2003; Marchant et al., 2004). In the setting of sub-Saharan Africa (SSA), the aetiology of anaemia is multifactorial (Crawley, 2004). Although the main cause is thought to be low dietary iron bioavailability, anaemia can also result from diseases, such as malaria (Menendez et al., 2000), helminth infection or schistosomiasis (Friedman et al., 2005), from chronic inflammatory disorders (Yip & Dallman, 1988), or from nutritional deficiencies of folate, vitamin B$_{12}$, or vitamin A (Suharno et al., 1993; Savage et al., 1994).

Proposed strategies in combating anaemia in developing countries include iron fortification, anthelmintic treatment, and malaria reduction; iron fortification is efficacious in experimental settings and is considered to be cost-effective (Baltussen et al., 2004). Recent recommendations put forth combined chemotherapy with praziquantel (against schistosome infections) and albendazole (against soil-transmitted helminth infections) to enhance control programme efficacy and cost-effectiveness in reducing the helminth burden (Savioli et al., 2002; Utzinger & Keiser, 2004; Hotez et al., 2006). Intermittent preventive treatment (IPT) as a means to reduce malaria has produced benefit in intervention trials, with the focus on pregnant women and infants (Egan et al., 2005; O'Meara et al., 2005; van Eijk et al., 2005; Cisse et al., 2006). IPT has recently been suggested as a strategy in malaria prevention in children in areas with high seasonal malaria transmission, arguing for a potentially reduced transmission pressure, and hence a reduction in overall malaria prevalence (Greenwood, 2006).
Introduction

The aim of this thesis was to investigate potential strategies to overcome anaemia. One of them is iron fortification; water-soluble compounds are well absorbed but cause organoleptic problems, whereas water-insoluble compounds are organoleptically more stable but are less well absorbed (Hurrell, 2002b). New and innovative iron compounds could overcome this dilemma. Thus, in parallel to field studies, it was aimed to develop highly bio-available, yet water-insoluble iron nanoparticles that cause less sensory changes. At the same time, two field studies were implemented, and some laboratory investigations were performed. The two field trials evaluated the influence of micronutrient deficiencies on iron metabolism, followed by a trial investigating the relative efficacy of iron fortification, malaria reduction, and anthelmintic treatment alone and in combination, to reduce anaemia in schoolchildren in south-central Côte d'Ivoire.

The thesis is presented in five chapters:

Chapter 1 is a literature review and gives an overview on the epidemiology and aetiology of anaemia in SSA followed by the assessment methods used for determining parameters that are associated with anaemia. The second part reviews current knowledge on iron metabolism, and given the importance of infection in the setting of tropical Africa, the alteration of this same metabolism during inflammation. The last section of the review gathers information on the limitation of currently used iron fortificants and the potential of nanoparticles in iron fortification to overcome iron deficiency. In the subsequent chapters, published articles and manuscripts in preparation are presented.

Chapter 2 presents the first published manuscript comparing two different methods of serum ferritin quantification; one automated enzyme-linked immuno-sandwich-assay (ELISA), the second a widely-used manual ELISA method.

The manuscript from chapter 3 focused on the influence of riboflavin deficiency on iron metabolism. This study was carried out in the same area as the one presented in chapter 4, in order to obtain as much information as possible on existing nutritional deficiencies other than iron deficiency.
In chapter 4, the results of a 6-month, double-blind, placebo-controlled trial, using a 2x2x2 factorial design in mildly anaemic schoolchildren is presented. Aim of the study was to evaluate the relative efficacy of iron fortification, IPT with sulfadoxine-pyrimethamine, and administration of praziquantel/albendazole, alone and in combination, to reduce anaemia in schoolchildren in south-central Côte d'Ivoire.

Chapter 5 deals with the synthesis of iron nanoparticles, the methods to appropriately characterize them, and finally, a comparison of their bioavailability in a rat model.
1 LITERATURE REVIEW

1.1 Anaemia in sub-Saharan Africa: definition, epidemiology and consequences

1.1.1 Definition

Anaemia, Greek for "without blood", is a reduction in the mass of red blood cells and the haemoglobin content of blood. It occurs if the production and destruction of the red blood cells is disturbed (Schnall et al., 2000). This disruption can be caused by various factors, the so-called multifactorial anaemia. Although several diagnostic methods have been used over the years, the most reliable parameter is haemoglobin (Hb). The World Health Organization (WHO) thus uses this parameter to define anaemia in different population groups. The proposed thresholds vary, because normal Hb distribution varies with age, sex, race, altitude, physiological status (pregnancy, lactation) and smoking (INACG, 2002). The difference in Hb due to differences in ethnicity has been a subject of discussion; estimates vary from 5 to 10 g/L lower Hb concentrations in black populations compared to Caucasians (Perry et al., 1992; Johnson-Spear & Yip, 1994; Beutler & West, 2005); however, no consensus was found and to date, WHO does not take into consideration ethnicity in their guidelines on cut-offs to use (WHO/UNICEF/UNU, 2001). Table 1 indicates the Hb thresholds to be used for different population groups.

<table>
<thead>
<tr>
<th>Population group</th>
<th>Hb threshold [g/L]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Children 0.5-4.9y</td>
<td>&lt;110</td>
</tr>
<tr>
<td>Children 5.0-11.9y</td>
<td>&lt;115</td>
</tr>
<tr>
<td>Children 12.0-14.9y</td>
<td>&lt;120</td>
</tr>
<tr>
<td>Non-pregnant women ≥15.0y</td>
<td>&lt;120</td>
</tr>
<tr>
<td>Pregnant women</td>
<td>&lt;110</td>
</tr>
<tr>
<td>Men ≥15.0y</td>
<td>&lt;130</td>
</tr>
</tbody>
</table>

For infants under 0.5 years of age, no threshold is defined and thus, it is difficult to obtain prevalence estimates for this age group.
Literature review

The severity of anaemia is important when it comes to differentiating between acute and chronic consequences. Acute anaemia is defined as an Hb value of <70 g/L for all age groups (WHO/UNICEF/UNU, 2001). Further, prevalence thresholds for classifying the severity of anaemia as a public health problem have been established and they are: <5% "no public health problem", 5-19.9% "mild public health problem", 20-39.9% "moderate public health problem", ≥40% "severe public health problem" (WHO/UNICEF/UNU, 2001).

1.1.2 Epidemiology

Estimates of global anaemia prevalence are out-of-date for many parts; the last comprehensive overview (WHO DB I) for all population groups was in 1985 (DeMaeyer & Adiels-Tegman). The "WHO global database on anaemia" was recently re-examined and for specific population groups (WHO DB III), i.e. pre-school children, non-pregnant and pregnant women, anaemia prevalence estimates were calculated by region. For the other population groups (school-aged children, men, and elderly), only world estimates are provided (WHO/CDC, in press). Table 2 shows the worldwide anaemia prevalence and the number of individuals affected.

Table 2: Worldwide anaemia prevalence and number of individuals affected, by age groups (WHO, 2008)

<table>
<thead>
<tr>
<th>Population group</th>
<th>Anaemia prevalence [%]</th>
<th>Affected individuals [million]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-school children</td>
<td>47.4</td>
<td>293</td>
</tr>
<tr>
<td>School-age children</td>
<td>25.4</td>
<td>305</td>
</tr>
<tr>
<td>Pregnant women</td>
<td>41.8</td>
<td>56</td>
</tr>
<tr>
<td>Non-pregnant women</td>
<td>30.2</td>
<td>468</td>
</tr>
<tr>
<td>Men</td>
<td>12.7</td>
<td>260</td>
</tr>
<tr>
<td>Elderly</td>
<td>23.9</td>
<td>164</td>
</tr>
<tr>
<td><strong>Total Population</strong></td>
<td><strong>24.8</strong></td>
<td><strong>1620</strong></td>
</tr>
</tbody>
</table>

The most affected population groups are pre-school children, with almost half of them suffering from anaemia, followed by pregnant and non-pregnant women with 42% and 30%, respectively. Although closely followed by the school-age children (25%), the database does not consider this group further.

Of particular interest for this review is SSA. In the WHO DB III, the WHO system for the definition of world regions is used (WHO, 2008). This group assignment brings
the advantage that Africa comprises almost exclusively SSA countries, since all North African countries (except Algeria) are assigned to the Eastern Mediterranean region (Egypt, Morocco, Somalia, Sudan, and Tunisia). Hence, the political WHO Africa region closely represents the geographic SSA area.

For African pre-school children, pregnant and non-pregnant women, estimated prevalences of anaemia are 67.6%, 57.1% and 47.5%, respectively; these prevalences correspond to 83.5, 17.2, and 69.9 million affected individuals, respectively.

The main target population of this review are school-age children; unfortunately, the WHO DB III only provides regional estimates for the above groups. In the WHO DB I, 52% of school-age children were estimated to suffer from anaemia, with 52% reported for North Africa, 55% for West Africa, 49% for East Africa, 3% for South Africa, and missing data for Middle Africa.

The presently available WHO database on anaemia (WHO DB II) also provides prevalence data, but in contrast to the WHO DB III does not give absolute number estimates. Table 3 shows prevalence data and mean Hb values by country for the WHO African region for school-age children. Countries not tabulated do not have prevalence data for this age group. Approximately 85 million African children aged 5 to 14 years were reported to suffer from anaemia (WHO/UNICEF/UNU, 2001), in absolute figures therefore presenting the biggest proportion of affected individuals as compared to 83.5, 17.2 and 69.9 million for pre-schoolers, pregnant women and non-pregnant women.
Table 3: African countries with available anaemia data on school-age children, data extracted from (WHO, 2007)

<table>
<thead>
<tr>
<th>Country</th>
<th>Survey level</th>
<th>Year</th>
<th>n subjects</th>
<th>Age range [y]</th>
<th>Mean Hb [g/L] (SD)</th>
<th>Hb cut-off [g/L]</th>
<th>Anaemia prevalence [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benin</td>
<td>District</td>
<td>1986</td>
<td>100</td>
<td>6-14</td>
<td>112 (18)</td>
<td>120</td>
<td>54</td>
</tr>
<tr>
<td>Botswana</td>
<td>National</td>
<td>1994</td>
<td>100</td>
<td>5-9</td>
<td>113 (14)</td>
<td>115</td>
<td>53</td>
</tr>
<tr>
<td>Burkina Faso</td>
<td>State</td>
<td>2001</td>
<td>1368</td>
<td>5-17</td>
<td>115</td>
<td>70</td>
<td>0.3</td>
</tr>
<tr>
<td>Cameroon</td>
<td>Local</td>
<td>1991</td>
<td>294</td>
<td>5-12</td>
<td>120 (12)</td>
<td>120</td>
<td>47</td>
</tr>
<tr>
<td>Chad</td>
<td>National</td>
<td>2000</td>
<td>1024</td>
<td>6-16</td>
<td>117</td>
<td>110</td>
<td>25.1</td>
</tr>
<tr>
<td>Cote d'Ivoire</td>
<td>Regional</td>
<td>1996</td>
<td>531</td>
<td>6-17</td>
<td>112 (15)</td>
<td>110</td>
<td>46</td>
</tr>
<tr>
<td>Ghana</td>
<td>National</td>
<td>1998</td>
<td>926</td>
<td>6-11</td>
<td>n/a</td>
<td>120</td>
<td>71.3</td>
</tr>
<tr>
<td>Guinea</td>
<td>National</td>
<td>2000</td>
<td>1450</td>
<td>5-10</td>
<td>n/a</td>
<td>115</td>
<td>51.6</td>
</tr>
<tr>
<td>Kenya</td>
<td>District</td>
<td>2002</td>
<td>f: 74</td>
<td>6-11</td>
<td>n/a</td>
<td>110</td>
<td>12</td>
</tr>
<tr>
<td>m: 84</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Madagascar</td>
<td>National</td>
<td>2000</td>
<td>883</td>
<td>6-15</td>
<td>n/a</td>
<td>120</td>
<td>38</td>
</tr>
<tr>
<td>Malawi</td>
<td>State</td>
<td>1998</td>
<td>n/a</td>
<td>7-12</td>
<td>n/a</td>
<td>115</td>
<td>40</td>
</tr>
<tr>
<td>Mali</td>
<td>District</td>
<td>2000</td>
<td>1112</td>
<td>6-20</td>
<td>115 (13)</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Mauritania</td>
<td>Local</td>
<td>1995</td>
<td>238</td>
<td>5-13</td>
<td>n/a</td>
<td>110</td>
<td>50.4</td>
</tr>
<tr>
<td>Mozambique</td>
<td>Local</td>
<td>2001</td>
<td>f: 789</td>
<td>10-19</td>
<td>121</td>
<td>120</td>
<td>45</td>
</tr>
<tr>
<td>Niger</td>
<td>Local</td>
<td>1992</td>
<td>174</td>
<td>n/a</td>
<td>n/a</td>
<td>120</td>
<td>59.7</td>
</tr>
<tr>
<td>South Africa</td>
<td>Local</td>
<td>1999</td>
<td>123</td>
<td>n/a</td>
<td>n/a</td>
<td>120</td>
<td>22</td>
</tr>
<tr>
<td>United Rep. of Tanzania</td>
<td>District</td>
<td>2000</td>
<td>3400</td>
<td>7-19</td>
<td>115 (14)</td>
<td>110/120</td>
<td>31.2/62.6</td>
</tr>
<tr>
<td>Zambia</td>
<td>Local</td>
<td>2001</td>
<td>406</td>
<td>7-16</td>
<td>130</td>
<td>n/a</td>
<td>n/a</td>
</tr>
</tbody>
</table>

A challenge in interpreting this Table 3 is that different thresholds for defining anaemia have been applied in the surveys. When available, mean Hb values are provided. Further limitations are the small numbers in some surveys and the survey level; only 5 of the countries provide nationally representative data. Finally, the biggest limitation is the lack of data on many countries, in particular very populated ones, e.g. Nigeria. A trend that can be observed is that the anaemia prevalence in Western and Central African countries is higher than in Eastern and Southern African countries. WHO considers anaemia prevalence above 40% as a severe public health problem; despite the lack of harmonization in the cut-offs, it is obvious that anaemia in this population group in many of these countries would be considered as a severe public health problem.
The above section dealt with global and SSA occurrence of anaemia, but as a part of evaluating the magnitude of the problem, subsequent health consequences of anaemic subjects have to be addressed. As above, this review starts with different population groups and then focuses on the main target group of the present thesis: school-age children.

In the literature, anaemia and IDA are often used interchangeably because of the generally accepted concept that approximately half of all anaemia cases are due to iron deficiency. There are often overlaps between multifactorial anaemia and IDA and thus, this section tries to integrate the different aetiologies to discuss the consequences of anaemia as such.

### 1.1.3 Health consequences

Although multifactorial in its nature, in clinical terms, anaemia is a haemoglobin concentration insufficient to meet the oxygen needs of the tissues. The consequences of this shortage are manifold and include reduced work capacity, poor pregnancy outcomes, increased maternal and perinatal mortality and morbidity, impaired cognitive performance and poorer educational achievement. In the following section, evidence for these statements is presented with anaemia as the epidemiological modulator and not necessarily the underlying causes.

**Perinatal outcomes**

The severity of anaemia surely plays a role in the impact on the subject's health status, and a very low Hb concentration can lead to a fatal event, e.g. due to severe malarial anaemia (Lamikanra *et al.*, 2007). However, the large majority of anaemia is of a milder form and most often, impairments are consequences of chronic mild-to-moderate anaemia. In a meta-analysis, the relation between anaemia during pregnancy and perinatal mortality was examined. For each 10 g/L increase in mean Hb, a relative risk of 0.75 (95% CI 0.62-0.89) and of 0.72 (0.65-0.81) was determined for maternal and perinatal mortality, respectively (Stoltzfus *et al.*, 2004). With regard to infant mortality, it is more difficult to extrapolate the impact of a generalized anaemia, since most of the studies were conducted in malaria-endemic countries and among severely anaemic mothers (Brabin *et al.*, 2001). A more recent study conducted in Tanzania found for children of severely anaemic mothers (<80 g/L) a
hazard ratio for infant mortality of 3.1 (95%CI 1.1-9.1), whereas neither ID nor malaria were significant predictors for child survival (Marchant et al., 2004). Birth weight has been shown to be influenced by Hb levels in a non-linear manner: higher relative risk (RR) for low birth weight for moderate to severe anaemia, low RR for normal birth weight for mild anaemia, and again increased RR for lower birth weight for no anaemia (Allen, 2000; Steer, 2000). Risk impact of anaemia on stillbirth is less well studied. In a recent re-analysis of 1988 U.S. delivery data, moderate but not mild anaemia in the first trimester was associated with increased risk for stillbirth in white women (Tomashek et al., 2006). A study conducted in Tanzania found for anaemic mothers an odds ratio for stillbirth of 3.74 (1.1-12.8), but maternal height and a history of past stillbirths were also significant predictors (Watson-Jones et al., 2007).

**Physical activity**

As for the pregnancy outcomes, publications often focus on separate aetiologies, when evaluating the impact of anaemia on work capacity or physical activity. In a comprehensive review, Haas and Brownlie assess the impact of ID and IDA on work capacity (2001). However, in the majority of the studies they looked at, Hb was used as the proxy for the degree of ID and thus the authors conclude that the reduction in various dimensions of work capacity/physical activity is mainly due to anaemia and subsequently, reduced oxygen transport. This assumption can be challenged, since iron as co-factor of enzymes in the respiratory chain plays an important role (Bollinger & Krebs, 2006). There, iron mainly interferes in electron transfer interactions. Nevertheless, it is a fact that many of the studies only assess anaemia and thus, we have to confine interpretation of the results to the only measured outcome. Two studies in non-anaemic ID subjects showed improved physical capacity after correction of ID (Hinton et al., 2000; Brownlie et al., 2004). Haemoglobin, but also ID with or without anaemia were predictors for total motor activity and locomotion (Siegel et al., 2005; Olney et al., 2007).

In school-age children, information on physical activity is scarce; available studies were mostly done in Asia (Bhatia & Seshadri, 1987; Satyanarayana et al., 1990; Sen & Kanani, 2006). One study investigated the effect of IDA on physical performance in adolescent girls from different races, but the number of subjects is too small for Afro-Caribbeans to draw conclusions (Nelson et al., 1994). The above studies tend to find
a negative impact of anaemia on physical performance, although all of them have their limitations: effects only on recovery time after exercise but not on exercise performance (Sen & Kanani, 2006); only one dimension assessed (running time; Satyanarayana et al., 1990). A study conducted in adolescents suffering from sickle cell anaemia found decreased physical activity but in terms of energy expenditure, results were less conclusive (Buchowski et al., 2002). Possible underlying mechanisms are discussed by Beard (2001). He proposes a concept, where the effect of anaemia (reduced oxygen transport) and ID (reduced oxidative metabolism) are overlapping.

**Mental development and cognition**

The assessment of the impact of anaemia among children on mental development or cognitive performance, often also termed intelligence or intelligence quotient (Connolly & Kvalsvig, 1993), is not straightforward, since in utero sub-optimal growth conditions might irreversibly alter postnatal brain development (Lozoff & Georgieff, 2006). Environmental or psychosocial factors equally play a role in the mental development of a child and are often difficult to separate from developmental disadvantages due to anaemia (or ID). These are some of the reasons for the difficulties in establishing clear links between interventions and impacts (Walker et al., 2007). Further, nutritional factors such as deficiencies of iodine, zinc, B vitamins and others or the consequences of malnutrition (stunting), influence the development (Bryan et al., 2004). An indirect consequence of (maternal) anaemia can be the retarded mental development in low birth weight children (Grantham-McGregor et al., 1998). Children who were stunted in early childhood showed to have slower development scores up to the age of 18 years (Holsapple et al., 2005). Although this data only deals with undernutrition, any mental retardation in early childhood might have an impact later in childhood, as supported by an intervention follow-up (Lozoff et al., 2000) and an observational study (Hurtado et al., 1999).

The biggest challenge in interpreting the impact of anaemia on cognitive performance is however the fact that the terms anaemia, ID, and IDA are used interchangeably.

For IDA children younger than 4 years, the overwhelming majority of studies reported poorer mental functional outcomes than in non IDA siblings (Grantham-McGregor 2001); of note, most of the intervention studies reviewed found improved mental
performance after correction of the IDA (with iron supplements), but inconsistent findings are reported in another meta-analysis (Sachdev 2005); these differing findings are likely to be due to different inclusion criteria for studies and within the selected studies, differences in subjects: some trials selectively included anaemic children, others included all apparently healthy children (mostly from anaemia endemic areas). Interestingly, the latter meta-analysis found a clear increase in intelligence scores in previously anaemic or ID children over 7 years of age after anaemia reduction or correction of ID; these findings are in agreement with a recent review, where causality was looked at (McCann & Ames, 2007).

If the focus is on anaemia due to parasites, such as malaria or helminths, the picture is unfortunately not sharper. Holding & Snow (2001) suggest malaria is likely to effect cognitive development. However, in a recent publication that critically reviews potential pathways of malarial damage to development and the assessment of the degree of developmental retardation (Holding & Kitsao-Wekulo, 2004), it becomes clear that the mechanisms that might interfere in the development are manifold. The evaluation difficulties already start at the level of disease severity, ranging from asymptomatic to severe disease, with or without cerebral sequelae. The majority of studies in this area focus on the clinical phase of malaria, but in humans, asymptomatic and clinical malaria coexist. To illustrate the magnitude of the problem, it seems thus more appropriate to present a few studies looking at malaria as the whole. There is some evidence that children with asymptomatic malaria undergo the negative consequences only on the long term (Al Serouri et al., 2000). The acute phase response in asymptomatic malaria is a potential mechanism. If malaria was prevented by administering chloroquine, school absenteeism was reduced, and both language and mathematics scores improved significantly; however, the potential bias of reduced absenteeism with regard to performance is not discussed as well as the fact that absenteeism did not follow a dose-dependent pattern (Fernando et al., 2006). Kihara and colleagues (2006) tried to categorize the magnitude of impaired cognition, and found rather solid data for uncomplicated clinical malaria, severe malaria and cerebral malaria but could not establish good evidence for the contribution of asymptomatic malaria, most likely due to scarcity of data.
After malaria, the second most common parasites worldwide, but particularly in developing countries, are helminths (Hotez et al., 2004). Anaemia due to infection with hookworms is likely to be due to blood loss and chronic infection, and thus, underlying mechanisms might be similar to IDA (blood loss) or malaria (chronic infection). In the 90's, two reviews covered this area with regard to cognitive outcomes and claimed a correlation between parasite density and cognitive impairment (Connolly & Kvalsvig, 1993; Watkins & Pollitt, 1997). A few more recent studies have investigated cognitive development after treatment with anthelminthics or the combination with iron supplements in pre-school children (Stoltzfus et al., 2001) and in schoolchildren (Boivin & Giordani, 1993; Simeon et al., 1995; Hutchinson et al., 1997; Jinabhai et al., 2001), and they provide mixed results. A heavily debated review (Dickson et al., 2000) questions the positive effect of anthelmintic treatment.

A common underlying mechanism for a potential benefit after anaemia reduction that is independent of its aetiology might be the light but chronic lack of oxygen transported to the tissue. Support for this statement can be found in the field of hypoxia research: for more pronounced forms of hypoxia, a clear association could be found, but also for milder forms, a link is likely (Bass et al., 2004); and from dialysis and transplantation research, where consistent improvements after therapeutic interventions are reported (Stivelman, 2000).

To link the physiological and mental consequences with economical costs seems very appropriate, but due to the complexity of the issue and the scope of the present review, this aspect is not discussed but the review continues with aetiologies of anaemia in the context of sub-Saharan Africa.

**1.1.4 Aetiology of anaemia in sub-Saharan Africa**

The aetiology of anaemia in children in this region is multifactorial (Crawley, 2004). Although the main cause is thought to be low dietary iron bioavailability, anaemia can result from other nutritional deficiencies of folate, vitamin B-12 or A (Suharno et al., 1993; Savage et al., 1994); or from diseases such as malaria (Menendez et al., 2000), helminth infection (Stephenson et al., 2000; Brooker et al., 2004),
schistosomiasis (Friedman et al., 2005), as a consequence from chronic inflammatory disorders (Yip & Dallman, 1988) or from genetic disorders such as haemoglobinopathies (Stuart & Nagel, 2004; Rund & Rachmilewitz, 2005), or glucose-6-Phosphate dehydrogenase deficiency (Cappellini & Fiorelli, 2008). Chapter 1.1.5 starts the aetiology discussion with several nutritional factors and the haemoglobinopathies that trigger anaemia development; however, the choice is not comprehensive, and there are more nutrients (iodine, folic acid, and other B-complex vitamins) that play a role but they are not in the focus of the present thesis. In chapter 1.1.7, the influence of a series of infectious diseases and the subsequent inflammation of anaemia is presented.

1.1.5 Nutritional deficiencies and haemoglobinopathies

Iron
The estimated proportion of anaemia attributable to iron deficiency has repeatedly numbered to be approximately 50%, likely due to the widely cited publication by De Maeyer and Adiels-Tegman (1985). In developed countries, where concomitant nutritional deficiencies or infections are less frequent, the contribution of ID to anaemia is higher. It is likely to be lower in the context of less developed countries, and to vary between different population groups. Contrasting evidence to this statement was presented a few years ago for West Africa, where an approximate relative contribution of 50% of ID to anaemia was estimated for school children (Staubli Asobayire et al., 2001). Stoltzfus questions this estimate that half of the anaemia is due to ID in a comment and a review: as an example, in pre-school and school children from Vietnam and Bolivia, over 80% of anaemia is attributable to ID, but among adolescents from Mali, only 20% could be attributed to ID (Stoltzfus, 2001; Stoltzfus et al., 2004). Similarly low percentages were found in Thai schoolchildren (Thurlow et al., 2005), but in Asia, hereditary haemoglobin disorders are very common.

In ID, the adequate formation of haem is prevented and this results in small, poorly haemoglobinised erythrocytes (Lilleyman, 1991). In a first stage, well prior to the onset of iron-deficiency anaemia, sub-clinical ID develops with iron store depletion
and subsequent iron-deficient erythropoiesis and only if pronounced, anaemia may result (Cook, 1999b). Iron is not only important as a major component in haem and thus for oxygen transport, but also functions as an enzyme cofactor and participates in the redox processes in energy metabolism (Beard, 2001).

An important cause of ID and IDA is insufficient uptake of iron due to low consumption or decreased absorption due to absorption inhibitors or infection. The influence of infection on iron metabolism is dealt with in detail in chapter 1.3 and thus, only nutritional factors are discussed here.

Dietary iron is consumed in two forms, heme iron and non-heme iron. The first originates from meat, poultry and fish, and its absorption pathway is different from that of non-heme iron (see chapter 1.3), and is hardly affected by iron absorption inhibitors (Hallberg, 1981). Absorption of non-heme iron - mainly from plant and dairy products and as fortification iron - on the other hand is influenced by its chemical form, the physiological iron status of the individual and the composition of the meal (Hurrell, 2002d; Moretti et al., 2006). Important enhancers of non-heme iron absorption are ascorbic acid and the protein fraction of muscle tissue, but inhibitors can counteract the positive effect; the most prominent ones are phytic acid, polyphenols and calcium (Lopez & Martos, 2004). Table 4 indicates the recommended nutrient intake of iron for children and adolescents. Of note, the above discussed factors to influence absorption are taken into account by introducing different levels of bioavailability estimations of 15%, (12%), 10% and 5% bioavailability.

Table 4: Recommended nutrient intakes of iron for children and adolescents, depending on bioavailability estimations of 15%, 10% and 5% (WHO/FAO, 2004)

<table>
<thead>
<tr>
<th>Population group</th>
<th>Age range [y]</th>
<th>Recommended nutrient intakes (RNI) [mg]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>15%</td>
</tr>
<tr>
<td>Children</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males, adolescent</td>
<td>11-14</td>
<td>9.7</td>
</tr>
<tr>
<td></td>
<td>15-17</td>
<td>12.5</td>
</tr>
<tr>
<td>Females, pre-menarche</td>
<td>11-14</td>
<td>9.3</td>
</tr>
<tr>
<td>Females, adolescent</td>
<td>11-14</td>
<td>21.8</td>
</tr>
<tr>
<td></td>
<td>15-17</td>
<td>20.7</td>
</tr>
</tbody>
</table>
To set the level of bioavailability, assumptions have to be made, but the best option is to conduct nutrition surveys and from there to calculate bioavailability using existing equations (Monsen & Balintfy, 1982; Tseng et al., 1997; Hallberg & Hulthen, 2000; Reddy et al., 2000).

Riboflavin

Riboflavin (vitamin B₂) is found predominantly in meat and dairy products, and to a lesser extent in fruit and vegetables (Powers, 2003) and thus, its deficiency is particularly common in regions and population groups where intakes of dairy products and meat are low (Neumann et al., 2003). Recommended nutrient intakes are: children 1-3 y (0.5 mg/d), 4-6 y (0.6 mg/d), 7-9 y (0.9 mg/d), female adolescents 10-18 y (1.0 mg/d), male adolescents 10-18 y (1.3 mg/d) (WHO/FAO, 2004).

For SSA, studies on riboflavin intakes in children have generally reported low intakes (Faber et al., 2001; Labadarios et al., 2005; Wegmuller et al., 2006) or adequate intakes (Kigutha, 1997; Vahatalo et al., 2005), and this is mostly reflected in studies where riboflavin was biomedically assessed. In Botswana 33-40%, in Kenya 24-34% and in the Gambia virtually all schoolchildren were deficient (no prevalence data), whereas in Côte d'Ivoire, 66% suffered from mild deficiency (Bates et al., 1994; Abrams et al., 2003; Siekmann et al., 2003; Wegmuller et al., 2006). One study questions all the above results because of methodological aspects (see 1.2.2) and found no riboflavin deficiency in malaria infected schoolers from Gabon (Traunmuller et al., 2003).

Due to its implication in many metabolic pathways, usually as a precursor of the flavin coenzyme (NAD) in redox reactions (McCormick, 1997), riboflavin deficiency has various consequences, such as impaired growth, cheilosis, angular stomatitis, glossitis, dermatitis, and impaired vision (Venkataswamy, 1967; Batey et al., 1992; FAO/WHO, 2001).

The role of riboflavin in the onset of anaemia was first described in animal experiments, where a marked decrease in erythrocytes was observed (Foy & Kondi, 1953; Foy et al., 1964), but up to date, the underlying mechanisms are debated. The main mechanisms derived from animal studies are i) decreased mobilisation of storage iron (Sirivech et al., 1974; Powers, 1995), ii) decreased iron absorption or
increased iron losses (Adelekan & Thurnham, 1986; Powers et al., 1988; Butler & Topham, 1993; Yates et al., 2001); iii) impaired iron utilisation in erythropoiesis (Powers, 1986; Powers et al., 1991). Riboflavin supplements given with iron improved the response to iron supplementation in pregnant and lactating women, adult males and school children (Buzina et al., 1979; Charoenlarp et al., 1980; Ajayi et al., 1990), but no enhanced positive effect was found in other studies within similar population groups (Decker et al., 1977; Powers et al., 1983; Powers et al., 1985). In adult men, a stable isotope study showed a greater increase in Hb with riboflavin/iron compared to iron only supplementation, but failed to demonstrate an increase in iron absorption, giving support to the mechanism that improvement in Hb may have been due to iron mobilization from liver stores (Fairweather-Tait et al., 1992). Alternatively, this effect could be due to stimulated globin production, as observed in a clinical study after riboflavin supplementation; a clear increase in conversion of vitamin B$_6$ was observed after riboflavin administration, which in turn increased α- and β-chain synthesis (Perry et al., 1980). Riboflavin is further involved in enzyme activities in the respiratory chain and the Krebs cycle (NADH dehydrogenase, succinate dehydrogenase) and thus, a certain influence on the iron metabolism can be postulated (Brody, 1999).

With the rather conflicting results from different studies, an estimation of the contribution of riboflavin deficiency to anaemia would be purely speculative and is thus not performed; however, evidence for a positive correlation between riboflavin status and malaria parasitaemia intensity exists (Dutta et al., 1986; Shankar, 2000), but mechanisms remain to be elucidated.
Literature review

**Vitamin A**

Vitamin A is important in the retina for vision, in particular for vision during low light hours (Sommer, 1995); for protein synthesis regulation affecting cell growth, differentiation and function (Ross, 1999); for immune function and reproduction (Underwood, 2004). As a consequence, its deficiency has been associated with a series of consequences such as increased severity of measles, malaria, diarrhoea, impaired growth, vision-related diseases (xerophthalmia), and anaemia (Barclay et al., 1987; Barreto et al., 1994; Sommer, 1996; West et al., 1997; Shankar, 2000; Cox et al., 2005).

Recommended nutrient intakes for vitamin A are: children 1-3y (400 μg RE/d), 4-6y (450 μg RE/d), 7-9y (500 μg RE/d), female adolescents 10-18 y (600 μg RE/d), male adolescents 10-18 y (600 μg RE/d) (WHO/FAO, 2004).

Vitamin A and its precursors of carotenoids occur in liver, dairy products, animal and to a lesser extent vegetable fats, yellow fruits and dark green leafy vegetables (Food and Nutrition Board, 2001). The prevalence of vitamin A deficiency (VAD) thus depends on the diet consumed. Rice-consuming societies are susceptible to VAD, but other populations with little consumption of dairy products are also at risk. In SSA, prevalence rates vary markedly; this depends not only on the population group but also on the methods used to assess vitamin A status (see chapter 1.2.3); Table 5 represents region estimates for SSA, as based on the WHO database on VAD (WHO, 2006a). The diversity of survey level (local, regional, state-wide, national), assessment method and age range complicate an interpretation, but in general, it can be stated that schoolchildren in Western Africa are likely to have a smaller risk of becoming VAD; this might be due to the high intakes of palm oil or other palm kernel products, since they contain considerable amounts of retinol-equivalents (Edem et al., 2002).
Table 5: prevalence estimates for vitamin A deficiency (VAD) among school-age children in SSA
(PSO, 2006a)

<table>
<thead>
<tr>
<th>Country</th>
<th>Survey level</th>
<th>n subjects</th>
<th>Age range [y]</th>
<th>Mean sR [g/L] (SD)</th>
<th>VAD prevalence [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Botswana</td>
<td>District</td>
<td>214</td>
<td>7-12</td>
<td>0.8 (0.4)</td>
<td>&lt;0.7: 47.2; &lt;0.35: 15.0</td>
</tr>
<tr>
<td>Burkina Faso</td>
<td>Regional</td>
<td>656</td>
<td>6-11</td>
<td>-</td>
<td>XN: 0.0-8.7; XB: 0.0-3.8</td>
</tr>
<tr>
<td>Cameroon</td>
<td>Local</td>
<td>231</td>
<td>6-16</td>
<td>-</td>
<td>&lt;0.7: 82.5; &lt;0.35: 6.5</td>
</tr>
<tr>
<td>Chad</td>
<td>State</td>
<td>665</td>
<td>6-11</td>
<td>-</td>
<td>XN: 1.7</td>
</tr>
<tr>
<td>Comoros</td>
<td>National</td>
<td>3537</td>
<td>6-12</td>
<td>-</td>
<td>XN: 1.9</td>
</tr>
<tr>
<td>Cote d’Ivoire</td>
<td>Local</td>
<td>152</td>
<td>5-16</td>
<td>1.2 (0.4)</td>
<td>&lt;0.7: 6.6</td>
</tr>
<tr>
<td>Djibouti</td>
<td>National urb.: 83</td>
<td>4-11</td>
<td>-</td>
<td>&lt;0.7: 21.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rur.: 114</td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.7: 12.3</td>
</tr>
<tr>
<td>Ethiopia</td>
<td>District</td>
<td>390</td>
<td>8-19</td>
<td>-</td>
<td>XB: 1.0</td>
</tr>
<tr>
<td>Gambia</td>
<td>Local</td>
<td>83</td>
<td>5-7</td>
<td>0.6 (0.0)</td>
<td>-</td>
</tr>
<tr>
<td>Kenya</td>
<td>Local</td>
<td>442</td>
<td>6-15</td>
<td>-</td>
<td>&lt;0.7: 90.6; &lt;0.35: 22.0</td>
</tr>
<tr>
<td>Malawi</td>
<td>National f: 308</td>
<td>6-13</td>
<td>0.8</td>
<td>&lt;0.7:41.1; &lt;0.7: 35.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>m: 295</td>
<td></td>
<td></td>
<td></td>
<td>0.9</td>
</tr>
<tr>
<td>Mali</td>
<td>State</td>
<td>538</td>
<td>6-11</td>
<td>-</td>
<td>XN: 9.5; XB: 0.2</td>
</tr>
<tr>
<td>Mozambique</td>
<td>Local</td>
<td>985</td>
<td>5-20</td>
<td>-</td>
<td>XN: 0.0</td>
</tr>
<tr>
<td>Niger</td>
<td>State</td>
<td>611</td>
<td>6-11</td>
<td>-</td>
<td>XN: 4.8; XB: 0.7</td>
</tr>
<tr>
<td>United Rep. of Tanzania</td>
<td>Local</td>
<td>562</td>
<td>6-16</td>
<td>-</td>
<td>XN: 9.1; XB: 0.5</td>
</tr>
<tr>
<td>Zambia</td>
<td>District</td>
<td>126</td>
<td>7-16</td>
<td>1.1 (0.3)</td>
<td>&lt;0.35: 0.0</td>
</tr>
<tr>
<td>Zimbabwe</td>
<td>National</td>
<td>657</td>
<td>6-15</td>
<td>1.0</td>
<td>&lt;0.7: 18.0; &lt;0.35: 2.0</td>
</tr>
</tbody>
</table>

<0.7 and <0.35 are respective serum retinol values in μMol/L for moderate and severe VAD; XN: night blindness, considered a public health problem in children <5 y, if prevalence >1%; XB: Bitot’s spots (keratin debris located superficially in the conjunctiva), considered a public health problem in children <5 y, if prevalence >0.5%; f: female; m: male; rur.: rural; urb.: urban

Evidence that vitamin A status impacts on anaemia was first obtained from observational studies, where an increased Hb was associated with higher vitamin A status (West et al., 2007), and subsequently from intervention trials, where it was discovered that concomitant supplementation with iron and vitamin A increased the response to iron status more markedly than iron supplements alone (Suharno et al., 1993; Ahmed et al., 2001; Semba & Bloem, 2002), and that anaemia can develop despite adequate iron supply if insufficient vitamin A is consumed (Hodges et al., 1978). Suggested underlying mechanisms for the contribution of VAD to anaemia are
various and equivocal due to findings in animal studies that could not consistently be confirmed in humans; they include compromised iron absorption (Layrisse et al., 1997), storage, transport and mobilization (Bloem, 1995; Fishman et al., 2000), reduced erythropoiesis (Roodenburg et al., 2000; Zimmermann et al., 2006a), or increased iron sequestration due to increased susceptibility to or intensity of infection (Wintergerst et al., 2007). Recently, evidence has been presented that iron absorption is maybe not decreased in VAD (Walczyk et al., 2003), but that the effect is rather on the side of hepatic iron mobilisation and erythropoiesis (Evans, 2005; Zimmermann et al., 2006a), although this statement is controversial (Garcia-Casal et al., 1998). In infants in a highly infection endemic area, iron absorption was even found to be reduced, if the diet was simultaneously supplemented with retinyl-palmitate (Davidsson et al., 2003).

Haemoglobinopathies

Congenital Hb disorders can be regrouped into those with an altered rate of globin chain synthesis and those with a modified structure of the globin chains. The first group is referred to as thalasaemias, whereof α- and β-thalasaemia are clinically the most relevant, although for SSA, almost exclusively α-thalasaemia is of significance (Serjeant, 1992). The second group is termed sickling disorders with many different forms, but the most common and clinically most important is HbS. In the text section, only epidemiologically relevant disorders in SSA are briefly discussed.

Alpha-thalassaemia is caused by the deletion of one or more of the originally four α-genes, which in turn leads to instability of haemoglobin and ineffective erythrocyte synthesis (Johnston, 2005). In Africans, the mild form of α+ - thalassaemia is relatively widespread, but in most cases this form has no clinically manifest consequences, except for mild hypochromic, microcytic anaemia (Serjeant, 1992). Subjects affected by α+ - thalassaemia stand a reduced risk of HbS traits (Steinberg & Embury, 1986).

Sickling red cells result from a protein substitution on the β-chain of adult HbA; subsequently, this modification changes the behaviour of the now termed HbS, rendering the red cells less pliable and deforming them into the sickle shape. The clinically relevant form is the homozygote of abnormal β-globins, HbSS. Carriers of the homozygous form suffer from various consequences and life expectancy is
markedly reduced; increased haemolysis causes anaemia per se, but occlusion of blood vessels due to HbS polymerisation, acute chest syndromes, aplastic crisis are severe diseases present in these subjects (Stuart & Nagel, 2004). Heterozygotes are less likely to suffer from obvious disability, if the other trait is HbA (resulting in HbAS); however, if the counterpart is HbC, another haemoglobin variant, the resulting HbSC causes similar clinical problems to sickle cell disease. The homozygous HbCC genotype suffers from a mild haemolytic anaemia (Serjeant, 1992). HbF, as HbAF, is predominant during foetal phase, but is due to disappear in the first years of life. Hereditary persistence results in HbAF, or HbSF, if a heterozygous sickle-cell trait is present. These subjects are normally not anaemic.

Table 6: haemoglobinopathies of significance in SSA and their impact on anaemia (Serjeant, 1992; WHO, 2006d)

<table>
<thead>
<tr>
<th>Type</th>
<th>Prevalence in SSA</th>
<th>Impact on anaemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>HbSS</td>
<td>1-2% in tropical Africa</td>
<td>+++</td>
</tr>
<tr>
<td>HbSC</td>
<td>regional, Ghana ~ 20%</td>
<td>+++</td>
</tr>
<tr>
<td>HbAS</td>
<td>15-30% in West Africa</td>
<td>(+)</td>
</tr>
<tr>
<td></td>
<td>0-45% in Central/East Africa</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0-5% in South Africa</td>
<td></td>
</tr>
<tr>
<td>HbAC</td>
<td>?</td>
<td>0</td>
</tr>
<tr>
<td>HbAF</td>
<td>?</td>
<td>? (negative correlation?)</td>
</tr>
<tr>
<td>HbCC</td>
<td>?</td>
<td>+</td>
</tr>
<tr>
<td>HbSF</td>
<td>?</td>
<td>(+)</td>
</tr>
<tr>
<td>α + -thalassaemia</td>
<td>~ 30% (heterozygous)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>~ 3-4% (homozygous)</td>
<td>0-(-+)</td>
</tr>
<tr>
<td>α -thalassaemia</td>
<td>extremely rare</td>
<td>++</td>
</tr>
<tr>
<td>β-thalassaemia</td>
<td>&lt;1% in Nigeria, Lagos</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>&lt;2% in Ghana</td>
<td></td>
</tr>
</tbody>
</table>

?: information not available/controversial results; 0: no effect; (+): weak effect; +: moderate effect; ++: strong effect; +++: very strong effect

Table 6 indicates the estimated prevalences of haemoglobinopathies in SSA and their relevance with regard to anaemia. The stronger the effect, the more negatively associated the disease is with Hb.
1.1.6 Infectious diseases

*Malaria*

An estimated number of up to 500 million individuals are infected with malaria each year (Snow *et al.*, 2005), with a total number of people at risk for infection with *Plasmodium falciparum* malaria of 2.5 billion (Guerra *et al.*, 2006). The endemicity of malaria is restricted to the tropics and subtropics, with over 70% of clinical episodes due to *Plasmodium falciparum* occurring in Africa (Hay *et al.*, 2004), and over 80% of the global burden concentrated in sub-Saharan Africa (WHO, 2004c). Other *Plasmodium* species are of less significance in SSA, but can be of importance for instance in Asia. The three other species regularly found in SSA are *P. malariae*, *P. ovalae* and *P. vivax* (Guerra *et al.*, 2006); due to the wide occurrence of *P. falciparum*, the review is focused on this species.

Pathogenic factors involved in malarial anaemia are not fully understood, but two different clinical patterns in the course of malaria emerge: i) clinical malaria, including complicated or severe malaria, where the biggest contribution of the disease to the onset of anaemia is likely to be due to haemolysis of infected and abnormal removal of uninfected erythrocytes (referred to as "symptomatic"), although it is debated that a reduced erythroid response might also contribute considerably (Jakeman *et al.*, 1999; Lamikanra *et al.*, 2007); ii) malaria parasitaemia (asymptomatic), where more subtle changes can lead to anaemia of inflammation (see chapter 1.1.7) or to alterations in iron metabolism (Ghosh, 2007). It is estimated that (severe) symptomatic malaria contributes to 18% of childhood deaths in SSA (Rowe *et al.*, 2006) and causes pregnancy complications (Greenwood, 1997). Its contribution of milder forms to long-term anaemia in schoolage children may be of relevance, but is less well described (Reyburn *et al.*, 2005). Due to the large numbers of asymptomatic cases in this age group, the relative contribution to anaemia might be of importance. For Tanzanian young children and pregnant women, an attempt to quantify the contribution of asymptomatic malaria to anaemia was undertaken and estimated that 3-4% of severe anaemia cases are due to the parasites (Carneiro *et al.*, 2006).
Since pathways leading to anaemia overlap for the acute and the asymptomatic form, the broader picture for both clinical patterns is presented here. Figure 1 summarizes the possible direct and indirect mechanisms of malaria to the onset of anaemia (Lamikanra et al., 2007). Although the authors discuss severe symptomatic malaria, most of the alterations also occur in asymptomatic malaria in a less pronounced manner (Ghosh, 2007).

Figure 1: proposed mechanisms of malarial anaemia (Lamikanra et al., 2007). Destruction of infected erythrocytes is a major consequence of parasite invasion; after subsequent schizogony (asexual reproductive process), both infected and uninfected erythrocytes are cleared by the macrophages (MΦ) to the spleen. Pigment-containing macrophages may release cytokines and other biological mediators (Tumor-Necrosis-Factor-α [TNF-α], Inteleukin-10 & 12 [IL], Hydroxy-Nonenal [HNE]), impacting negatively on erythropoiesis and EPO-response. Together with other parasite products, erythropoiesis is disturbed. Evidence levels: yellow boxes from mouse and human studies, pink from mouse studies, blue from human trials. GPI, glycophosphatidylinositol; HZ, hemozoin, both catabolites of the malaria parasite.

The following mechanisms have been proposed to lead to decreased Hb values: i) loss of infected erythrocytes through parasite maturation and recognition by macrophages (Casals-Pascual & Roberts, 2006); ii) loss of uninfected erythrocytes due to reduced half-life and increased level of macrophages (Omodeo-Sale et al.,
2005); iii) perturbed (reduced) erythropoiesis as an indirect cause of increased macrophage levels and acute-phase response (Howard et al., 2007) or due to metabolic side-products from haemoglobin destruction, e.g. haemozoin (Giribaldi et al., 2004); iv) functional ID as a consequence of increased macrophage levels or acute-phase responsive elements, and increased iron demand of the parasites (Oppenheimer, 1989).

Soil-transmitted helminths

Infections with soil-transmitted helminths (Ascaris lumbricoides, hookworm [Necator americanus, Ankylostoma duodenale] and Trichuris trichiura) are highly endemic across SSA (de Silva et al., 2003; Horton, 2003). For each of the three soil-transmitted helminths (STH), the estimated number of people affected ranges from 160 to 200 million cases for SSA alone, with over a billion persons affected worldwide (Bethony et al., 2006). These infections are predominant in tropical and sub-tropical regions, in particular of the developing world, where adequate water, sanitation and concrete floors are lacking, and where climate is appropriate for parasite survival (Brooker et al., 2006).

Of note, infection intensity often follows a certain age pattern, with peaking intensity in school-age to adolescent children for T. trichiura and A. lumbricoides (Galvani, 2005), whereas for hookworms, the peak may be shifted to individuals of 20 to 25 years (Stephenson, 1987; Hotez et al., 2004). In a recent study, Brooker and colleagues (2007) estimated the number of school-going children in SSA infected with hookworm, T. trichiura, and A. lumbricoides, and found numbers of 45, 30, and 25 million, respectively; however, these numbers cannot be added up, since co-infection is likely to occur. Data from Côte d'Ivoire suggests around 30 to 55% of school-aged children be affected by hookworms (Raso et al., 2004; Matthys et al., 2007).

The burden of STH infection depends on intensity of infestation, and many observational studies found an association between STH and Hb (Saint-Martin & Dussault, 1957; Stephenson, 1987; Stoltzfus et al., 1997b; Albonico et al., 1998; Dreyfuss et al., 2000; Stoltzfus et al., 2000). In schoolchildren, anthelmintic treatment with albendazole or mebendazole gave generally positive but very mixed results, but
anaemia prevalence and iron status at baseline varied considerable (Robertson et al., 1992; Bhargava et al., 2003). In a regional deworming programme with mebendazole, a non-significant decrease in Hb was found if anthelmintics were given only twice a year; however, only a non-significant Hb increase resulted, if anthelmintics were given three times (Stoltzfus et al., 1998). Overall, it can be stated that treatment of hookworms, *T. trichiura*, and co-infections thereof has a weak but intensity-dependent relationship to anaemia (Bates et al., 2007; Gulani et al., 2007).

With regard to hookworms, two mechanisms can cause anaemia: the direct pathological consequence is intestinal blood loss; as a consequence of infestation, chronic inflammation may lead to the onset of anaemia of inflammation, which is discussed in further detail in chapter 1.1.7 (Loukas et al., 2005). Severe hookworm infection may result in loss of appetite and generalized mal-/undernutrition (Stephenson et al., 2000). Blood loss results from the attachment of adult parasites to the mucosa and sub-mucosa of the small intestine (Hotez et al., 2004). The onset of anaemia is not only dependent of infection intensity but also on host iron status and physiological requirement (Diemert et al., 2008) and thus, the number of adult parasites required to cause IDA varies. Calculations from the WHO assume that 1000 eggs per gram feces convert into worm loads of 11 and 32 for *A. duodenale* and *N. americanus*, respectively, and as a consequence into daily blood losses of 2.2 and 1.3 mL or 1.1 and 0.65 mg iron (World Health Organization, 1991). These values in turn would result in a theoretical daily loss (assuming no compensation by the iron metabolism) of 0.32 g Hb for *A. duodenale* and of 0.19 g Hb for *N. americanus* (Braunitzer, 1964). It is clear that the disease burden not only depends on host iron status but also on parasite species.

**Schistosomiasis**

Globally, 779 million individuals are estimated to be at risk of schistosomiasis, whereof over 200 million are infected, 120 million are symptomatic and 20 million suffer from the severe disease (Chitsulo et al., 2000). Most of the present schistosomiasis burden is concentrated to (sub-Saharan) Africa with over 160 million cases (Fenwick, 2006), and due to age-dependent infection patterns, highest
prevalences and intensities are found in school-age children and adolescents (Woolhouse, 1998). For Côte d'Ivoire, 5.6 million individuals were calculated to be infected, representing 40% of the population (Raso et al., 2004), a prevalence that has been confirmed by recent regional estimates (Steinmann et al., 2006).

Since schistosomiasis is a waterborne disease, its distribution patterns are closely related to proximity of water sources and behaviour that favours contact with infected water and hence, local distribution may vary considerably (Chitsulo et al., 2000). For a small area in Western Côte d'Ivoire, infection distribution was mapped, where this strong variation in infection patterns is visible (Raso et al., 2006). There are various species of schistosomes, but for SSA of particular interest are S. mansoni (Sm), S. haematobium (Sh) and, to a lesser extent, S. intercalatum. The two main species Sm and Sh are affecting different organs with regard to the onset of anaemia, despite similar life cycles: adult worms of Sm deposit their eggs in the large bowel, whereas Sh discharge them into the bladder. Due to their passage through skin and lung and maturation phase in the liver, acute manifestations such as fever and skin rash are common; chronic consequences include diseases of liver and gastrointestinal (Sm) or urinary tract (Sh; Ross et al., 2002). The relationship between schistosomiasis and anaemia has recently been reviewed by Friedman et al. (2005). Observational studies are limited in that many confounders weaken outcomes, so that they might be masked. Thus, for Sm, observational studies could not establish an association (Sturrock et al., 1996). In contrast, one randomized trial identified an increase of 2.1 g/L in Hb 8 months after single administration of albendazole/praziquantel (Friis et al., 2003); other studies using combined chemotherapy against schistosomes and hookworms found a more pronounced impact, but there, effect separation is impossible, in particular since treatment efficacy was more pronounced for hookworms (Koukounari et al., 2006). In the case of Sh, the situation is similar for randomized trials, where concomitant treatment is the norm rather than the exception. One trial with a complicated baseline pattern and study design found no clear association (Olds et al., 1999). Observational studies claimed an association between Hb, iron status and infection intensity (Prual et al., 1992; Tatala et al., 1998). A recent meta-analysis looked quantitatively into the effects of i) schistosomiasis infection; ii) schistosomiasis treatment; iii) schistosomiasis infection intensity on anaemia. It found for almost all groups consistent associations: i) lowered Hb in infection, ii) increased Hb after treatment, iii) intensity dependent decrease of Hb
(King et al., 2005). For Sh, Stephenson et al. calculated a infestation-intensity dependent blood loss of 0.6 to 1.3 mL/d (Stephenson et al., 1985); this range converts to 0.3 – 0.65 mg iron loss/d and would – again assuming no homeostatic interactions – translate into a Hb decrease of 0.09 – 0.18 g Hb. Blood loss is thus one of the possible mechanisms leading to anaemia. Sm may cause splenomegaly, which decreases half-life, likely due to autoimmune haemolysis, and splenic sequestration of erythrocytes (Mahmoud & Woodruff, 1972; Friis et al., 2003). Similar to the STH, schistosomes cause infection that leads to anaemia of inflammation; increased acute-phase proteins and cytokines were observed for S. japonicum, but wait to be confirmed for other species (Coutinho et al., 2005). The phenomenon of anaemia of inflammation will be discussed below.

1.1.7 Anaemia of inflammation

Anaemia of inflammation, also termed anaemia of chronic disease, is one form of anaemia that develops as a consequence of altered iron metabolism, which can originate from abnormal iron absorption, distribution, and utilisation (reduced erythrocyte half-life, and impaired erythropoiesis; Weiss, 2002). Thus, the anaemia of inflammation will be discussed in the chapter 1.3 “iron-infection interactions”.

1.2 Assessment of nutritional and parasitaemic status

1.2.1 Iron status

As discussed above, global estimates of ID are often based on Hb values, assuming that half of the anaemia is due to ID. However, the assessment of ID, in particular in SSA is more complicated than this, because various stages of iron depletion occur before the onset of anaemia and because inflammation complicates the diagnosis. This chapter is structured in two parts: assessment of iron status in healthy school-age children, and particularities in the setting of endemic infections. With regard to the below discussions, Table 7 gives an overview of the assessment methods. It indicates normal values and those signalling different levels of ID, respectively.

Iron stores

Iron stores are affected in an early stage of ID, when no clinical signs have developed yet. The gold standard for the assessment of iron stores is the bone
marrow aspiration (haemosiderin), but due to the invasiveness, its use is only justified in patients presenting with clinical symptoms (Cook, 1994).

Table 7: choice of parameters and threshold values for assessment of different stages of iron deficiency in children 5 to 14 y old [modified from (Pippard, 1996)]. Parameters in dark grey have lost of their importance, partly due to the development of newer assessment methods. The grey shaded areas indicate the stage at which the parameters of concern begin to be altered.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal</th>
<th>Depleted</th>
<th>Fe-deficient</th>
<th>IDA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron stores</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bone marrow examination</td>
<td>present</td>
<td>trace</td>
<td>absent</td>
<td>absent</td>
</tr>
<tr>
<td>sF [µg/L]</td>
<td>15-300</td>
<td>15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>TIBC* [µmol/L]</td>
<td>45-70</td>
<td>70</td>
<td>75</td>
<td>&gt;75</td>
</tr>
<tr>
<td>Tissue iron supply</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>serum iron [µmol/L]</td>
<td>10-30</td>
<td>20</td>
<td>&lt;10</td>
<td>&lt;7</td>
</tr>
<tr>
<td>Tf saturation (%)</td>
<td>35&lt;sup&gt;h&lt;/sup&gt;</td>
<td>30&lt;sup&gt;h&lt;/sup&gt;</td>
<td>&lt;15&lt;sup&gt;h&lt;/sup&gt;</td>
<td>&lt;15&lt;sup&gt;h&lt;/sup&gt;</td>
</tr>
<tr>
<td>TIBC saturation [%]</td>
<td>16-60</td>
<td>30</td>
<td>&lt;16</td>
<td>&lt;10</td>
</tr>
<tr>
<td>sTfR [mg/L]</td>
<td>2.8-8.5</td>
<td>≤8.5</td>
<td>≤8.5</td>
<td>&gt;8.5</td>
</tr>
<tr>
<td>marrow sideroblasts [%]</td>
<td>30-50</td>
<td>30-50</td>
<td>≤10</td>
<td>&gt;10</td>
</tr>
<tr>
<td>ZPP [µmol/mol haem]</td>
<td>&lt;40&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&lt;40&lt;sup&gt;b&lt;/sup&gt;</td>
<td>≥ 40&lt;sup&gt;b&lt;/sup&gt;</td>
<td>≥ 40&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Erythrocyte production</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>haemoglobin [g/L]</td>
<td>5-11 y: &gt;115&lt;sup&gt;a&lt;/sup&gt;</td>
<td>do.&lt;sup&gt;a&lt;/sup&gt;</td>
<td>do.&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5-11 y: ≤115&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>12-14 y: &gt;120</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>haematocrit [L/L]</td>
<td>40&lt;sup&gt;g&lt;/sup&gt;</td>
<td>40&lt;sup&gt;g&lt;/sup&gt;</td>
<td>40&lt;sup&gt;g&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>mean corpuscular volume [fl]</td>
<td>80-92</td>
<td>80-92</td>
<td>80</td>
<td>&lt;80</td>
</tr>
<tr>
<td>mean red cell</td>
<td>27-32</td>
<td>27-32</td>
<td>27</td>
<td>&lt;27</td>
</tr>
<tr>
<td>haemoglobin [pg]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Continuous values</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sTfR/log(sF) ratio</td>
<td>&lt;100&lt;sup&gt;d&lt;/sup&gt;</td>
<td>&lt;500&lt;sup&gt;d&lt;/sup&gt;</td>
<td>&lt;2000&lt;sup&gt;d&lt;/sup&gt;</td>
<td>≥2000&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>[µg/µg]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>body iron (stores) [mg/kg]</td>
<td>&gt;5&lt;sup&gt;e,f&lt;/sup&gt;</td>
<td>0&lt;sup&gt;e&lt;/sup&gt;</td>
<td>&lt;0&lt;sup&gt;e&lt;/sup&gt;</td>
<td>&lt;0&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*total iron-binding capacity

<sup>a</sup> (WHO/UNICEF/UNU, 2001); <sup>b</sup> on washed erythrocytes (Hastka et al., 1994); <sup>c</sup> on unwashed erythrocytes (Punnonen et al., 1997; WHO/UNICEF/UNU, 2001); <sup>d</sup> (Baynes, 1996); <sup>e</sup> (Cook et al., 2003); <sup>f</sup> suggested value for adult Caucasian population; <sup>g</sup> (Weatherall, 1996); <sup>h</sup> (Herbert, 1987).
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Since the sF concentration is proportional to stainable marrow iron in healthy individuals, it indicates iron store depletion; the threshold for children >5 years of age is below 15 µg/L (WHO/UNICEF/UNU, 2001); other cut-off values have been discussed (for adults), e.g. 30 µg/L, for improved sensitivity and specificity (Mast et al., 1998), but so far, there is not enough population-based evidence to support this threshold in healthy subjects.

Measurement of sF is becoming more and more routine, but until now, remains costly and larger amounts of blood (i.e. venipuncture) are still required. sF is often determined using immunoradiometric (IRMA) or enzyme-linked immunosorbent (ELISA) assay. Efforts to decrease cost and field applicability have been undertaken but to date, they have not been used on larger scale (Ahuwalia et al., 1998; Cook et al., 1998; Erhardt et al., 2004). Despite sF currently being the most important and widely used indicator for early ID, its biggest limitation in the context of SSA is its function as acute-phase protein and subsequently, elevated values leading to false-negative diagnosis, e.g. (Beutler et al., 2003). TIBC (total iron-binding capacity) is of less importance due to diurnal variation and the overlap between ID and iron sufficient populations (Institute of Medicine, 2001).

Tissue iron supply

At the stage of iron-deficient erythropoiesis, similar limitations apply to serum iron, transferrin saturation, and TIBC saturation: diurnal variation (WHO/UNICEF/UNU, 2001), interindividual variation (Worwood, 1997), and in the case of transferrin saturation (defined as the ratio of serum iron: TIBC), lack of specificity, e.g. in the case of anaemia of chronic disease (Cook, 1999a). Marrow sideroblasts comprise the same problem as bone marrow stains: their investigation requires marrow invasion. Zinc protoporphyrin (ZPP) is reflective of insufficient iron supply for Hb synthesis: due to a lack of available iron, other divalent metals, mainly zinc, are incorporated into the protoporphyrin ring; fluorescence of the protoporphyrin is dramatically reduced, if iron is incorporated, whereas it is unaffected by zinc (Hastka et al., 1992). Susceptibility to inflammation, lead poisoning and lack of consensus for methodology/threshold are of concern in this parameter (Hastka et al., 1993; Zimmermann et al., 2006b; Counter et al., 2007). Two principal methodological approaches with subsequent different cut-off values exist: fluorometric analysis on unwashed or washed erythrocytes. Currently, the analysis on unwashed cells by
Literature review

fluorometry is the WHO recommended approach, but debates on measurement interference have been described (Garrett & Worwood, 1994), including acute-phase proteins. In the context of chronic infection, equivocal findings are reported, ranging from no influence of (asymptomatic) malaria on ZPP in school-children (Stoltzfus et al., 1997a) or samples from adults (Hiremath et al., 2006) to a generalized influence of inflammation (WHO/UNICEF/UNU, 2001). Advantages of this method are that it is relatively cheap (except apparatus cost) and, if analysis on the whole blood proves to be valid for different settings, the ease of field applicability, since no venous blood and no centrifuge are required.

Transferrin receptor (TfR) regulates the uptake of transferrin iron to cells, and is thus produced in proportion to the body’s iron requirement in order to compete more effectively for the reduced availability of iron (Cook et al., 1994). Currently, manual or automated assays using monoclonal antibodies or enzyme-linked immunsorbent assays are the most widely used (Cook, 1999b). Besides the high analysis cost, a major limitation of sTfR assays is the lack of an internationally certified reference material, which makes comparison of different assays difficult. This is one of the reasons, why WHO has not issued cut-off values in their recently published guidelines (WHO, 2006b). On the other hand, the advantage of sTfR that has been discussed is its non-responsiveness to inflammation (Ferguson et al., 1992), although, for asymptomatic malaria, a certain influence has been observed in infants (Stoltzfus et al., 2000; Menendez et al., 2001; Verhoef et al., 2001).

Erythrocyte production

Haemoglobin (Hb) is still the most widely used parameter to assess iron status at this late stage, although for most of the world’s regions, this approach for diagnosis of ID cannot be justified, as discussed in section 1.1.5. It is a very important parameter from the public health point of view, but for diagnosing anaemia rather than ID. The reason for its use is the uncomplicated but sufficiently accurate diagnose, nowadays directly in the field using a Hemocue® (Burger & Pierre-Louis, 2003) or in a relatively simple laboratory setting for the cyanmethaemoglobin-method requiring a spectrophotometer (National Committee for Clinical Laboratory Standards, 2000). Studies comparing the Hemocue® method with others such as the direct cyanmethaemoglobin found varying results, likely due to differences in blood sampling in the case of capillary blood. A study in adult women reported slightly
elevated Hb concentrations as compared to the direct cyanmethemoglobin method (Sari et al., 2001), contrasted by a rather small study in children: lower Hb concentrations were found and in consequence, elevated prevalence estimates of about 3% (Neufeld et al., 2002). As further methods, even automated cell counters can be used in a relatively simple setting, which can provide supplementary information on erythrocytes, such as haematocrit or mean corpuscular volume. It is noteworthy that the interpretation of Hb values has to be done in relation to age, sex, physiological status, and maybe even race (INACG, 2002). Very similar information to Hb yields the haematocrit (or packed cell volume), although its response to impaired Hb formation is slightly delayed and thus, despite lowered Hb values, haematocrit might be normal in an early stage of anaemia (Graitcer et al., 1981). As for Hb, the main disadvantages of this value for detecting ID are low specificity (due to multifactoriality of anaemia) and poor sensitivity (only detects IDA and not earlier stages of ID). Besides automated cell counters, haematocrit can be determined by centrifuging small capillary tubes (heparinised).

As a measure of average erythrocytes size, the mean corpuscular volume (MCV) is a parameter that can be helpful in narrowing down underlying causes of anaemia: macrocytosis can be indicative of vitamin B\textsubscript{12} or folic acid deficiency, whereas microcytosis points at iron or vitamin B\textsubscript{6} deficiency, but could also be due to chronic inflammation, haemoglobinopathies or lead poisoning (Begemann, 1999).

Assessment of MCV is performed on automated cell counters.

Mean red cell haemoglobin is defined as the average haemoglobin content of the red blood cells, and provides similar information to MCV: in ID its value is lowered, but in vitamin B\textsubscript{12} and folic acid deficiency it is elevated (Gibson, 2005).

Continuous values
The ratio of sTfR to sF has been proposed to provide information over the total range of iron status, because sF is predictive of storage iron and sTfR of functional iron (Baynes, 1996). With progressive depletion of iron stores, sF values are constantly reduced, but sTfR values are unaltered to a certain stage; only after complete depletion, their values rise (Punnonen & Rajamaki, 2005). Physiologically, the depletion of iron stores triggers the production of sTfR in order to increase turnover efficiency (Skikne et al., 1990).
Body iron calculation has recently been proposed as a useful tool mainly in intervention trials to calculate the absorption of the additional iron in the intervention (Cook et al., 2003). The physiological concept is similar to the sTfR/sF ratio and thus, both these methods do have one important limitation: since sF as an acute-phase protein is increased during inflammation, they both have to be interpreted with caution in the situation of inflammation. Nevertheless, the WHO recently recommended the use of body iron calculation to assess population-based intervention efficacy (WHO, 2004a).

**Particularities during infection**

Several clinical disorders influence iron metabolism and mimic manifestations observed in IDA, but the most common is inflammation that hampers iron utilisation (Cook, 2005). Thus in areas like SSA where chronic disorders leading to inflammation are common, this aspect is of important consideration. Despite the many advantages of sF as indicator for storage iron, the big drawback is its second function as an acute-phase protein and thus, sF concentration can be increased despite low iron stores (Abraham et al., 2003; Beard et al., 2006). For children <5 years of age, the use of a threshold of 30 µg/L has been recommended to correct for this elevation (WHO/UNICEF/UNU, 2001). For school-children in infection-endemic areas, no such modified cut-offs are issued despite the likely similar pattern in this age group (Stoltzfus et al., 1997a). From a study in elderly adults, much higher sF thresholds of 70 µg/L were postulated to correct for subclinical inflammation (Guyatt et al., 1990). Two other studies reported higher sensitivity and specificity in anaemic adults with or without inflammation if using sF values of ≥30 µg/L, indicating that maybe the current thresholds tend to be low (Mast et al., 1998; Thomas & Thomas, 2002).

It is recommended to determine acute-phase proteins concomitantly with iron status assessment to identify the infected individuals. The most commonly used indicator for subclinical inflammation is C-reactive protein (CRP), but α1-acid-glycoprotein (AGP) and α1-antichymotrypsin have been postulated to better coincide with increased sF concentrations over time due to inflammation (Thurnham et al., 2005). The lack of consensus on thresholds for inflammation and on the processing of data
from individuals with inflammation complicates their use and a more standardized procedure would ease the comparison of different trials.

The usefulness of ZPP during inflammation is equivocal but likely to be reduced, in particular if analysis is performed on unwashed erythrocytes (Beutler et al., 2003). If assayed on washed cells, interferences are less pronounced (Hastka et al., 1992). A study comparing school-age children from an infection-endemic area and a region with low sub-clinical inflammation from different ethnic origin proposed higher cut-off values for ZPP on washed erythrocytes in order to increase specificity and sensitivity (Zimmermann et al., 2005). Of note, in this study, children with elevated CRP values were excluded from analysis and despite this, differences in optimal cut-offs persisted.

sTfR has been reported not to be influenced by inflammatory state (Carriaga et al., 1991; Ferguson et al., 1992; Staubli Asobayire et al., 2001; Hanif et al., 2005) but several other reports have contrasted this statement for asymptomatic or mild malaria due to malaria-associated haemolysis (Mockenhaupt et al., 1999; Menendez et al., 2001; Verhoef et al., 2001), and this might limit the usefulness of this parameter in a malaria-endemic setting. Another important limitation of sTfR in assessing the impact of iron interventions is that it might not respond to the intervention despite other indicators showing improvements (Mei et al., 2005).

In a study that confirmed the low susceptibility of sTfR to inflammation, further diagnostic improvement in differentiating between IDA and anaemia of inflammation was reported to be obtained using the combination of sTfr/log(sF) as an indicator for storage and utilizable iron (Punnonen et al., 1997). Based on the concept of combination of iron compartments, Cook and colleagues (2003) presented the equation of body iron stores, which is as follows:

\[
\text{body iron (mg/kg) = } \frac{-[\log(TfR*1000/sF)-2.8229]}{0.1207}
\]

This equation allows expressing body iron proportionate to body weight and hence, extrapolating to various age (weight) groups. This approach however, is still limited by the difficult interpretation during inflammation.
The discovery of the function of hepcidin as a regulatory protein in iron metabolism and as an acute-phase reactant may show to be useful in future for assessing iron status and differentiating between IDA and anaemia of inflammation but to date, no standardized and commercially available tests exist (Anderson et al., 2002; Nemeth et al., 2003).

Overall, it has to be stated that lack of standardization of tests and harmonization of thresholds for defining ID add on to the difficulty of the limitation of these parameters during inflammation. Whereas the former is difficult to overcome, the latter could be solved by pooling studies with comparable methodology and population groups of different ethnicity and optimize cut-off values.

1.2.2 Riboflavin status

Currently, the most widely used procedure is that of erythrocyte glutathione reductase activation coefficient (EGRAC), an in-vitro stimulation of the erythrocyte glutathione reductase by FAD (flavin adenine dinucleotide), but prior to the availability of the EGRAC-test, assessment of urinary riboflavin levels was common (Sauberlich, 1999). Other methods, most of which were more recently developed, include liquid chromatographic or microbiologic determination of riboflavin, flavin mononucleotide (FMN) and FAD in blood, plasma or erythrocytes (Hustad et al., 2002; Graham et al., 2005; Hautem et al., 2006).

Since riboflavin is light and temperature sensitive, correct sampling and handling is of great importance: immediate freezing after blood drawing with protection from light, standardized thawing conditions for all assays and for the EGRAC method, a pre-incubation time as well as harmonized lysis time (Dror et al., 1994). For the African context, limitations that are worth considering are the relatively widespread prevalence of glucose-6-phosphate dehydrogenase deficiency, malaria and haemoglobinopathies; these diseases have been reported to artificially increase prevalences (Flatz, 1971; Yawata & Tanaka, 1974; Traunmuller et al., 2003). Furthermore, the lack of consensus on threshold values for the EGRAC, but also most other methods, introduce difficulties in the interpretation of population surveys.
In order to establish commonly accepted thresholds, larger samples from different population groups and ethnicities should be taken. Subjects should be apparently riboflavin sufficient individuals, i.e. meat and dairy product consumers. The current thresholds for the EGRAC method of <1.2 "normal status", 1.2-1.4 "marginal deficiency" and >1.4 "deficiency" were established on 430 well nourished American children, 6 adult men and in a depletion-repletion study in 8 Indian adults (Bamji, 1969; Sauberlich et al., 1972; Tillotson & Baker, 1972). Since then, discussions on the threshold have not ceased but to date, different values for the same method are used. In the actual situation, the best method to be used despite all its limitations is the EGRAC using the above thresholds, since the comparability to earlier studies is possible.

1.2.3 Vitamin A

The liver is the main storage compartment of the fat-soluble vitamin A and thus, the gold standard for determining vitamin A status is a liver biopsy, which by definition determines VAD if liver stores fall below 20 µg retinol/g tissue (Sommer & Davidson, 2002). Due to its invasiveness, this method is not adequate for population studies. There are several other proxies that can be used to assess either clinical or subclinical signs of VAD. Important clinical signs are related to changes in the eye due to the important role of vitamin A in vision. Of note, these changes occur in a later stage of VAD and thus, present the tip of the iceberg. Among these eye-related consequences of VAD are the two used in the WHO VAD database (WHO, 2006a) to assess the degree of VAD, night blindness (XN) and Bitot’s spots (XB or X1B). For children under 6 years of age, VAD is considered a public health problem, if night blindness is prevalent in >1% or if Bitot’s spots occur in >0.5% of the infants/children (Sommer & Davidson, 2002). For older children, no such prevalence thresholds have been issued. In order to detect an earlier stage of VAD that already increases the risk of negative consequences, analysis of serum retinol (sR) seems to be the best currently available parameter (de Pee & Dary, 2002; Tanumihardjo, 2004). The following sR concentrations indicate severe, moderate and mild VAD, respectively: <0.35 µmol/L (<10 µg/dL), <0.7 µmol/L (<20 µg/dL), <1.05 µmol/L (<30 µg/dL), respectively (WHO, 1996). Major limitations of this parameter is its susceptibility to inflammation leading to lowered sR values and hence, overestimation of prevalence (Thurnham et al., 2003); and the fact that venous blood sampling is required. Method
of choice for sR analysis is HPLC due to sufficient precision at low concentrations (de Pee & Dary, 2002), which is a further challenge in setting up this method. As for some of the iron status indicators, measurement of sR on dried blood spots from capillary blood has been investigated, but so far, this approach did not experience a major breakthrough (Craft et al., 2000; Erhardt et al., 2002). Measurement of retinol-binding protein (RBP) and the further development of concomitant transthyretin (TTR) analysis are well correlated with sR levels and could be an alternative (Rosales et al., 2002; Hix et al., 2004).

1.2.4 Malaria
The diagnosis of malaria relies on clinical examination and the presence of parasites in the blood. The accuracy of clinical diagnosis is very limited due to its low specificity and relatedness to many other disease symptoms (Talisuna & Meya, 2007). For children in a high risk malaria setting, criteria for positive diagnosis are fever and history of fever without alternative explanation of fever (WHO, 2005). The clinical assessment alone leads to overestimation of malarial cases and subsequent malaria treatment (Reyburn et al., 2004). As a consequence, risks for drug intolerance and parasite drug resistance are unnecessarily increased. Assessment of asymptomatic but due to the above reasons also of symptomatic malaria should therefore be based on biomedical methods. Among the two most commonly used methods for biomedical diagnosis of malaria are light microscopy and rapid diagnostic tests (RDT). RDTs detect specific antigens produced by malaria parasites and released into the host blood. In species-ignorant tests, this antigen usually is the histidine-rich protein-2, but newer tests are capable of differentiating between species and use a variety of antigens. Of note, RDTs indicate recent history of malaria parasitaemia, because antigens can be present even after parasite clearance (WHO, 2004b). Although light microscopy is considered to be the gold standard, it brings along a very important disadvantage: operator dependency. A comparison between the two above methods is provided by the WHO (2000). Advantages of light microscopy, if performed by a well trained, experienced operator are the quantitative assessment, low detection limit, species typing and low operating costs. For the use of RDT, the training is simple and technical skills are not necessary. However, since they do not allow quantification of parasite burden, the result needs to be combined with a clinical diagnosis. RDT tend to be more expensive than light microscopy. With recent
development and decrease in cost, RDT's have become an alternative to light microscopy for detection of *P. falciparum*, but for other species, microscopy still remains the method of choice (Ochola *et al*., 2006). Highly objective and reliable methods appear to be molecular assays (polymerase chain reaction, quantitative nucleic acid sequence based amplification), but their use in field settings is very limited (Mens *et al*., 2007).

1.2.5 Helminths

Diagnosis of soil-transmitted helminths and other gastro-intestinal parasites (e.g. *S. mansoni*) is most often done by visualisation of eggs in the stool, that of *S. haematobium* by microscopy of filters, with a previous filtration of a defined volume of urine. For analysis of stool samples, several preparatory methods are available, but due to its ease of use and low cost, the Kato-Katz technique is the most widely applied and is recommended by WHO for diagnosis of helminth infestation (Katz *et al*., 1972; WHO, 1991). Recent improvements include the preparation of stool slides in duplicates in order to reduce the effect of inhomogeneity of eggs (Booth *et al*., 2003). Another disadvantage of the Kato-Katz technique is the time-dependency of the reading for detecting eggs and cysts. With this regard, the also widely used ether-based concentration method is more solid, in particular, if microscope readings cannot be readily performed (Allen & Ridley, 1970; Goodman *et al*., 2007). A very recent study investigated a newly developed method, where a relatively large amount of stool sample can be observed, thereby reducing sampling inhomogeneity (Utzinger *et al*., 2008). The method, termed FLOTAC, has shown to be more sensitive for qualitative diagnosis (uninfected vs. infected) as compared to the two previously described methods, but yielded lower egg counts.

For detection and quantification of *S. haematobium*, the filtration method is used, but problems of inhomogeneity also persist, and diurnal variation in egg secretion may impair prevalence and intensity estimates (Braun-Munzinger & Southgate, 1992). Antibody detection assays are very sensitive, but as for RDT for malaria, may provide information on recent and not only current infection. However, specific antigens for circulating schistosomes have recently become available and thus, may improve diagnostic performance (Salah *et al*., 2006). Dipstick tests for haematuria are very simple to use but are not specific; however, in a setting where other diseases leading to haematuria are minimal, it can be a useful test (Kotb *et al*., 1996).
1.3 Iron-infection interactions

In the above sections, it has become apparent that the contribution of nutritional iron deficiency per se may be overestimated in the aetiology of anaemia. However, functional iron deficiency or iron deficiency due to iron losses plays a central role in the onset of anaemia. It is therefore justified to look in more detail at the healthy or undisturbed iron metabolism in a first instance to compare it later on with the altered state during chronic or acute inflammation.

1.3.1 Normal iron metabolism

The importance of iron in numerous biological and cellular processes, such as oxygen transport, electron transfer, DNA synthesis, and as a co-factor of enzyme systems make it indispensable for human life (Halliwell & Gutteridge, 1986). These important functions are due to its high redox potential enabling iron to rapidly change from the ferrous to the ferric state, but it is this characteristic that also renders iron potentially dangerous for proteins, nucleic acids, and fatty acids (Ganz & Nemeth, 2006a). Iron cannot be actively excreted from the body and thus homeostasis is regulated at the level of absorption; additionally, iron in the body has to be well controlled in order to avoid free – i.e. highly reactive – iron (Andrews, 1999).

The total amount of iron in the body depends on age, gender, nutritional, and general physiological state. For an adult male it is estimated that 35-45 mg iron/kg body mass are present, whereof two thirds are incorporated into haemoglobin and myoglobin, the remainder being stored in the liver. Only small amounts are involved in enzyme activities (Conrad et al., 1999) or circulate in the plasma bound to transferrin or even as non-transferrin bound iron, which can be free, but is mostly chelated to organic acids (Chua et al., 2007).

After its absorption, iron is distributed to different organs or tissues, depending on the host iron status. Based on needs, iron can be released from storage compartments and carried to the target tissues (Mackenzie & Garrick, 2005; Petrak & Vyoral, 2005). At all levels, regulatory mechanisms are involved, and recent discoveries have helped to better understand them. Most of the models of iron metabolism are derived from absorption studies in adults, human haemochromatosis (HH) disease, or mutant
animal studies. It is debatable whether the evidence available can be extrapolated to children of school-age; a recent review highlights that in infancy and early childhood, iron homeostasis is maintained differently, since needs differ (Lonnerdal & Kelleher, 2007), but it might be speculated that school-age children have a decreased iron need in relation to body mass and thus, regulation is similar to the models developed for male adults.

Absorption
Predominantly, proteins implicated in the iron absorption pathway are located in the upper part of the duodenum and thus, most iron is absorbed there (Carpenter & Mahoney, 1992). Dietary iron occurs in two forms, haem iron and non-haem iron, which are absorbed via different pathways (Figure 2). Although more efficiently absorbed, haem iron only accounts for 10-15% of daily iron intake due to the limited consumption of meat products (Mackenzie & Garrick, 2005).

Despite different uptake into enterocytes, both iron forms subsequently enter a common pool, and are transferred to the basolateral side of the enterocyte and released into the bloodstream.

Haem iron is thought to be taken up as intact metalloprotoporphyrin into the enterocyte via the haem carrier protein 1 (HCP1; Shayeghi et al., 2005), and afterwards broken down by haem oxygenase 1 (HO1) into ferrous iron and biliverdin (Raffin et al., 1974). It could further be shown by Shayeghi et al. (2005) that HCP1 is post-transcriptionally regulated by iron stores. Recent data however questions this model of haem iron absorption because it assumes HO1 and HCP1 to be co-localized during uptake phases, an assumption that could not be confirmed in an animal model (West & Oates, 2008). It is likely that HCP1 is not the only uptake protein for haem iron. It has recently been reported to have high affinity to folate and thus, other, yet undiscovered haem transporters may be present (Qiu et al., 2006).

The absorption of non-haem iron is realized by a different pathway: the iron ions – if present in the ferric form – are reduced to the ferrous form importantly by duodenal cytochrome B (DcytB) assisted by ascorbic acid or the NADH/FMNH₂ complex as electron donor, but ascorbic acid may perform this reduction enzyme-independent (Gunshin et al., 2005b). The ferrous form of iron is taken up into the enterocyte by a
divalent metal transporter (DMT1; Fleming et al., 1997). DMT1 (also termed DCT1 or Nramp2) is a membrane protein that non-specifically transports divalent metal ions by a proton symport mechanism in exchange to Na+/H+ (Fleming et al., 1997).

![Diagram of iron absorption by the enterocyte](image)

Figure 2: Iron absorption by the enterocyte, including haem and non-haem iron [modified from (Zimmermann & Hurrell, 2007); courtesy R. Biebinger]. Haem iron is absorbed via haem-carrier protein 1 (HCP1) by an endosomal process; the porphyrin ring is separated from the ferrous iron by haem oxygenase 1, the iron subsequently being exported from the endosome into the cytosol by the divalent metal transporter 1 (DMT1; Chua et al., 2007). Non-haem iron is – if necessary – reduced by the ferrireductase DcytB and then transported in the ferrous state across the brush border via DMT1. Once in the enterocyte, both iron forms are either stored as ferritin or transferred by ferroportin1 (FPN1) across the basolateral membrane. At this site, the iron oxidized by hephaestin and bound to circulating apo-Tf (iron-empty transferrin; Tf) in the bloodstream, if hepcidin has not down-regulated FPN1-activity. The major regulatory mechanisms are described in green.

A third absorption mechanism using directly available ferric iron has been discussed over the last years, but its significance and the detailed pathway are still insufficiently understood (Cremonesi et al., 2002). According to this model, ferric iron is reduced after internalization. Membrane transfer is realized via mucin, Fe reduced by β3-integrin and transferred to mobilferrin or the paraferritin complex. Thus, this mechanism has been termed integrin- or integrin-mobilferrin-paraferritin pathway (Conrad et al., 2000). This alternative pathway seems to be of some significance, if dietary ferric iron is the predominant form. However, blocking of β3-integrin does not
lead to IDA, whereas inactivation of DMT1 does, and this information indicates that the latter might be essential, whereas the \( \beta_3 \)-integrin pathway might be complementary (Mackenzie & Garrick, 2005; Beaumont et al., 2006).

After uptake into the enterocyte, iron is collected in a common cytosolic pool. From there, it can either be stored as ferritin in the enterocyte, where it is lost upon enterocyte exfoliation (Ma et al., 2002) or transferred to the basolateral side and into the bloodstream (McKie et al., 2000). With increased body iron needs, the iron flux to the basolateral side is increased and there, a concerted action of ferroportin1 (FPN1; IREG1; MTP1) and membrane bound hephaestin (and maybe caeruloplasmin) takes place to release iron into the bloodstream (Vulpe et al., 1999; Cherukuri et al., 2005). Over-expression of FPN1 increases iron release from the cytosol and the ferritin stores (Donovan et al., 2000). FPN1 is also highly expressed in hepatocytes and macrophages of liver, spleen, and bone marrow (Abboud & Haile, 2000).

The role of hephaestin and caeruloplasmin is to oxidize the ferrous form, thus allowing sequestration by circulating Tf in the plasma, a process yet poorly understood (Wessling-Resnick, 2006). Notably, FPN1 and hephaestin co-localize in the basolateral membrane, where they are closely associated to TfR (Han & Kim, 2007).

Three main regulatory models to control iron absorption on a molecular level have evolved: the "erythropoietic regulator" (a), the "stores regulator" (b), and the so-called "mucosal block" (c). With the discovery of key regulatory proteins, models (a) and (b) seem not to be mutually exclusive anymore. The dominant role of hepcidin has been more and more evidenced, but still, many of the details in the regulation of iron remain to be elucidated (Fleming & Britton, 2006). The third and older model (c) is of relevance, if high oral doses of iron are administered.

a) Erythropoietic regulator

This model postulates the rate of erythropoiesis to affect the regulatory pathway. Iron absorption is increased, if iron is lost or ineffective erythropoiesis is occurring. Erythropoietin (EPO) concentration is modulated in these states, very similar to hypoferric anaemia.

b) Stores regulator
For the “stores regulator”, two hypotheses have evolved and these seem also to better fit together since the discovery of the key role of hepcidin. The “crypt-cell hypothesis” postulates regulatory mechanisms at duodenal crypt enterocytes. Within immature crypt enterocytes, intracellular iron level reflects iron status. The close interaction of HFE (gene products of hereditary haemochromatosis) with TfR1 in the crypts suggests that it acts as a sensor of iron stores by monitoring transferrin saturation (Fleming & Britton, 2006). A reduction in HFE would then predispose subsequent crypt cells to a relative ID and results in an increased expression of proteins of the iron absorption pathway (DMT1, DcytB, FPN1; Dupic et al., 2002).

The “hepcidin hypothesis” postulates that body iron sensing is controlled in the liver. Decreased hepcidin levels in HH (hereditary haemochromatosis) patients with mutations in HFE and TfR2 suggest that these two proteins are involved in the regulation of hepcidin. The binding of diferric Tf (Tf-Fe2) to TfR1-HFE and TfR2 in hepatocytes results in an up-regulation of hepcidin expression (Bridle et al., 2003; Nemeth et al., 2005). The increased hepcidin production and binding of hepcidin to FPN1 results in a blockage of iron efflux, by internalization and degradation of FPN1 (Nemeth et al., 2004b; Frazer & Anderson, 2005).

c) Mucosal block

The so called mucosal block was observed, when high oral doses of iron were administered. On the molecular level, the expression of DMT1 and DcytB is rapidly down-regulated, whereas neither FPN1 nor hephaestin are affected. This indicates that the block occurs on the apical side before iron is taken up; down-regulation is thought to be due to increased intra-enterocyte iron, and thus increased ferritin levels that trigger the expression (Chua et al., 2007). Recently, hepcidin has also been discussed to be directly involved at this stage of regulation, but the mechanism remains elusive. A cell model postulates hepcidin involvement by inhibiting DMT1 transcription (Mena et al., 2008).

Transport

Ferrous iron, once absorbed, is relocated to the outside of the basolateral membrane, where it is oxidized to the ferric form and transferred onto circulating apo-
Tf. The partly or completely saturated Tf supplies most of the iron required by the tissues (Chua et al., 2007). Only minor amounts are present as non-Tf-bound iron (NTBI). Sequestered by Tf, the iron can be shuttled either into storage or directly to the main site of utilisation. Principal storage occurs in hepatocytes, spleen macrophages, bone marrow, and skeletal muscles, but all nucleated cells are able to store minor amounts of iron in the form of ferritin (Lynch, 2007b).

The two isoforms TfR1 and TfR2 bind mono- or diferric Tf (Tf-Fe), each TfR allowing the docking of two holo-Tf (Tf-Fe), because of its much higher affinity to holo-Tf, TfR1 is thought to be more important for the transport of iron, whereas TfR2 may be involved in regulatory issues (Hentze et al., 2004).

Whereas iron-free Tf is not recognized by TfR, the Tf-Fe-complex is endocytosed. The phagocytosis is realized through invagination of clathrin-coated pits and the formation of endocytic vesicles, so-called endosomes (Alvarez et al., 1990b; Alvarez et al., 1990a). Upon acidification of the endosome, a conformational change in Tf is induced that triggers iron release. The emptied Tf (apoTf) remains bound to TfR at mildly acidic pH, and as the complex is returned to the cell surface, the higher extracellular pH leads to the dissociation of the Tf-TfR-complex (Bacon & Tavill, 1984). Intracellular Tf-Fe separation might also be facilitated by an oxidoreductase or a ferrireductase (Steap3; Ohgami et al., 2005). This receptor-mediated uptake of iron holds for most of the cell types. The cytoplasmic iron is then either directly used as functional iron, stored in ferritin or haemosiderin, or shuttled into the mitochondria (Hentze et al., 2004).

**Utilisation**

Although most of the iron used in the bone marrow is not newly absorbed, this tissue remains the largest "user" of iron. Erythroid cells express membrane-bound TfR that incorporate the Tf-Fe-complexes as above. It is likely that this is the only uptake pathway in these cells. TfR-mutant animals that are unable to express TfR develop a severe anaemia that eventually is fatal. Interestingly, it was found that most non-haematopoietic tissues were iron-sufficient, indicating that these are capable of using different uptake pathways (Levy et al., 1999). Iron released from the endosome is moved to the mitochondrion to be incorporated into protoporphyrin IX by
Literature review

ferrochelatase. Import into the mitochondrion has recently been described to be facilitated by mitoferrin (SLCC25A37; Shaw et al., 2006).

The process of incorporation of iron into muscle cells for myoglobin production remains to be elucidated. Neither a lack of TfR nor of DMT1 causes a meaningful reduction in the synthesis of myoglobin (Andrews & Schmidt, 2007).

Recycling and storage

Most of the available iron originates from the recycling of iron from senescent erythrocytes, after their intracellular lysis and release of haem. Free Hb binds to haptoglobin, and this complex is subsequently scavenged by macrophages (Latunde-Dada et al., 2006). Phagocytosis is initiated by a receptor termed CD163. After ingestion, the complex is broken down (Kristiansen et al., 2001) and iron is released via a FPN1-mediated mechanism (Donovan et al., 2005).

Macrophages do have some iron storage capacity, but most of the surplus iron is stored in spleen and dominantly in the hepatocytes (Andrews & Schmidt, 2007). For iron uptake into hepatocytes, not only the Tf-TfR-pathway can be utilized. In serum iron abundance, other transferrin-bound iron (TBI) and NTBI-uptake pathways can become important, but remain to be properly described (Kielmanowicz et al., 2005).

The Tf-TfR-pathway is upregulated as a consequence of increased TfR1 expression in ID hepatocytes, and downregulated in iron repletion. Further, the absence of HFE increases hepatocyte iron uptake. Nitric oxide and IL-6 trigger TfR1 expression, another suggested mechanism for upregulation of the Tf-TfR-pathway (Kobune et al., 1994; Barisani et al., 1999). Since the circulating plasma Tf concentration is much higher than that needed to saturate TfR1, other TBI uptake mechanism have been postulated: holo-Tf binding to hepatocyte surface sites other than TfR1, with subsequent endocytosis and DMT1-mediated endosome transfer (Morgan et al., 1986); iron release on the outside of the membrane from Tf, followed by a ferroxidase (caeruloplasmin) step and transmembrane shuttling mediated by an iron transporter (Thorstensen & Romslo, 1988).

The pathways of the NTBI uptake are mostly unknown, but they do not necessarily involve DMT1 (Gunshin et al., 2005a). NTBI is chelated to a ligand, mostly organic
acids, but irrespective of the type of ligand, is taken up after reduction into the hepatocyte via a transporter (Trinder & Morgan, 1998). NTBI occurs in minute amounts, usually undetectable quantities but certainly below 2% of the serum iron. In pathological state, the concentration can be increased (Van Der A et al., 2006).

Ferritin is the main storage protein in humans. It can take up ferrous iron and oxidize it to the ferric form, thus protecting the body from the Fenton- and Haber-Weiss reactions. Ferritin is mainly present in the cell cytoplasm although mitochondrial ferritin has also been found, but its role in there remains to be fully understood (Corsi et al., 2002). Ferritin gene transcription is regulated by endogenous and exogenous factors, such as oxidants. This upregulation has to be seen as part of the cellular defence mechanisms against excess iron ( Hintze & Theil, 2006). Cytokines and thyroid hormones have further been reported to increase transcription (Iwasa et al., 1990; Wei et al., 1990). During inflammation, macrophage ferritin and its degradation product haemosiderin are increased, potentially to remove reactive iron by converting it into hydrated ferric iron (Liu & Theil, 2005).

Cellular and systemic regulation of iron metabolism
This section gives an overview of the regulatory mechanisms involved in iron metabolism. It is organized around a schematic (Figure 3) modified from Steele et al. (2005) and Fleming & Bacon (2005).

Iron is needed to accomplish basal functions, but the presence of free iron has to be avoided. The regulation at the cellular level is predominantly governed by cytoplasmic iron regulatory proteins (IRP1 and IRP2). IRP1 and IRP2 might exert different actions, but each of them can modulate the transcription, and in combination, they appear to be more efficient (Wang et al., 2007).

If cells show low iron status, IRPs adhere to the iron regulatory elements (IRE) of mRNA of proteins involved in iron metabolism, such as ferritin, TfR, DMT1 (Kato et al., 2007), but also in enzymes involved in red blood cell haem synthesis, such as the erythroid-specific form of aminolaevulinic acid synthase (ALAS-E; Schranzhofer et al., 2006).
Figure 3: Schematic representation of iron homeostasis regulation. Dietary iron is taken up and transferred across the enterocyte as described in Figure 2. Tf-Fe reaches hepatocytes or other cells, where mainly TIR1 and TIR2 phagocytose Tf-Fe, releasing iron into cytoplasm and Tf extracellularly. For macrophages, other uptake mechanisms have been proposed, where Hb is bound to haptoglobin prior to scavenging (CD163; Kristiansen et al., 2001). TIR2, HFE and haemojuvelin are thought to sense iron status and trigger hepcidin production. Hepcidin is high during iron sufficiency, and mainly exerts its regulatory function by blocking ferroportin (FPN1). This process occurs in enterocytes, hepatocytes, and macrophages. Other signalling pathways may be the hypoxia-inducible factor (HIF), expression of which is elevated in case of low oxygen pressure; it may regulate hepcidin and caeruloplasmin expression (Peyssoniaux et al., 2008). EPO mainly acts in the bone marrow, but is also thought to upregulate divalent metal transporter (DMT1) and hephaestin (Hph) activity (Kong et al., 2008). On the cellular level, iron regulatory proteins (IRP) influence translation of proteins by adhering to iron responsive elements (IRE): in case of low iron demand, translation of DMT1 and TfR is reduced, but that of ferritin increased. Regulation within erythropoietic cells remains to be further elucidated, but EPO, IRP/IRE and TfR may play a role; ferrochelatase is thought to incorporate iron into protoporphyrin IX and is regulated by IRP/IRE. Within the enterocyte, iron concentration might downregulate FPN1 activity (Ganz, 2006).

At low intracellular iron concentration, IRP bind to IRE in the 5'-'untranslated region (UTR) of ferritin mRNA and block translation of the ferritin protein. IRPs also bind to IREs located in the 3'-UTR of TfR and DMT1 mRNA, thereby stabilizing the message against enzymatic cleavage and thus increasing the uptake of Tf-bound iron.
Although this iron fraction is poorly characterized, it is thought that the labile iron pool within the cell holds the regulatory function (Pantopoulos, 2004). However, it has also been postulated that haem plays a role in the regulation of ferritin, and very recent studies propose its control at the transcriptional level via antioxidant responsive elements and the haem binding transcriptional repressor Bach1 (Hintze et al., 2007).

As briefly mentioned above, the protein involved in hereditary haemochromatosis, HFE, is involved in the regulation of TfR1. HFE and Tf-Fe can simultaneously bind to TfR1, but decreased affinity of TfR1 to Tf-Fe results from this quasi-competitive binding (Giannetti & Bjorkman, 2004). Besides its role as iron transporter, TfR2 has also been postulated to serve as a signal transducer. TfR2 protein expression is regulated by Tf-Fe, and transferrin saturation is thus sensed by TfR2, which then loops back to hepcidin expression in the hepatocytes. This information is thought to be transported via a kinase complex (Calzolari et al., 2006). Whether TfR2 needs HFE for iron sensing needs further clarification (Fleming & Britton, 2006; Goswami & Andrews, 2006).

Hepcidin is synthesized and secreted mainly by hepatocytes and circulates in plasma (Park et al., 2001). Plasma hepcidin levels are reduced in the state of hypoxia, anaemia, and iron depletion, but are elevated during inflammation, infection, and iron repletion (Pigeon et al., 2001; Nicolas et al., 2002b). When liver hepcidin expression is elevated, iron absorption/release by the enterocytes and iron export by the macrophages is decreased (Nicolas et al., 2002a). The regulation at this level is predominantly exerted by binding to ferroportin, causing its internalization and degradation (Nemeth et al., 2004b). Differences in speed of regulation have recently been reported, hepcidin to act much faster on macrophages than on enterocytes (Chaston et al., 2008).

Despite a relatively clear model of the impact of hepcidin on iron homeostasis, it remains to be explained in detail how iron regulates hepcidin production. Many of the models trying to explain regulation of hepcidin are derived from knockout mice models or HH disorders (Ganz & Nemeth, 2006b). Low levels of liver hepcidin have been observed in humans and mice of HFE- and TfR2-related HH despite increased liver iron stores (Bridle et al., 2003; Kawabata et al., 2005). A third type of HH, the
haemojuvelin-type has been described to reduce hepcidin response to iron overload (Andriopoulos et al., 2007). TfR2 and haemojuvelin seem to have the most important impact on hepcidin blockage, and it has thus been postulated that HFE may be a signal modulator from the iron sensor to hepcidin, but is not essential for the pathway itself (Ganz & Nemeth, 2006b). This iron sensor might be TfR2, being triggered by transferrin saturation (Johnson & Enns, 2004). Hepcidin expression appears to be largely regulated at the transcriptional level: haemojuvelin is a member of the repulsive guidance molecule family, which also includes the bone morphogenetic protein (BMP), and haemojuvelin has been described to serve as a BMP co-receptor (Babitt et al., 2006; Lin et al., 2007). This mechanism and its potential gene inhibitor (SMAD) are nicely reviewed in Andrews & Schmidt (2007). A very recent study postulates the soluble HJV to act as antagonist of the membrane bound haemojuvelin for downregulation of hepcidin expression (Lin et al., 2008).

Hepcidin production is suppressed by anaemia and hypoxia: phenylhydrazine-induced haemolysis leading to anaemia in mice held in hypobaric chambers showed lower levels of hepcidin, followed by increased EPO release. Both of these mechanisms allow the organism to more efficiently produce haem in the situation of anaemia or low oxygen (Atanasiu et al., 2007). It is likely that anaemia or hypoxia dominate downregulation of hepcidin over iron status, as could be seen in cell cultures (Nemeth & Ganz, 2006). In the case of β-thalassaemia associating anaemia and iron overload simultaneously, the regulation tends to be governed by anaemia rather than the iron condition (Camberlein et al., 2008), but stronger evidence is needed to give support to this finding. An alternative hypothesis postulates the growth differentiation factor-15 (GDF-15) be this mediator of erythropoietic regulation (Tanno et al., 2007); it was found that β-thalassaemia patients had up to 150x higher GDF-15 serum concentrations, and that these concentrations were positively correlated with sTfR, EPO and sF.

The pathways that regulate iron metabolism during hypoxia remain elusive, but to date, there is some evidence that the so called hypoxia-inducible factor (HIF) is a candidate. A recent review presents the evidence for HIF as the governing factor in the regulation of iron metabolism during hypoxia (Peyssonnaux et al., 2008). In Figure 3, these potential influences are depicted in light blue. It is hypothesized that
in the presence of oxygen, HIF is hydroxylated and then degraded, but anaemia might reduce this hydroxylation and even increase the lifespan of HIF by destabilizing an iron-dependent tumour suppressor protein involved in HIF degradation. This hypothesis is experimentally on weak grounds but would argue for iron sensing capacities of HIF in the liver. HIF might further interfere with the regulation on the gene level: it is thought to influence genes for caeruloplasmin within the hepatocytes, genes for TfR, and hepatic FPN1. The role of HIF in the regulation of EPO is thought to be performed in the cortex of the kidney, but mechanisms are equivocal: gene enhancer models during hypoxia and EPO gene expression inhibitor roles are discussed (Imagawa et al., 1997; Vaux et al., 2001), but also the direct inhibitory effects of HIF on hepcidin gene expression in the hepatocyte are proposed (De Domenico et al., 2008).

1.3.2 Iron metabolism during inflammation

Inflammation is the central response of the immune system to an antigen. Any foreign substance or organism entering the human body encounters a series of defence mechanisms. In the first line, these mechanisms include defensins (antimicrobial peptides) that can destroy bacteria and even some viruses but also modulate inflammatory response. The degradation products of “invaders” or body's own damaged cell material induce inflammation (Aarbiou et al., 2002). These products cause a whole cascade of inflammatory response to be started, including regulatory proteins, peptides and enzymes expressed as a result of activation of inflammatory genes (Abreu & Arditi, 2004). It would, however, be beyond the scope of this review to summarize the whole inflammatory pathway, and the focus is directed towards acute-phase reactants that play a role in iron homeostasis; for comprehensive reviews, see Delves & Roitt (2000b; 2000a). It is also noteworthy, that with regard to this issue, iron-overload disorders are very closely linked. However, main goal of this review is to present knowledge on anaemia and iron deficiency and thus, the iron-overload diseases are only discussed if appropriate for the understanding of the onset of iron deficiency or anaemia.

With a very few exceptions, all living organisms are essentially dependent on iron for their survival and functioning. This holds equally for invasive organisms, such as
human pathogens. No matter whether the pathogens live (and proliferate) in the gastro-intestinal tract, the bloodstream or in particular body organs, they need iron from their host. Thus, there has been an evolutionary competition between the host and the pathogen to withhold iron from the pathogen or in the opposite case, to defeat these mechanisms to access iron (Ratledge, 2007). Therefore, it is comprehensible that the invasion of a pathogen or its by-products causes a response that includes reduction of freely available iron.

It is known that agents of oxidative stress, such as nitric oxide, hydrogen peroxide or other potential radicals can impact on iron metabolism by increasing binding affinity of IRP-1 to TfR mRNA and thus stabilizing it. Or, that cytokines affect iron homeostasis by changing the production patterns of these radicals and are thus influencing the transcription/translation of sF, TfR, DMT1 or FPN1 (Pantopoulos & Hentze, 1995; Weiss et al., 1995). A rapid drop in serum iron was observed in subjects after injection of endotoxins, and oral or parenteral iron administration did not importantly change this (Thurnham & Northrop-Clewes, 2007). In cancer patients given tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and melaphan (a cancer chemotherapy constituent), interleukin-6 (IL-6) levels immediately increased, peaking after 4 hours, CRP peaked at day 2, AGP and α1-antitrypsin peaked at day 3; albumin and Tf decreased and rose to normal after 2 days, whereas sTfr levels decrease to a minimum after 8 hours, coinciding with increased sF levels (Feelders et al., 1998). These changes taken together may cause hypoferraemia (functional ID) that is not necessarily a genuine ID but can occur despite normal iron stores; parameters of iron metabolism in patients with inflammatory disease as compared with iron deficiency are summarized in Table 8.

Inflammation causes many, mostly important changes in iron metabolism. One parameter described in Table 8 is decreased tissue iron release causing a reduction in circulating iron levels and decreased iron availability of iron for haematopoiesis. This is why, on the longer run, inflammation will lead to decreased haematocrit, Hb and increased ZPP due to iron-deficient erythropoiesis. Reduced iron absorption as a consequence could be shown in rats and patients with rheumatoid arthritis (Hershko et al., 1974; Lynch, 2007a).
Literature review

Table 8: parameters of iron metabolism in patients with inflammatory disease as compared with iron deficiency [modified from (Konijn, 1994)]

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Inflammation</th>
<th>ID</th>
</tr>
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<tr>
<td>Tissue iron release</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>Tissue storage iron</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>Iron absorption</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>Serum Iron</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>TIBC</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>Hb</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Haematocrit</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Mean corpuscular vol.</td>
<td>↓ or →</td>
<td>↓</td>
</tr>
<tr>
<td>ZPP</td>
<td>↑ or →</td>
<td>↑</td>
</tr>
<tr>
<td>sF</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>sTfR</td>
<td>↓ or →</td>
<td>↑</td>
</tr>
<tr>
<td>stainable marrow iron</td>
<td>→</td>
<td>↓</td>
</tr>
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</table>

The acute phase response that is characterized by various local and systemic effects, is mainly orchestrated by the cytokines IL-1, TNF-α, IL-6 and IFN-γ (Baumann & Gauldie, 1994). These cytokines are released at the site of inflammation by endothelial and mast cells, as well as by macrophages (Konijn, 1994). As mentioned above, it was long believed that these “macroscopic” modifications are regulated at the cellular level by influencing the IRP/IRE mechanism, or by direct impact of the cytokines on iron metabolism proteins, such as sF and sTfr.

In response to infection or inflammation, a pattern of increased synthesis of proteins involved in host defence and the selective suppression of synthesis of other secreted proteins in the liver is importantly involved.

The discovery of hepcidin helps to understand the molecular mechanisms of how the processes during inflammation are controlled. The hepcidin gene encodes a propeptide of 84 amino acids, whereof the 25 amino acids containing hepcidin is formed (Park et al., 2001). It appears that the last five amino acids of the N-terminus are essential for interaction with FPN1 (Nemeth et al., 2006). A drop in serum iron following an injection of hepcidin in mice could be observed within an hour, but also administration of turpentine – an inflammation inducing agent – caused hypoferremia (Nicolas et al., 2002b; Rivera et al., 2005). Expression of hepcidin is up-regulated in chronic inflammation via an IL-6 and IL-1 mediated STAT3 signal transduction pathway, STAT3 being a transcription factor that binds to the hepcidin promoter.
region (Wrighting & Andrews, 2006; Falzacappa et al., 2007). In a recent editorial, the postulated pathway for IL-6 has been elegantly summarized (Fleming, 2007): IL-6 is received by IL-6 receptor, followed by a dimerization of GP130, a subunit with signal transduction capability. This dimerization "attracts" janus kinase, which in turn phosphorylates the GP130 and activates the STAT3 (signal transducer and activator of transcription) mediated pathway, but also the MAPK (mitogen-activated protein kinase) pathway. Both of these pathways influence *hamp* (hepcidin gene) expression.

Other cytokines, such as IFN-β may be involved, too, but so far, most of the evidence is derived from rat studies and whether this can be extrapolated to humans, remains to be elucidated (Lee et al., 2005). In cell cultures, leptin was found to increase hepcidin expression, a finding that could potentially show its relevance in the field of inflammatory response due to overweight (Chung et al., 2007); a tumour-suppressor (p53) was shown in cell lines to target *hamp* for transcription, which would deprive cancer cells from iron (Weizer-Stern et al., 2007). A study in rats used turpentine oil to induce an acute phase response and looked at the gene expressions of IRP within the liver but also in other tissues (Sheikh et al., 2007); IL-6 and IL-1β were strongly upregulated in the muscle, whereas in the liver, IL-1β and TNF-α but not IL-6 were upregulated. Hepatic hepcidin expression was increased and gene expression of Tf, Tfr1, Tfr2, and the H-subunit of ferritin was upregulated, IRP-1 and -2, and DMT1, while decreased in haemojuvelin, FPN1, DcytB, and hephaestin. This indicates that the liver is probably not the only tissue, where cytokines attach and initiate the altered iron regulation. Most of the above experiments describe modulations at the level of gene expression; this however, does not automatically include the translation into the active protein.
Figure 4: pathophysiological changes of iron metabolism as a consequence of an acute phase reaction. It is thought that the main trigger for hepcidin production during inflammation is IL-6, for which a specific receptor in the hepatocytes has been identified (Nemeth et al., 2004a; Lee et al., 2005). At the enterocyte, iron uptake from lumen is reduced by the influence of hepcidin either directly or via IRP/IRE pathway (Mena et al., 2008). Iron export into blood stream is blocked by hepcidin adhered to FPN1 and thus, degradation of the latter (Nemeth et al., 2004b). Cytokines, in particular IL-10, trigger the expression of TfR and ferritin (Weiss & Goodnough, 2005), likely by their influence on the IRP/IRE-complex. Expression of Tf is also increased in inflammation, but mechanisms by which its expression is stimulated are unknown (Sheikh et al., 2007). Iron efflux from hepatocytes and especially macrophages is blocked at the site of FPN1 by hepcidin. Within the macrophages, important changes occur: TfR1 and DMT1 are highly expressed, and the uptake of haem iron/senescent erythrocytes is increased at the site of CD163. HIF maybe destabilized, and in turn, induce a reduced EPO expression in the kidney, which in turn leads to a decreased erythropoiesis (Weiss & Goodnough, 2005; Dallalio et al., 2006). Whether hepcidin directly interferes in erythropoiesis, is debated (Kato et al., 2008).

Valore & Ganz (2008) could show that other factors, such as the hepatic prohormone convertase furin, are involved in the protein processing of hepcidin. An inhibitory effect of hepcidin translation was described. However, in convertase free cell
cultures, HIF alone could not induce an inhibition of translation. Noteworthy is a very recent study conducted in humans, where hepcidin mRNA expression was found to be increased in patients with anaemia of inflammation and correlated with reduced FPN1 expression (Theurl et al., 2008). These results indicate the importance of hepcidin within the macrophages with regard to iron sequestration. Taken together with findings from Wardrop & Richardson (1999) that *nramp* expression (for DMT1) is targeted to the membrane of the phagosome (the vacuole around the absorbed bacterium), the initially mentioned hypothesis of a defence mechanism by excluding pathogens from iron, may be extended: it is not only general deprivation outside of the macrophages but also iron poisoning of scavenged pathogens.

For the onset of anaemia of inflammation, the above described disturbance has to persist, but it does not fully explain the extent to which for instance erythropoiesis is modified; in general, anemia of inflammation is defined by three characteristics: a) the iron flow between tissues is altered; b) erythroid precursors respond poorly to
erythropoietin and proliferation of erythroid progenitor cells is impaired; c) half-life of circulating erythrocytes is decreased (Roy et al., 2003).

Figure 5 gives an overview on different steps involved in the onset of anaemia of inflammation. Although it is hypothesized that hepcidin may modulate some of these mechanisms and some cell culture evidence exists, this remains to be validated for humans. EPO responds to hypoxia and anaemia, probably mediated by HIF (Ratcliffe, 2002). In anaemia of inflammation, this response is blunted directly by interference of IL-1 and TNF-α due to cytokine-mediated reactive oxygen species that affect transcription factors (Weiss & Goodnough, 2005). The proliferation and differentiation of erythroid progenitors (burst-forming and colony-forming units) are impaired, predominantly by IFN-γ. Here, the underlying mechanism might include apoptosis, but direct toxic effects of cytokines (nitric oxide generation) may play a role as well (Weiss, 2002). The lifespan of circulating erythrocytes is decreased due to more aggressive phagocytosis; how cytokines exert their effect remains debatable (Roy & Andrews, 2005).

1.3.3 Malaria-iron interaction

It has been suspected for several decades now that severe malnutrition might be protective against malaria. Haemoglobinopathy prevalence data suggest that there might be a selection for such carriers in malaria-endemic regions. With regard to iron deficiency and reversal of the latter by iron supplementation, recent data from a big intervention trial in young children give raise to the hypothesis that there is a parasite-host link between host iron status and parasite development but also between parasite invasion and host iron status (Sazawal et al., 2006). In a recent review, Prentice and colleagues (2007) gathered available information on observational and intervention studies investigating whether alterations in iron status influence malaria status, and on the interconnection between malaria and haemoglobinopathies. The former shall be summarized in the following paragraph, but aim of this section is to look at the mechanistic host-parasite interactions with regard to iron homeostasis, with the focus on children.

The few observational studies in moderately malnourished infants/children yield an inconclusive picture, with studies demonstrating an increased, neutral or decreased effect on malaria risk with improved iron status (Prentice et al., 2007). Numerous studies using iron supplementation to prevent anaemia are reported, many of them in
malaria-endemic areas. In the three meta-analyses and reviews cited, a total of 50 studies (partly overlapping) were reported (Shankar, 2000; Oppenheimer, 2001; Gera & Sachdev, 2002). Heterogeneity of dose, duration, supplementation regimen, age group studied and inclusion criteria of the original articles may weaken the results of these analyses. Overall, there was a non-significant trend to increased risk ratios for clinical malaria attacks and for parasitaemia. Results of the individually assessed trials are equally equivocal (Prentice et al., 2007). If studies including children of schoolage are looked at, the above discrepancies do not disappear: one study administering a bolus dose of iron (900mg as FeSO4/d for 30d) to initially non-parasitaemic anaemic individuals (11-60y) reported an odds ratio of 14.8 for suffering from clinical malaria despite the small sample size (approx 70/group; Murray et al., 1978); a study in 8-12y old children given 200mg iron as FeSO4/d for 16 weeks (n=318; intervention vs. placebo) found overall decreased parasitaemia, but reduction was smaller in the iron treated group (Harvey et al., 1989); a smaller study (n=87; intervention vs. placebo) administering 150mg iron as FeSO4/d for 14 weeks reported no negative effects on malaria morbidity (Lawless et al., 1994). Although conducted in a different age group, it is interesting to note that in the ID subgroup of the study conducted by Sazawal et al. (2006), iron supplementation did not increase risk for morbidity and mortality but significantly decreased it.

The human host is infected with malaria by the byte of an infected female Anopheles mosquito; 60 out of 400 Anopheles species worldwide are malaria vectors, but only four malaria species can infect humans (Tuteja, 2007). Figure 6 depicts the whole life cycle of the malaria parasite. For simplicity and because it is the predominant malaria sub-species in Africa, the figure depicts the stages of P. falciparum (Pf), which is similar for P.malariae, P. vivax and P.ovalae. For the two latter, the main difference is that they can latently persist in the liver and cause delayed blood stream release. Pf is not only the most widespread species in Africa, it generally has the most severe consequences: anaemia is most severe with this species; small blood vessels can be obstructed by heavily infected erythrocytes (a pathogenic mechanism that may cause cerebral malaria) and even uninfected erythrocytes show reduced deformability, which in turn can lead to clearance of uninfected erythrocytes (Miller et al., 2002). The pathogenesis of malarial anaemia has already been briefly discussed in 1.1.6. and thus, only aspects with regard to alteration in iron metabolism are discussed.
The infection with malaria parasites has several potential interferences with the iron metabolism: a) the parasites cause the launch of the acute phase response, maybe already at the time of injection, but at the latest during their liver passage (Ghosh, 2007), leading to increased cytokine levels, in particular of IL-6 (Wenisch et al., 1999; Lyke et al., 2004); b) in the liver, sporozoites pass through several hepatocytes before parasite maturation (Mota et al., 2001); although there is no clear evidence, the spatial co-localization of the parasite and the "heart" of iron metabolism regulation, allow the possibility of interactions; c) invasion and rupture of erythrocytes, and surface modification of uninfected erythrocytes, followed by macrophage phagocytosis (Lamikanra et al., 2007).

As with regard to malarial anaemia, the disturbance of the iron metabolism is dependent on the degree of malaria disease (asymptomatic vs. symptomatic), on the host immunity and other factors (Wickramasinghe & Abdalla, 2000). However, general patterns can be extrapolated for the different conditions.

Cytokine release impairs erythropoiesis in the first place (see Figure 5), but decreased erythroid progenitor production (burst-forming unit, colony-forming unit) and abnormal morphology has been described (Lamikanra et al., 2007). The secretion of hemozoin, a catabolite of haemoglobin produced by malarial parasites may be involved in impaired erythropoiesis as well, but mechanisms need to be elucidated (Ghosh, 2007).

With regard to erythropoietin production, data seems to be conflicting with studies reporting increased or reduced EPO production (Wickramasinghe & Abdalla, 2000). It is possible that these differing findings might be partly explained with age dependency of the host reaction to malaria infection (Nussenblatt et al., 2001). High EPO levels have been reported to increase survival in a cell culture (Wiese et al., 2008) and to reduce the complications of malaria in children (Casals-Pascual et al., 2008).
Literature review

Figure 6: Life cycle of *Plasmodium falciparum* [from (Center of Disease Control, 2008)]. Malaria sporozoites are transferred from *Anopheles* to the human during blood sucking 1. Sporozoites infect liver cells 2 and mature into schizonts 3, which rupture and release merozoites 4. After this initial replication in the liver (exo-erythrocytic schizogony A), the parasites undergo asexual multiplication in the erythrocytes (erythrocytic schizogony B). Merozoites infect red blood cells 5. The ring stage trophozoites mature into schizonts, which rupture releasing merozoites 6. Some parasites differentiate into sexual erythrocytic stages (gametocytes) 7. Blood stage parasites are responsible for the clinical manifestations of the disease. The gametocytes, male (microgametocytes) and female (macrogametocytes), are ingested by an *Anopheles* mosquito during a blood meal 8. The parasites’ multiplication in the mosquito is known as the sporogonic cycle C. While in the mosquito's stomach, the microgametes penetrate the macrogametes generating zygotes 9. The zygotes in turn become motile and elongated (ookinetes) 10 which invade the midgut wall of the mosquito where they develop into oocysts 11. The oocysts grow, rupture, and release sporozoites 12, which make their way to the mosquito’s salivary glands. Inoculation of the sporozoites 1 into a new human host perpetuates the malaria life cycle.
There is evidence that cytokines are involved in the sequestration of iron during malarial infection. In the above section, the importance of hepcidin as a mediator substance between cytokines and iron sequestration was illustrated. The extrapolation to the case of malaria seems plausible, but up to date only two publications addressing this issue could be identified. Oliveras-Verges & Espel-Masferrer hypothesize that elevated hepcidin levels in the liver inhibit the development of malaria infection (2008). They reason as follows: during hepcidin abundance, hepatocytes sequester iron, which inhibits sporozoite development in the liver. Unfortunately, this publication is purely theoretical and no additional evidence is presented. Extension of this hypothesis to macrophages, when schizonts have invaded erythrocytes, and increased phagocytosis occurs, could lead to the conclusion that increased iron import into macrophages occurs to cause iron poisoning of the schizonts. Interestingly, in a cross-sectional study a stronger correlation between urinary hepcidin and parasitaemia than between urinary hepcidin and haemoglobin was found, which supports the above hypothesis (Howard et al., 2007). This study is limited in that no iron status parameters were assessed. Further studies are needed to elucidate the role of hepcidin in malaria.

**Impact of host iron status on malaria parasites**

As stated at the beginning of this chapter, the malaria parasites depend on iron (and other nutrients) for their survival in the human body. A comprehensive review of the iron metabolism of Pf is given in Mabeza and colleagues (1999). The intraerythrocytic parasite is embedded within a vacuole and obtains the nutrients by ingesting host cell cytoplasm. Further, by modifying the erythrocyte membrane surface, it increases influx of several metabolites. Among these, Hb is proteolyzed into haem via falcipain, a cysteine protease, to obtain essential amino acids (Krugliak et al., 2002). The liberated haem is polymerized to form hemozoin, a non-toxic haem cristal. Over 95% of the iron biochemistry of Pf occurs in this acidic digestive vacuole, but evidence suggests that parasite-bioavailable iron originates from a labile iron pool in the erythrocyte rather than from the abundant haem iron (Scholl et al., 2005). The uptake mechanisms of this functional iron into the parasite are not very clear, but uptake from a labile intra-erythrocyte iron pool has been suggested (Mabeza et al., 1999). Information on how Pf absorbs iron during the liver cycle phase, where the first parasite replication takes place, is scarce. It appears that more iron-dependent
pathways are being carried out in the trophozoite stadium. Especially enzymes involved in DNA-synthesis (ribonucleotide reductase), Hb proteolysis, haem synthesis (δ-Aminolevulinate synthase) during the erythrocytic life stage require high amounts of iron (Mabeza et al., 1999).

The mechanisms of how iron deficiency influences parasite development remains unclear, since results from rat or mouse studies are equivocal. Hershko & Peto failed to demonstrate an effect of ID on malarial onset in rats (1988), whereas a recent study did not only find a marked decrease of P. berghei from 54% to 33% but also found an increased rate of clearance of infected erythrocytes from circulating blood in ID mice (Koka et al., 2007). The correlation between malaria reduction and shortened erythrocyte life span might be explained by similar findings in sickle-cell traits and β-thalasaemia: premature ageing and apoptosis leads to an over-proportional clearance of the ring stage-infected erythrocytes and subsequently interrupts the replication cycle (Ayi et al., 2004). Higher amounts of NTBI in rats did not increase susceptibility to malaria infection, indicating that iron originates from the inside of the erythrocytes (Hershko, 2007). The main limitation of animal studies with regard to malaria research is that Pf or other human malaria does not cause malaria in rats/mice and thus, other species need to be utilized, a practice that might limit extrapolation.

A further mechanism that is discussed to reduce schizont replication, is the inhibition of hemozoin crystal formation by ZPP IX (Iyer et al., 2003). The authors describe a manifold increase in ZPP IX due to thalasaemia, ID, increased haemolysis and chronic inflammation. Although this might not hold for malarial infection, where a recent study reported no increase in ZPP after infection (Hiremath et al., 2006), this theory could help to explain the potential protective effect of ID.

Looking at the influence of the host iron status on the evolution of malarial disease, the nutritional immunity hypothesis may help to elucidate some aspects. However, iron plays various roles in the defence system: it helps producing the nitric oxides that are thought be important in pathogen defence but a the same time might weaken the immune response of some interleukins (Weiss et al., 1997; Nyakeriga et al., 2005). It is important to note that other micronutrients, such as vitamin A, vitamin E, riboflavin, folate, copper, selenium and zinc play a role in the defence cascade.
against malaria, and since deficiencies in several micronutrients often co-exist, a more holistically look at the immune hypothesis could contribute to a better understanding of its importance (Nussenblatt & Semba, 2002).

1.3.4 Impact of helminths

As discussed in chapter 1.1.6., all helminths relevant for SSA cause anaemia by inducing blood loss from the bladder (S. haematobium) or from the gastrointestinal tract (S. mansoni, soil-transmitted helminths). Outcomes from observational or intervention studies yielded equivocal results: in 120 Jakarta school-children treated with albendazole to clear infection with A. lumbricoides and T. trichiura, plasma concentrations of IL-1, IL-6, TNF-α and CRP were normal before and 10 days post treatment. Drug efficacy for the latter species was relatively weak; despite high prevalences of both species, infection intensity was relatively low (Karyadi et al., 1996). In a similar setting in younger children (high prevalence of A. lumbricoides and T. trichiura, low intensity), bimonthly anthelmintic treatment over the course of one year resulted in lowered AGP and total plasma protein. The study did not individually randomise and thus, baseline conditions of the treatment vs. placebo group were different (Northrop-Clewes et al., 2001). The two studies assessed different markers in that the first one used more markers of acute phase response, while the latter used slower reacting markers. From these studies, it could be hypothesized that the effect of helminths on inflammation is of low intensity, but might persist over a longer period of time.

The life cycle of helminths occurs in different anatomic sites, including skin, lung, intestinal mucosa and bladder. In order to pass from one tissue to another without being attacked by the host immune system, helminths are said to secrete immunomodulatory molecules to assure their survival in the host. This includes two conditions: the invasion of the helminths should not cause reactions with fatal consequences for the host, and the invader should not be killed. Two extensive reviews on immunology of helminths infections describe the secretion of these immunomodulators and hence, the creation of a helminth friendly environment in detail (MacDonal et al., 2002; Loukas et al., 2005). Cytokines involved in the host-response to most helminths infections include IL-4, -5, -9, -10 and -13, as well as IFN-γ. The latter was discussed above to be directly involved in the onset of anaemia of inflammation by impairment of erythroid progenitor production.
Literature review

At the site of iron absorption, a local inflammation short after infection was reported, but here again, the situation is not clear. On the longer term, it might even be that somehow, iron absorption is increased in order to keep the host alive (Crompton & Nesheim, 2002). With regard to the balance between optimal immune functions and reduced parasite iron availability, a recent study in hamsters found that severe dietary iron restriction impaired hookworm development, whereas mild restriction or adequate supply enhanced host susceptibility to disease (Held et al., 2006).

1.4 A strategy to overcome iron deficiency: nanoparticle approach

The above chapters revealed that anaemia in SSA is multifactorial and that maybe, the relative contribution of ID to the onset of anaemia is overestimated. Nevertheless, it surely plays a prominent role in the public health burden in SSA, since ID per se has detrimental effects, even before anaemia develops. This chapter shall briefly introduce into practices currently applied to reduce ID; subsequently, the focus shall be directed to one of these strategies, food fortification. Challenges of iron fortification and limitations of currently used fortificants will be addressed, followed by the discussion of a new type of fortificant, iron nanoparticles.

1.4.1 Rationale and objective

In general, four strategies to reduce micronutrient malnutrition and ID in particular are widely accepted: a) dietary modification and diversification; b) micronutrient supplementation; c) public health measures; and d) food fortification (WHO, 2006b).

a) A monotonous diet is usually poor in intake of varied macro- and micronutrients. Thus, increasing diversity and quality of consumed food brings advantages. To achieve this, three obstacles have to be overcome: i) varied food items have to be available on the local market, in the case of ID meat products and fruit; ii) this wider variety should not be remarkably more expensive, else the needy people cannot afford it; iii) target populations need to change their behavior. This strategy is thought to take considerable time to be implemented, but once working can be sustainable (Ruel, 2003).

b) Supplementation as an approach to reduce micronutrient deficiencies is regarded as being efficacious, but implies the disadvantage of continuous efforts needed to maintain such a programme functional. Supplementation is
an approach giving relatively high doses of one or several micronutrients in regular intervals, usually as pills, capsules, drops or syrups (WHO, 2006b). Often, supplementation is targeted, i.e. supplementation of a particularly vulnerable population group, such as infants or pregnant women. Such programmes have shown to be successful for delivering vitamin A to infants, children and postpartum women, and iron and folic acid to pregnant women. Vitamin A can be administered in bolus doses, as this is a fat-soluble vitamin and a body reserve can be established, which reduces administration of vitamin A capsules to 2-3 times per year. For a micronutrient that cannot be suitable stored by the body, more regular doses are required. In the case of iron, weekly supplementation of a relative high dose was proposed. However, with the recent results from a malaria-endemic region showing increased morbidity and mortality after iron supplementation, universal administration to children <5y is highly questionable in malaria endemic regions and thus, no longer recommended by WHO (2006c).

c) Improvement of the overall health situation of a given population by controlling parasites and ameliorating hygiene and sanitation results in reduced infections and overall morbidity. Particularly in the case of ID, a reduction of inflammation and blood loss may improve body iron homeostasis

d) The addition of micronutrients to a food item is termed fortification. Ideally, the fortified food is consumed in sufficient quantities so that the amount of the totally consumed micronutrients can have an effect, and the micronutrient added does not change the food, so that acceptability remains high; further, the fortificant should not considerably increase product costs. An example of a successful fortification programme is the worldwide salt iodization, where inadequate iodine intake could be reduced in many countries (Andersson et al., 2005). As stated above, the food item that is fortified (vehicle) should be consumed in amounts that allow adequate addition of the micronutrient (fortificant); the term “adequate” needs further refinement. Adequate means here that the effectively absorbed micronutrient should improve the respective micronutrient status. With regard to iron in school-age children (7 to 10 years), to illustrate this, should have a daily amount of 0.71 mg of iron effectively absorbed (WHO/FAO, 2004). Thus, besides the quantities of fortificant in the
vehicle, its bioavailability is also crucial for the equation that results in the amount of absolutely taken up micronutrients, in the above example 0.71 mg.

1.4.2 Limitations of conventional iron fortificants

Iron is probably the most challenging mineral in food fortification: to add a sufficiently absorbed compound in adequate quantities to meet the requirements and not to change the organoleptic properties of the vehicle, has shown to be a dilemma that is difficult to overcome (Hurrell, 2002c). Thus, to screen for usefulness of an iron fortificant, in a first instance, its bioavailability should be assessed. For this, a human absorption or rat depletion-repletion studies can be performed to yield a so-called relative bioavailability value (RBV), but other, faster methods can be used to screen for usefulness a range of compounds (Lynch et al., 2007). For the assessment of the RBV, results of the investigated compounds are compared to ferrous sulphate (FeSO₄) as the reference substance.

In a second step, the absolute iron absorption from the fortified food has to be calculated. This takes into account the RBV and the amount of iron added to the food to achieve adequate levels of fortification. Consequently, iron compounds with low RBVs need to be added in higher amounts per unit of food so that an appropriate level is warranted (Hurrell, 2007).

It is important to note that the availability in humans not only depends on the iron compounds, but also on the food matrix, where iron absorption enhancers and inhibitors can interfere, and on the iron and general health status of the host.

Currently available iron compounds can be assigned to five groups [modified from (Hurrell, 2002a)]:

a) Water-soluble compounds: these compounds have a high RBV and are usually cheap compound, but tend to cause the most organoleptic changes; examples are FeSO₄, ferrous gluconate, ferrous lactate, ferrous ammonium citrate.

b) Poorly water-soluble compounds: these are readily soluble in dilute acid and hence, usually show acceptable RBV, but a lower impact on sensory properties; examples are ferrous fumarate, ferrous saccharate and ferrous succinate.

c) Water-insoluble compounds: these are poorly soluble in dilute acids and therefore, may be not dissolved in the gastric juice; however, the dissolution
pattern of a given compound depends on its physical and chemical characteristics, such as particle size, surface area, purity. Organoleptically, these fortificants are stable, but their RBV is generally low: examples: elemental iron (electrolytic, H-reduced, carbonyl, CO-reduced, atomized) and ferric phosphate (orthophosphate and pyrophosphate).

d) Chelates: including sodium iron ethylenediaminetetraacetic (NaFeEDTA), ferrous bisglycine or ferric glycinate. In these compounds, iron is chelated and thus not vulnerable to absorption inhibitors, such as phytic acid. Iron chelators, particularly NaFeEDTA show very high absorption rates in inhibitory meals (high phytic acid or polyphenols), but cost and toxicity (EDTA) are issues, and in baked products organoleptic problems might occur.

e) Encapsulated iron-compounds: these are iron compounds that have been developed to optimize the RBV, while at the same time yielding good organoleptical properties. Usually, water-soluble iron compounds are embedded into a fat or carbohydrate matrix to build a physical/chemical barrier; capsule size is critical for many manufacturing processes (e.g. sieving during flour processing), and the capsule: iron ratio needs to be high enough to ensure adequate RBV; cost may be an issue.

For elemental iron compounds and iron pyrophosphates, several studies have been conducted to investigate the influence of particle size on RBV. In early studies, it was shown for elemental iron that reduction of mean particle size (as determined by dynamic light scattering) of approximately 60% to 7-10 μm increased RBV in rats by 50% (Motzok et al., 1975; Verma et al., 1977). Similar observations were made in the case of ferric pyrophosphates: in a rat study, three commercially available compounds were compared (Wegmuller et al., 2004). A reduction in mean particle size from 21 μm to 2.5 μm and 0.3 μm resulted in respective RBVs of 59%, 69% and 95%, although the first two were not significantly different from each other. In humans, the mean particle size reduction from 12.5 μm to 6.7 μm resulted in a non-significant trend of increased RBV of 42% and 52%, respectively (Fidler et al., 2004). The above findings and the intuitive assumption that a reduction in particle size and thus, increase in specific surface area, show that the rate of dissolution in the acidic milieu of the stomach is accelerated. This in turn means higher absorption rates and therefore, a drastic reduction of particle size down to the nanoscale of poorly water soluble or water-insoluble compounds would increase their bioavailability. How this
relates to the sensory aspects and influences the potential risks of toxicity, shall be addressed in the subsequent section. To start with, a short introduction in synthesis technologies of iron nano-compounds is given.

1.4.3 Synthesis technologies of iron nano-compounds

Nanoparticle synthesis is a field that has application in a wide range of products. With regard to this review, food and pharmaceutical applications are of prominent interest. In pharmaceutical drug development, the poor water solubility of many drugs is a challenge, and drug dissolution enhancement is of special interest (Rasenack & Muller, 2004).

Nanosized metal material comprises metals with particle sizes below 100 nm, a range that also modulates surface reactivity and other material properties (Li et al., 2006). There are two basic approaches to synthesize iron nanoparticles, a) physical and b) chemical methods.

a) physical methods: these include inert gas condensation whereby a metal is evaporated in an inert gas atmosphere and condensed, high energy ball-milling and ultrasound shot peening where high-frequency ultrasound is applied to cause mechanical loads.

b) chemical methods: these include reverse micelle synthesis, controlled chemical precipitation, chemical vapour condensation whereby a metal is evaporated in a reactive gas atmosphere and reacts with it before condensation, and liquid flame spray (flame spray pyrolysis; FSP).

Often, the method chosen depends on the desired compounds: iron nano-compounds with an organic component will require milder method, such as the micelle synthesis or the controlled chemical precipitation. Elemental iron nanoparticles can be achieved for instance by ultrasound shot peening or inert gas condensation; however, due to their high reactivity, elemental iron particles need to be kept in an inert gas atmosphere. Iron oxides and phosphates and iron compounds integrating other metals (Mg, Zn, etc.) are best produced by flame technology.

Today, about 90% of the value and volume of fine particles are generated by flame technology in the gas phase (Strobel & Pratsinis, 2007), probably due to the ease of scale-up and the wide range of elements nanoparticles can be produced of (Mueller et al., 2003).
FSP allows production of single or mixed metal oxide powders of a wide range in particle size, its scale-up is relatively easy and likely to be at low cost (Madler et al., 2002). This comprehensive publication reviews the physical background of FSP. A schematic is reproduced in Figure 7. The liquid precursor is composed of a combustible and the precursor substance(s) that is (are) dissolved in the combustible itself or in an additional solvent. The precursor is pumped through a fine capillary and at the capillary exit, fine dispersion of the liquid into small droplets occurs through the dispersion gas that flow at a high rate. Simultaneously, the droplets are ignited at the methane/oxygen flamelets. Through the heat and diminishing space due to reducing particle diameter, a chemical reaction can occur, if several metals are mixed in the precursor, or, after evaporation, metals are oxidized if the dispersion gas is oxygen. If the dispersion gas is an inert gas, oxidation can be minimized and other particle composition can be obtained. Primary particles subsequently aggregate or agglomerate, before they reach the filter, where they are collected.

Figure 7: schematic of a set-up of flame spray pyrolysis using an oxygen-assisted nozzle and two supporting CH4/O2 flamelets [modified from (Madler et al., 2002)].
Literature review

Particle size is adjusted by changing the flow ratio of liquid precursor: dispersion gas; as a thumb rule the following holds: the higher the ratio, the larger the particles. However, this relationship is non-linear and has its limits: towards smaller diameters, the amount of combustible may become insufficient to promote evaporation, the limit of larger diameters is due to an oversaturated flame, where precursor reaches either the filter as a liquid or the flame emits soot.

1.4.4 Nanoparticles in nutrition

Potential benefits in nutrition

Nanoparticles find broad applications in the food industry, to improve physical properties of packaging material, as sensors of toxicity or bacterial growth in packaging, as texture building components, as flavour enhancer; but also in the field of functional foods and nutrition, a wide range of potential opens up: enhanced bioavailability of poorly absorbed compounds or encapsulation to protect and targetedly deliver food ingredients to specific site of actions (Chen et al., 2006a). However, many of the developments originate from pharmaceutical research and may be too expensive to implement on a commercial scale (Tarver, 2006). Nevertheless, it was prognosed in 2006 that the market volume of nanofood would develop from approximately 7 billion US$ in 2006 to more than 20 billion US$ in 2010 (Tiju & Morrison, 2006).

In this review, only potential nutritional applications are discussed, and the first field is targeted delivery. A delivery system must fulfil several functions: it is a vehicle to the functional ingredient and carries it to the site of action; it builds a physical/chemical barrier to protect the ingredient from degradation during processing, storage or utilisation prior to release; it may need to be capable of controlled release, such as release rate or release triggered by specific environmental conditions (pH, temperature, ionic strength); it should be compatible with the surrounding food product in terms of qualitative attributes, such as sensorial aspects (Weiss et al., 2006). Examples of this type of application are the nano-emulsions, which in principle are nothing but small micellled emulsions. They are used to render lipophylic substances hydrophilic by surrounding them with bipolar surfactants, a procedure that allows using hydrophilic substances in an aqueous system, or vice versa for
hydrophilic substances in oil-based systems. The advantage of nano-sized micellae in comparison to conventional ones is the increased surface area and thus increased interaction with the human body (McClements, 2005).

In contrast to the above micro- and nanoemulsions for food ingredient delivery, the nanoscaling of food ingredients to enhance their bioavailability has not found broad applications yet. However, in pharmaceutical drug development, this concept has gained importance and a relationship between particle size (and surface area) to bioavailability has clearly been established (Kesisoglou et al., 2007). A contrast to the high potential of improving applications stands the potentially increased health risk of nanoparticles, an issue that is discussed in the next section.

_Potential risks for human health_

As discussed above, downsizing of material to the nanoscale gives it unusual physicochemical properties due to the small size together with increased surface area, particle size distribution, surface reactivity, solubility, shape and aggregation behaviour. It is these properties of nanomaterials that raise concerns about adverse effects on biosystems, such as humans (Nel et al., 2006). From ambient nanoparticles, a reasonable amount of research on risk assessment is available with regard to particle inhalation (Oberdorster et al., 2005; Nel et al., 2006), and often, risk assessments yielded elevated risks for health (Araujo et al., 2007; Gong et al., 2007; Bernstein et al., 2008). The difficulty when talking about risk assessment of nanomaterial is that this material is often made of well characterized substances, but what is different, is “only” their behaviour due to the small size. Thus, extrapolation from the macromaterial to the nanomaterial may be indicative for potential substance toxicity, but effective toxicity of the nanosized material cannot be predicted. The same accounts for extrapolation from one nanomaterial to another: many parameters can change (size, shape, surface reactivity) that modify the effect of the human organism (Tran et al., 2007). From research on inhaled particles, it is known that transcytosis across epithelium and transport into blood occurs, but largely depends on surface chemistry and _in-vivo_ modification (Hoet et al., 2004).
Less knowledge is present about consequences of ingestion, but it is widely recognized that particle uptake through the gastrointestinal-tract is possible, although to a restricted extent, since elimination of inert particles through gut is high (Yamago et al., 1995); surface charge, size and composition of the nanoparticle determine the ease of uptake (Hussain et al., 2001). It was shown that small, inert nanospheres of polystyrene reached the apical membranes of the GI surface epithelium in a very short time, but then stuck to them (Szentkuti, 1997). Further pathways for inert particles are described in a review, where transcellular uptake through normal enterocytes and Peyer’s Plaques take place (Hussain et al., 2001). Several studies on the influence of size and surface charge on the absorption of inert material have been published, and a size dependency could clearly be shown (Hodges et al., 1995; Hillyer & Albrecht, 2001). All the above literature deals with inert or biologically non-reactive nanomaterials such as polystyrene. Information on how nanomaterial that interacts with an organism behaves is very scarce. An acute toxicity study in mice that were administered gastrointestinally a metallic zinc nanoparticle (approx. 60nm) versus zinc microparticle (1080nm) suspension of high concentrations found a higher lethality for the nanoparticles (Wang et al., 2006). Metallic zinc is very poorly soluble and thus, the compound used in this study was probably biologically inert. For acute toxicity of copper particles of varying sizes, a high correlation between size distribution and oral toxicity was found in rats (Chen et al., 2006b). Information on chronic toxicity assessment after ingestion via the gastrointestinal tract could not be found.

An important difference between iron and another mineral that should exert its biological role and the above materials is that the iron has to be dissolved in the gastric juice. Thus, what is increased by the down-scaling to nanosize is primarily the solubility due to increased surface area. The potential toxicity originates from a particle that has not been dissolved prior to reaching the jejunum, or has passed cell membranes during chewing and swallowing process and is thus absorbed as particulate material. Risk assessment trials for oral uptake are limited, since the majority concentrated on uptake kinetics (Jani et al., 1990), but methods are derived from other fields of particle research. The classical approach is to investigate behavior and translocation microscopically and to estimate the potential hazard caused by this translocation (Hodges et al., 1995; Simon et al., 1997). Nel and colleagues (Nel et al., 2006) postulate particle-induced oxidative stress as the base for studying nanomaterial
toxicity, as nanoparticles create reactive oxygen species. However, their theory is mainly based on lung exposure and is then extrapolated to other uptake pathways. Suggested markers are lipid peroxidation and cytokines as well as signs of inflammation. Recently, the impact of nanoparticles on gene expression has been discussed as a valid tool for low dose-long term exposure (Cui, 2007).
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2 COMPARISON OF MANUAL AND AUTOMATED ELISA METHODS FOR SERUM FERRITIN ANALYSIS

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Abstract

Serum ferritin concentration is a sensitive measure of body iron stores. The aim of this study was to compare the performance of two commercially available enzymelinked immunoassays (ELISAs) for serum ferritin: a widely used manual assay kit (Spectro Ferritin MT®), and a new fully automated assay (ImmunoLite®). We analyzed serum samples from Moroccan school-aged children (n=51) from a rural area with a high prevalence of iron deficiency anemia (IDA). Four replicates of each sample were analyzed using both assays. For the manual method, the interassay repeatability was 24%, 22%, and 11%, and intraassay precision was 18.3%, 9.2%, and 9.1% at increasing serum ferritin concentrations. Using the automated assay, the interassay repeatability was 7%, 6%, and 6%, and intraassay precision was 1.5%, 5.4%, and 5.5% at increasing serum ferritin concentrations. The two assays were well correlated (y=1.16x+1.83; r=0.98). However, the limits of agreement (LOAs) were wide, particularly at low concentrations. A comparison of the assay results with recommended cutoffs for serum ferritin generated sharply different estimates of the prevalence of iron deficiency (IDA) in the sample. We conclude that the automated assay has several potential advantages compared to the manual method, including better precision, less operator dependence, and faster sample throughput.
Introduction

Iron deficiency anemia (IDA) is a major public health problem worldwide (1). Serum ferritin concentration is the recommended screening test to identify iron deficiency (ID), and a serum ferritin <15 μg/L in the presence of anemia indicates IDA (1, 2). The utility of serum ferritin for identifying ID is well established, and its sensitivity and specificity may be as high as ≈92–98% compared to bone marrow biopsy (3). Several assays for serum ferritin are available, all of which are based on immunochemical principles. Few data are available comparing the performance of the available assays (4), particularly in children. In our previous large surveys of iron status in west and north Africa (5, 6), we used a manual enzyme-linked immunoassay (ELISA) method to measure serum ferritin, but the assay is labor-intensive and highly operator-dependent, and its precision is variable at low concentrations. A fully automated, ELISA-based method has recently become available. In this study the performance of the automated assay was compared with a widely used manual serum ferritin assay.

Materials and methods

Sample Characterization

Whole blood was collected by venipuncture into EDTA-containing tubes in October 2003 from children in northern Morocco during a large cross-sectional screening of iron status. The mean age of the children was 9 years (range=6–14 years). Blood samples were transported on ice to the regional hospital laboratory. After centrifugation on the day of collection, serum samples were aliquotted and frozen at −20°C until they were analyzed. The samples were defrosted and analyzed with the two serum ferritin methods on the same day.

Laboratory Analyses

From the screening, we selected serum samples (n=51) that represented a range of serum ferritin values of ≈0–100 μg/L. For the comparison study, serum ferritin was measured using two methods: a manual method (Spectro Ferritin MT®; Ramco Laboratories, Houston, TX), and a fully automated method (Immulite®; Diagnostics Products Corporation, Los Angeles, CA). Both assays were done following the
manufacturer's instructions. For the Ramco assay, three-level WHO reference controls (WHO control serum; Ramco Laboratories, Houston, TX) were used (15±6 μg/L, 83±21 μg/L, and 330±99 μg/L) as external controls. For the Immulite assay, three-level reference controls provided by the manufacturer were used (34±3 μg/L, 135±10 μg/L, and 287±16 μg/L). The samples were analyzed in four replicates for each method, except for the controls, in which duplicates were analyzed.

**Statistical Analyses**

Data processing and statistics were done using Excel 2002 (Microsoft Inc. Redmond, WA) and SPSS 10.0 (SPSS Inc., Chicago, IL). Statistical analysis was done according to the method of Bland and Altman (7), with slight modifications. The absolute differences of the values in the samples analyzed by the two methods were calculated. The difference of the highest to the lowest value obtained with each method within the four replicates, as well as the difference of the center values of the four replicates was calculated. Limits of agreement (LOA) were calculated using

\[
\delta - 2s = LOA_{\text{low}}
\]

\[
\delta + 2s = LOA_{\text{high}}
\]

where \(\delta\) is the mean of the difference between the two methods, and \(s\) is the standard deviation (SD) of this difference. Interassay precision expressed as RSD of the mean of all performed measurements was calculated using the quality control sera for different levels. Intraassay precision expressed as RSD was based on four consecutive measurements of the same sample.

**Results**

The interassay repeatability (n=30) was 24%, 22%, and 11%, and the intraassay precision (n=4) was 18.3%, 9.2%, and 9.1% for control sera at levels of 15, 83, and 330 μg/L using the manual assay. The interassay repeatability (n=14) was 7%, 6%, and 6%, and the intraassay precision (n=4) was 1.5%, 5.4%, and 5.5% for control sera at levels of 34, 135, and 287 μg/L using the automated assay. The slope (±SD), intercept (±SD), and correlation coefficient of the linear regression between the two methods was \(y=1.16 (±0.03)x+1.83 (±1.67)\) and \(r=0.98\) (manual=y, automated=x; individual data points calculated from the median of four replicates). Figure 1 shows
the difference in the medians of the two methods (based on four replicates), including the LOAs (−8.5±16.8).

Figure 1. Difference of medians of two methods (based on 4 replicates), including the limits of agreement (-----).

To investigate the stability of the replicate results, and hence the stability of the method, we calculated the difference of the extreme replicate values and the central values (x₁ and x₄ are the highest and lowest replicate values, respectively; Table 1). All reference control values were within the acceptable range, except for one high control measured by the manual assay.
Table 1. Means calculated for the difference between the highest and the lowest replicate values, the two center values, and the standard deviation.

<table>
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<tr>
<th></th>
<th>Ramco (μg/L)</th>
<th>Immulite (μg/L)</th>
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<td>$x_1-x_4^b$</td>
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</tr>
<tr>
<td>SD$^b$</td>
<td>3.49</td>
<td>0.54</td>
</tr>
</tbody>
</table>

$^a$Mean calculated for all samples (n=51).
$^b$Mean calculated for samples with ferritin concentration=20 μg/L (n=16).

Discussion

The LOAs between the two assays were wide, particularly considering that the recommended WHO cutoff value for serum ferritin for identifying ID is 15 μg/L (1). In screening for ID in our sample using this cutoff, the two assays produced sharply different estimates of prevalence: the prevalence of ID was 13.7% using the manual assay, and 27.5% using the automated assay. With the use of these assays, measurements of longitudinal changes in iron status within a population group are minimally affected by bias. Our findings suggest that the automated ELISA method for serum ferritin has several advantages over the manual assay, including 1) better precision, which is particularly important at low serum ferritin concentrations; 2) less operator dependence; and 3) faster sample throughput.

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3 MILD RIBOFLAVIN DEFICIENCY IS HIGHLY PREVALENT IN SCHOOL-AGE CHILDREN BUT DOES NOT INCREASE RISK FOR ANAEMIA IN CÔTE D'IVOIRE

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Abstract

There are few data on the prevalence of riboflavin deficiency in sub-Saharan Africa, and it remains unclear whether riboflavin status influences the risk for anaemia. The aims of this study were to: (1) measure the prevalence of riboflavin deficiency in children in south-central Côte d'Ivoire; (2) estimate the riboflavin content of the local diet; and (3) determine if riboflavin deficiency predicts anaemia and/or iron deficiency. In 5- to 15-year-old children (n=281), height, weight, haemoglobin (Hb), whole blood zinc protoporphyrin (ZPP), erythrocyte glutathione reductase activity coefficient (EGRAC), serum retinol, C-reactive protein (CRP) and prevalence of Plasmodium spp. (asymptomatic malaria) and Schistosoma haematobium (bilharziosis) infections were measured. Three-day weighed food records were kept in twenty-four households. Prevalence of anaemia in the sample was 52%; 59% were iron-deficient based on an elevated ZPP concentration, and 36% suffered from iron deficiency anaemia. Plasmodium parasitaemia was found in 49% of the children. Nineteen percent of the children were infected with S. haematobium. Median riboflavin intake in 5- to 15-year-old children from the food records was 0.42 mg/d, ~47% of the estimated average requirement for this age group. Prevalence of riboflavin deficiency was 65%, as defined by an EGRAC value >1.2. Age, elevated CRP and iron deficiency were significant predictors of Hb. Riboflavin-deficient children free of malaria were more likely to be iron deficient (odds ratio; 3.07; 95% CI 1.12, 8.41). In conclusion, nearly two-thirds of school-age children in south-central Côte d'Ivoire are mildly riboflavin deficient. Riboflavin deficiency did not predict Hb and/or anaemia, but did predict iron deficiency among children free of malaria.
Introduction

Riboflavin (vitamin B₂) is required for many metabolic pathways, usually as a precursor of the flavin coenzyme, NAD, in redox reactions (McCormick, 1997). Riboflavin deficiency is particularly common in regions where intakes of dairy products and meat are low (Neumann et al. 2003). Deficiency causes impaired growth, cheilosis, angular stomatitis, glossitis, dermatitis and impaired vision (Venkataswamy, 1967; Batey et al. 1992; FAO/WHO, 2001). Schoolchildren, in both developing and industrialised countries, are an age group at high risk for riboflavin deficiency (Powers, 2003).

In Western Africa, anaemia affects 50–80 % of the children (ACC/SCN, 2000; WHO/UNICEF/UNU, 2001). There are many aetiologies of anaemia in African children. These include malaria, blood loss from parasitic infections, vitamin A deficiency and diets low in bioavailable iron (ACC/SCN, 2000). Riboflavin deficiency may also impair erythropoiesis and contribute to anaemia (Foy & Kondi, 1953; Foy et al. 1964). Suggested mechanisms for this effect of riboflavin deficiency are decreased mobilisation of iron from stores (Sirivech et al. 1974; Powers, 1995), decreased iron absorption and increased iron losses (Adelekan & Thurnham, 1986; Powers et al. 1988; Butler & Topham, 1993). Whereas these mechanisms have been investigated in animals, there are few data from human studies. Riboflavin supplements given concurrently with iron improve the response to iron supplementation in pregnant and lactating women, adult males and schoolchildren (Buzina et al. 1979; Charoenlarp et al. 1980; Ajayi et al. 1990). A stable isotope study in adult Gambian men showed a greater increase in Hb values with riboflavin supplementation compared with controls (both groups received iron supplements), but failed to demonstrate an increase in iron absorption, suggesting that the improvement in Hb may have been due to iron mobilisation from stores (Fairweather-Tait et al. 1992).

The aims of the present study were to: (1) determine the prevalence of riboflavin deficiency in school-age children in southern Côte d'Ivoire; (2) estimate the riboflavin content of the local diet; and (3) investigate if riboflavin status contributes to anaemia or iron deficiency in this population.
Subjects and methods

Study site
The study sites were two villages in Côte d'Ivoire from geographically distinct regions. Orbaff is a rural village in the Dabou district of Côte d'Ivoire, 10 km from the southern coast. The climate is tropical, with a temperature of ≥ 27°C and a relative humidity of ≥ 80 % much of the year. Plantain, rice, yam and dried, smoked fish are eaten regularly. Bringakro is further north (District Toumodi), about 180 km northwest of Abidjan. The region is situated in the transition zone from rainforest to savannah, with a mean temperature of 27°C and a relative humidity of 70 %. Staple foods in this area are yam, plantain, cassava and red palm oil. The surveys were conducted in May 2004 in Orbaff (Wegmuller et al. 2006), and in May 2005 in Bringakro.

Subjects and enrolment
In both villages, a random sample was taken from children attending grades 1–5 in primary schools, with 50 and 28 % of children in these grades enrolled in Bringakro and Orbaff, respectively. Informed written consent was obtained from the school directors and the parents of the children. The Ethical Committee of the Swiss Federal Institute of Technology in Zürich, Switzerland, and the Scientific Council of the CSRS in Abidjan, Côte d'Ivoire gave ethical approval for the study. A total sample size of 280 children was calculated assuming a change in Hb of 3 g/l as clinically relevant, a standard deviation (sd) of Hb of 12 g/l, a significance level of 95 %, and a power of 75 % for a two-sided outcome.

Screening
Weight and height were measured upon registration (surname, first name, sex, date of birth) of the child. For these measurements, children removed their shoes and wore light clothing. Weight was recorded to the nearest 0·1 kg (Skyline, Soehnle-Waagen GmbH & Co. KG, Murrhardt, Germany), and height to the nearest 0·5 cm (Person check®, KaWe, Asperg, Germany) by the local nurse and an experienced technician. Age was obtained from the school registers, where age is taken from birth certificates. Venous blood samples (7·5 ml) were drawn in the morning into EDTA-treated evacuated tubes, and blood samples were kept in the dark on ice during transport to a nearby laboratory. A morning casual spot urine sample was

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collected for measurement of microhaematuria (as an indicator for bilharziosis). After the screening, children with iron deficiency were given supplemental oral iron for 12 weeks (daily 60 mg of iron as FeSO₄), and children with bilharziosis were given a single dose of praziquantel (40 mg/kg).

**Dietary assessment**

Three-day weighed food records were collected in Orbaff in 24 randomly selected households. The records were done on three consecutive days (including two weekdays and one weekend day). In the village, family meals are traditionally eaten from shared bowls, but to estimate individual food intake during the 3 days of recording, families ate from individual bowls. Edible portions of all food and beverages were weighed using a Soehnle scale (Vera 67 002, Soehnle-Waagen GmbH & Co. KG, Murrhardt, Germany) accurate to ± 1 g during preparation and consumption. Meals typically consisted of a cooked staple and a sauce. When the meal was ready, the total weight of the cooked staple and sauce was weighed. After the meal, the food that remained uneaten was weighed and the proportion of staple to sauce was estimated by a team of experienced investigators. Subjects absent during the day were asked about their food intake and amounts eaten outside the home; these estimations were then added to the food records. Riboflavin intake was calculated using food composition tables (Toury *et al.* 1965; FAO, 1968; Bundesinstitut für gesundheitlichen Verbraucherschutz und Veterinärmedizin, 1996) and food analysis software EBISpro (University of Hohenheim/Stuttgart, Germany).

**Laboratory analysis**

In Orbaff, Hb measurement was performed on the day of blood sampling on an AcT8 Counter (Beckman Coulter, Krefeld, Germany) using three-level controls provided by the manufacturer. In Bringakro, because the AcT8 Counter was not available, the Hemocue system was used (Hemocue, Angelsborg, Sweden) using two-level controls provided by the manufacturer. Venous blood was used for both methods; values obtained by these two Hb methods from venous blood are highly correlated (Morris *et al.* 2001). Zinc protoporphyrin (ZPP) was measured on washed red blood cells within 7 d of blood sampling in duplicate with a haematofluorometer (Aviv Biomedical, Lakewood, NJ, USA) (Hastka *et al.* 1992), with standards provided by
the manufacturer. C-reactive protein (CRP) in samples from Bringakro was measured using an automated chemiluminescent high sensitivity immunoassay system (IMMULITE®, Diagnostic Products Corporation, Los Angeles, CA, USA), and Orbaff CRP samples were measured using nephelometry (TURBOX®, Orion Diagnostics, Espoo, Finland). Anaemia was defined as Hb < 120 g/l in children aged ≥ 12 years, and Hb < 115 g/l in children aged 5–12 years (WHO/UNICEF/UNU, 2001). Iron deficiency was defined by a ZPP value >40 µmol/mol haem (Hastka et al. 1992). CRP values < 10 mg/l were defined as normal (Pepys, 1981). Serum retinol (SR) was measured by HPLC (Merck-Hitachi, Tokyo, Japan) according to Tanumihardjo et al. (2004) with reference material from the National Institute of Standards and Technology (Gaithersburg, MD, USA); vitamin A deficiency was defined as an SR < 0·70 µmol/l, and low vitamin A status as an SR < 1·05 µmol/l (WHO, 1996). Riboflavin was measured by the erythrocyte glutathione reductase activity coefficient (EGRAC) assay using a modification of the method of Dror et al. (1994). In our laboratory, the inter- and intra-assay CV of the EGRAC method is 3 and 4 %, respectively. Although there is no consensus for EGRAC cut-off values for riboflavin deficiency (Tillotson & Baker, 1972; Prasad et al. 1992; Sauberlich, 1999), we used cut-offs specified for this EGRAC assay (Dror et al. 1994), i.e. values >1·2 indicated marginal deficiency and values >1·4 indicated clear deficiency (Sauberlich, 1999). For statistical analysis, EGRAC values >1·2 were used. On the day of blood sampling, whole blood was used to prepare a thick and a thin smear for malaria parasites by the Giemsa coloration technique (WHO, 1990). Parasites were counted against leucocytes; fields containing >200 leucocytes were counted and if < 10 parasites were identified, the counting continued up to 500 leucocytes. These counts were converted to the number of parasites per µl of blood, assuming a leucocyte count of 8000/µl (WHO, 1990). On the day of blood sampling, haematuria, as an indirect marker for infection with Schistosoma haematobium, was assessed semiquantitatively by dipstick (Combur 7, Roche Diagnostics GmbH, Mannheim, Germany).

Statistical analysis
Data processing and statistical analysis were done using Excel (XP 2002; Microsoft, Redmond, CA, USA) and SPSS 13.0 (SPSS Inc., Chicago, IL, USA). Height-for-age and weight-for-age Z-scores were calculated using Epi-Info (Epi-Info Version 3.3.2,
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Centers for Disease Control and Prevention, Atlanta, GA, USA). All data except ZPP were normally distributed and are presented as means and their SD. ZPP data were skewed and are presented as medians (ranges); for statistical analysis, these values were log-transformed. One-way ANOVA was used to test for differences for all scaled variables among the different geographic areas; the Mann–Whitney test was applied for ordinal data. Two-tailed Pearson's correlation coefficients were used to examine the relationship between iron status or anaemia and riboflavin status, malaria, inflammation, SR and haematuria. Stepwise linear regression on Hb and logZPP was performed with age, sex, EGRAC, SR, CRP, malaria and Hb or logZPP as independent variables. Odds ratios for the risk of anaemia or iron deficiency in riboflavin deficiency were calculated. P values < 0.05 were considered statistically significant.

Results

Three day weighed food records
The 3 d weighed food records were kept in 24 families comprising 207 subjects (median age: 16 years; range: 2–81 years); the results for children 6–15 years of age (n 71) are reported here. Daily intakes of riboflavin ranged from 0.17 to 1.16 mg, with a median of 0.42 mg, representing 70 and 47 % of the estimated average requirement for children 6–8 years of age and those aged 9–13, respectively (Institute of Medicine, 2001). Riboflavin intake was low due to infrequent consumption of animal foods, with the exception of smoked fish, and the negligible riboflavin content of cassava, the dietary staple. Median (range) iron intake was 9.8 mg (3.6–19.8) mg/d, well above the estimated average requirement of 6.3 mg/d for 6- to 15-year-old children. Only 21 % of children had iron intakes below the estimated average requirement. However, the bioavailability of the dietary iron was probably low, as 90 % of iron was non-haem iron.

Anthropometry
Mean age of the screened children was 10.2 (SD 2.3) years, mean weight and height were 31.8 (SD 10.1) kg and 1.38 (SD 0.15) m, respectively. Forty-four percent of the children were female. As shown in Table 1, the children from the two villages differed significantly in sex ratio, age, weight and height. However, height-for-age as well as
weight-for-age Z-scores were not significantly different. Eight percent of the children were stunted (height-for-age Z-score less than –2 SD) and 11% were undernourished (weight-for-age Z-score less than –2 SD).

Table 1: Anthropometric, nutritional and health characteristics of Ivorian children in two villages of Côte d'Ivoire: Orbaff, in the southern tropical zone, and Bringakro, in the savannah transition zone

<table>
<thead>
<tr>
<th></th>
<th>The two villages (n 281)</th>
<th>Orbaff (n 171)</th>
<th>Bringakro (n 110)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age* (years)†</td>
<td>10.2 (2.3)</td>
<td>9.9 (2.3)</td>
<td>10.8 (2.2)</td>
</tr>
<tr>
<td>Height* (m)†</td>
<td>1.38 (0.15)</td>
<td>1.37 (0.14)</td>
<td>1.41 (0.17)</td>
</tr>
<tr>
<td>Weight* (kg)†</td>
<td>31.8 (10.1)</td>
<td>30.4 (8.7)</td>
<td>33.9 (11.6)</td>
</tr>
<tr>
<td>Height-for-age Z-scores</td>
<td>-0.3</td>
<td>-0.2</td>
<td>-0.4</td>
</tr>
<tr>
<td>Prevalence of stunted children, less than –2 SD (n (%))</td>
<td>23 (8)</td>
<td>14 (8)</td>
<td>9 (9)</td>
</tr>
<tr>
<td>Weight-for-age Z-scores</td>
<td>-0.6</td>
<td>-0.6</td>
<td>-0.7</td>
</tr>
<tr>
<td>Prevalence of undernourished children, less than –2 SD (n (%))</td>
<td>27 (10)</td>
<td>15 (9)</td>
<td>12 (11)</td>
</tr>
<tr>
<td>Sex ratio (female:male)†</td>
<td>0.8</td>
<td>0.7</td>
<td>1.0</td>
</tr>
<tr>
<td>Hb (g/l)*</td>
<td>116 (12)</td>
<td>117 (12)</td>
<td>115 (12)</td>
</tr>
<tr>
<td>Zinc protoporphyrin (mmol/mol haem)††</td>
<td>49.5 (17-296)</td>
<td>53.0 (24.296)</td>
<td>45.2 (17-289)</td>
</tr>
<tr>
<td>Prevalence of anaemia (n (%))</td>
<td>145 (52)</td>
<td>83 (48)</td>
<td>62 (56)</td>
</tr>
<tr>
<td>Prevalence of iron deficiency anaemia (n (%))</td>
<td>101 (36)</td>
<td>61 (36)</td>
<td>40 (36)</td>
</tr>
<tr>
<td>Prevalence of iron deficiency anaemia (n (%))</td>
<td>167 (59)</td>
<td>112 (66)</td>
<td>55 (50)</td>
</tr>
</tbody>
</table>
Prevalence of anaemia, iron deficiency and iron deficiency anaemia

A high prevalence of anaemia (52 %) was found in the studied population (Table 1). Nearly 60 % of all children had iron deficiency, and iron deficiency was present in 69 % of the anaemic children. Iron deficiency anaemia was present in 36 % of all children. As expected, ZPP and Hb were significantly correlated (Table 2), but no other parameter alone influenced these two variables. Vitamin A deficiency is thought to contribute to anaemia (Reddy, 1998), but only 2 % of the children in the sample were vitamin A deficient. Comparing riboflavin-deficient (n 182) and riboflavin-sufficient (n 99) children, ZPP concentrations were 51 (17–296) and 46 (20–49) μmol/mol haem, the prevalence of iron deficiency was 63·2 and 52·5 %, and the prevalence of iron deficiency anaemia was 35·7 and 36·4 %, respectively.
Table 2: Pearson's correlation coefficients between nutrition and health-related variables in school-aged children in two villages of Côte d'Ivoire

<table>
<thead>
<tr>
<th></th>
<th>ZPP</th>
<th>CRP</th>
<th>EGRAC</th>
<th>Malaria</th>
<th>Schisto</th>
<th>SR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb</td>
<td>-0.175†</td>
<td>-0.062</td>
<td>0.112</td>
<td>-0.010</td>
<td>0.060</td>
<td>0.093</td>
</tr>
<tr>
<td>ZPP</td>
<td>0.037</td>
<td>0.054</td>
<td></td>
<td>0.041</td>
<td>-0.117</td>
<td>0.055</td>
</tr>
<tr>
<td>CRP</td>
<td>-</td>
<td>-</td>
<td>-0.018</td>
<td>-0.041</td>
<td>0.066</td>
<td>-0.164*</td>
</tr>
<tr>
<td>EGRAC</td>
<td>-</td>
<td>-</td>
<td></td>
<td>0.043</td>
<td>-0.110</td>
<td>0.022</td>
</tr>
<tr>
<td>Malaria</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td>-0.045</td>
<td>0.214*</td>
</tr>
<tr>
<td>Schistosoma</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td>-0.003</td>
</tr>
</tbody>
</table>

ZPP, zinc protoporphyrin; CRP, C-reactive protein; EGRAC, erythrocyte glutathione reductase activity coefficient; SR, serum retinol. * Correlation is significant at the 0·05 level. † Correlation is significant at the 0·01 level.

Prevalence of malaria parasites and microhaematuria

Despite a high prevalence of malaria parasites (49 %), the severity of infestation was low, with only 8 % of the children having a parasite load >1000/μl blood, and only two children having clinical malaria, as defined as a parasite load >5000/μl blood (Trape, 1985). * Plasmodium* load was positively correlated with SR, but with no other variable (Table 2). Microhaematuria, an indicator for *S. haematobium* infection, was present in 19 % of the children.

Prevalence of inflammation

The prevalence of inflammation or infection, as indicated by elevated CRP concentrations, was moderate in both villages. Only about 15 % of the children had an elevated CRP despite a considerably higher prevalence of *Plasmodium* parasitaemia. This discrepancy may at least partly be explained by the generally low malaria severity.

Prevalence of riboflavin deficiency

Two-thirds of the children suffered from riboflavin deficiency, but the degree of deficiency was mild. Only 2 % of children had EGRAC values >1·4, suggesting clear deficiency. Of the riboflavin-deficient children, 7·5 % showed EGRAC values >1·4. EGRAC was not correlated with any other measured variable (Table 2).

Regression analysis and odds ratios

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In the stepwise linear regression on Hb, age \((P < 0.001)\), log ZPP \((P < 0.001)\) and CRP \((P < 0.05)\) were significant predictors. With logZPP as the dependent variable, Hb \((P < 0.001)\) and EGRAC \((P < 0.05)\) were significant predictors. Overall, correlations were modest.

In riboflavin-deficient subjects, the odds ratios (95% CI) for anaemia and iron deficiency were 0·69 (0·42, 1·13) and 1·55 (0·94, 2·55), respectively. Because EGRAC values may be influenced by malaria (see below), odds ratios were also calculated after excluding subjects with malaria. In riboflavin-deficient subjects (EGRAC >1·2) without *Plasmodium* infection \((n 80)\), the odds ratio for iron deficiency was significant (3·07; 1·12, 8·41).

**Discussion**

Mild riboflavin deficiency was very common in the study children, with nearly two-thirds of the children affected. A previous Ivorian study reported EGRAC values >1·68, but no information on the age of the subjects or the prevalence of riboflavin deficiency was provided (Arnaud *et al.* 2001). A food survey in central Côte d’Ivoire found low consumption of dairy products and meat, the usual major dietary sources of riboflavin, but riboflavin intakes were not reported (Staubli-Asobayire, 2000). Although there are few data on the riboflavin status of children in sub-Saharan Africa, the few published studies suggest that riboflavin deficiency may be common. A study in Botswana reported that 33–40 % of the examined children had an EGRAC ≥ 1·4 (Abrams *et al.* 2003). In Kenya, approximately one-third of the children were riboflavin deficient, measured as red blood cell riboflavin (Siekmann *et al.* 2003).

In 2- to 5-year-old South African children, intakes of riboflavin were only 49 % of the RDA (Faber *et al.* 2001).

In this study, although malaria was endemic, with 49 % of the children infected, the parasite load was generally low. This prevalence is similar to that reported in a previous Ivorian study, but the severity of infection was higher in that study (Staubli Asobayire *et al.* 2001). A study conducted in eastern Côte d’Ivoire found that malaria affected 74–86 % of 7- to 11-year-old children and 68 % of children >12 years old (Girardin *et al.* 2004). Microhaematuria as an indicator of *S. haematobium* infection
varied markedly between villages, with nearly one-third of the children affected in Bringakro. This high prevalence of *S. haematobium* infection is consistent with a previous report from this region of Côte d'Ivoire, where 24.6% of the children had microhaematuria, although data varied markedly depending on the nearby presence of standing water (N'Goran *et al.* 1998). Anaemia prevalence in the present study (52%) was similar to that found in an earlier report in Ivorian children (46%) (Staubli Asobayire *et al.* 2001). However, in that study, the prevalence of elevated ZPP levels was higher (83%) than in the present study (59%).

There are several limitations to this study. First, the use of a single measure of iron status (ZPP) probably reduced our ability to define iron deficiency accurately (Zimmermann *et al.* 2005). Previous studies have suggested that ZPP may be elevated by infection and/or inflammation, and thus iron deficiency using ZPP may be overestimated in areas of endemic infection (Staubli Asobayire *et al.* 2001). However, in the present study, there was no correlation between ZPP and CRP, presence of malaria or suspected schistosomiasis. It has been suggested that acute malaria may spuriously elevate EGRAC (Anderson *et al.* 1994; Traunmuller *et al.* 2003). However, acute malaria was rare in our study population, and there was no relationship between EGRAC and malaria, so our estimate of riboflavin deficiency based on EGRAC is likely to be valid.

The public health impact of widespread riboflavin deficiency in schoolchildren is unclear; but it may influence iron metabolism (Sirivech *et al.* 1974; Adelekan & Thurnham, 1986; Powers *et al.* 1988; Butler & Topham, 1993; Powers, 1995). In our sample, anaemia and iron deficiency were common, with prevalences >50%, and two-thirds of the anaemic children were iron deficient. Neither linear regression nor calculation of relative risk identified riboflavin deficiency as a significant predictor of Hb or anaemia. Thus, our data do not support a detrimental effect of riboflavin deficiency on anaemia, as suggested by earlier studies (Foy & Kondi, 1953, 1958), a finding that might be explained by the low severity of the riboflavin deficiency in our sample. However, the risk of iron deficiency was increased 3-fold in children with riboflavin deficiency and without malaria. There are two likely explanations for this association. First, poor overall quality of the diet may have led to multiple micronutrient deficiencies; however, there was a very low prevalence of vitamin A deficiency, and iodine status appeared to be adequate. Secondly, the results could
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reflect impairment of iron absorption and/or metabolism by riboflavin deficiency, as suggested by earlier studies (Powers et al. 1988; Butler & Topham, 1993; Powers, 1995). In three trials in children and pregnant women (Charoenlarp et al. 1980; Powers et al. 1983; Suprapto et al. 2002), riboflavin and iron supplementation produced a greater increase in Hb compared with iron supplements given alone, although the results may have been confounded by concomitant folic acid supplementation in two (Charoenlarp et al. 1980; Suprapto et al. 2002). In contrast, another trial reported no additional benefit of riboflavin plus iron compared with iron supplementation alone in young adults (Powers & Bates, 1987). A study in a small number of pregnant women reported that riboflavin supplementation alone or in combination with iron supplementation did not prevent a decrease in Hb, although the decrease was less in the treated group than in the control group (Powers et al. 1985). Similarly, no effect could be found in a riboflavin supplementation trial in Croatian schoolchildren with adequate Hb levels (Suboticanec et al. 1990). Taken together, these data suggest that the effect of riboflavin status on Hb is variable, and may be confounded by the multifactorial aetiology of anaemia, particularly in countries in sub-Saharan Africa.

In conclusion, there is a high prevalence of mild riboflavin deficiency in Ivorian schoolchildren, but severe deficiency was rare. Riboflavin deficiency was associated with iron deficiency in children free of malaria parasites. However, there was no significant association between riboflavin status and anaemia.

Acknowledgements

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Siekmann, JH, Allen, LH, Bwibo, NO, Demment, MW, Murphy, SP & Neumann, CG (2003) Kenyan school children have multiple micronutrient deficiencies, but increased plasma vitamin B-12 is the only detectable micronutrient response to meat or milk supplementation. J Nutr 133, 3972S–3980S.


3.1 Addendum: a precautionary note on the possible need to re-evaluate result from the study on iron-riboflavin interaction

As described in the literature review, the EGRAC-method is the most widely used method to assess riboflavin status in humans. Riboflavin deficiency is defined as $EGRAC \geq 1.4$, with a marginal risk for $1.2 < EGRAC < 1.4$ (Sauberlich 1999). The in-house procedure for measuring EGRAC is based on modifications of the method as described by Dror et al. (1994). In-house validation of the method yielded acceptable intra- and interassay reproducibility (CV: <6.4% and 4.1%, respectively; Wälchli 2004). No commercial standardized reference material is available.

In a more recent work performed in India departing from the same in-house protocol, the method was further optimized (Räber, 2007). The incubation was performed at $37^\circ C$ instead of the earlier used temperature of $23^\circ C$. As shown in Figure 1, lower EGRAC values were obtained for the in-house reference material when measured at the higher temperature. This translates into better riboflavin status and based on these findings the results published as presented in chapter 3 are put into perspective.

In order to elucidate these conflicting outcomes, the following points need to be considered:

a) since only one level of reference material was available, the method validation would have to be extended in order to be able to calculate the slope and y-axis offset. This equation is needed to introduce a correction factor for the re-assessment of the results presented in chapter 3.

b) in order to exclude matrix interference and to establish the spearman coefficient, several samples ranging from riboflavin sufficient to severely deficient would need to be measured.

The best option would in theory be to re-measure all samples from the survey presented in chapter 3. However, since samples were collected in 2004 and 2005, results of the re-analysis might be biased due to the extended storage time.
Figure 1: Impact of different pre-incubation times on EGRAC values, measured at 25 and 37°C. Expected value and acceptable ranges for EGRAC measured at 37°C are mean EGRAC value from intra-day repeatability testing (n=10) and ± 2SD [from (Raber, 2007)].


4 IRON FORTIFICATION, ANTHELMINTHIC TREATMENT, AND INTERMITTENT PREVENTIVE TREATMENT OF MALARIA FOR ANAEMIA CONTROL IN AFRICAN SCHOOLCHILDREN: A RANDOMISED CONTROLLED TRIAL

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Summary

Background Anaemia is common among children in sub-Saharan Africa and its aetiology is multifactorial. Three of its major causes are low bioavailability of dietary iron, malaria and helminth infection. We compared the effect of iron fortification, intermittent preventive treatment (IPT) of malaria, and anthelminthic treatment on haemoglobin (Hb) and anaemia prevalence among schoolchildren in rural Côte d'Ivoire.

Methods Schoolchildren (aged 6 to 14 years, n=1034) were screened for anaemia using the HemoCue® method. Anaemic children (n=591) were then enrolled in a randomized placebo-controlled trial using a 2x2x2 factorial design to one of eight groups. The three interventions were: 1) iron-fortified biscuits containing 20 mg/day 4x/week as electrolytic iron (the iron compound used in the Ivorian national flour fortification program); 2) IPT of malaria with sulfadoxine (500 mg)-pyrimethamine (25 mg) (SP) at 0 and 3 months; and (3) anthelminthic treatment with albendazole (400 mg) and praziquantel (40 mg/kg) at 0 and 3 months. At baseline and after 6 months, Hb (assessed by an automated cell counter), plasma ferritin, soluble transferrin receptor, zinc protoporphyrin, c-reactive protein and a1-acid-glycoprotein, malaria parasitaemia and helminth infection were assessed. Data analysis was on per-protocol basis, with Hb and anaemia prevalence as the primary outcomes. The trial is registered with controlled-trials.com, number: ISRCTN21782274.

Findings At baseline, the prevalence of iron deficiency, helminth infection, malaria parasitaemia and anaemia was 9%, 54%, 58%, and 68% respectively. Less than 10% of children were lost to follow-up during the 6 month trial. Iron fortification did not improve iron status, IPT of malaria had no effect on malarial burden, and neither had an impact on Hb concentration or anaemia. There were no treatment interactions. Anthelminthic treatment significantly reduced the prevalence and intensity of helminth infections. Relative to placebo, this treatment increased mean Hb by approximately 3.0 g/L and/or decreased anaemia prevalence (odds ratio 0.3, 95% confidence interval 0.1, 0.8).
**Interpretation** To control anaemia in school-aged children in central Côte d'Ivoire, food fortification with electrolytic iron and IPT of malaria are ineffective. In contrast, regular administration of anthelminthics could be an effective public health strategy to control anaemia in this age group.
Introduction

Anaemia is a significant risk factor for perinatal and maternal mortality, impairs cognitive and physical development in children, and reduces work capacity in adults. In the developing world, more than half of the school-aged population suffer from anaemia; in sub-Saharan Africa alone there are an estimated 85 million school-aged children afflicted. The aetiology of anaemia is multifactorial, including iron deficiency (ID), malaria, helminth infection, haemoglobinopathies, as well as other nutritional deficiencies.

To control ID in developing countries, iron fortification and/or supplementation can be efficacious and cost-effective strategies. Because untargeted iron supplementation of children in areas highly endemic for malaria may increase mortality, targeted supplementation and/or iron fortification are recommended. The recently launched food fortification programme in Côte d'Ivoire mandates the addition of electrolytic iron to wheat flour (decree 025, issued on January 18, 2007). Together with similar mandates in South Africa, Nigeria, and Lesotho, electrolytic iron is now recommended for flour fortification for nearly 1/3rd of the total population of sub-Saharan Africa. Iron fortification may be efficacious in reducing ID and anaemia in areas with low inflammation rates, but conflicting results have been reported from different settings in sub-Saharan Africa. Because inflammation impairs iron absorption and utilization, it may be necessary to control parasite infections to ensure iron bioavailability from fortified foods.

Presumptive chemotherapy targeting soil-transmitted helminths (i.e. Ascaris lumbricoides, hookworm and Trichuris trichiura; usually with the drugs albendazole or mebendazole) and schistosomes (with the drug praziquantel) in highly-endemic areas is increasingly promoted by the World Health Organization. A recent systematic review concluded anthelmintic treatment may reduce anaemia, especially in children with high helminth infection intensities, and albendazole is more efficacious than mebendazole against hookworm infections in terms of cure rates. IPT of malaria with sulfadoxine-pyrimethamine (SP) is beneficial in pregnant women and infants, and preliminary results suggest that this strategy is also effective in school-aged children.

The aim of this study was to evaluate the relative efficacy of iron fortification, IPT of malaria using SP, and regular administration of albendazole plus praziquantel,
administered alone or in combination, to reduce anaemia in school-aged children in a rural part of Côte d'Ivoire.

Methods

Study area and participants
The study population consisted of 6 to 14-year-old children attending primary schools in five villages (Aluminakro, Bringakro, Gbohua, N'Da Kouassikro, Tafissou), located 15-20 km south of Toumodi, a district capital in south-central Côte d'Ivoire. Most families live on subsistence farming (cassava, plantain and yam) with occasional consumption of bushmeat and fish. There is limited cash crop production (i.e. cocoa and coffee). The area is situated in the transition zone from rainforest to savannah with an annual mean temperature of 27°C. The wet season usually lasts from March to November, with a dry spell in the months of July and August and the heaviest rains between March and May.

The study was carried out between November 2006 and July 2007 matching the school year, in order to maximise compliance. Village authorities, parents and legal guardians of eligible children were informed by two of the investigators (FR, EKN) about the purpose, procedures and potential risk of the study during village gatherings. Subsequently, household visits were done by trained local health workers to provide further specific information about the study and the children's right to withdraw at any time without further obligations. Written informed consent was sought from parents or legal guardians of the participating children. Illiterate, but consenting parents/guardians were asked to provide a fingerprint; children consented orally.

All consenting children were invited to a selection session with assessment of haemoglobin (Hb) on capillary blood using the HemoCue® apparatus (HemoCue AB, Angelholm, Sweden). Children with Hb <80 g/L were referred to the hospital, and children aged <12 years and ≥12 years with Hb ≥80 g/L and ≤115 g/L and ≥80 g/L and ≤120 g/L, respectively, were further retained. The inclusion criteria were: anaemia according to the criteria above; for girls, non-pregnant as assessed by a female medical doctor; absence of major systemic illnesses; anticipated residence in the study area for the entire study duration; no known or reported hypersensitivity to SP, albendazole or praziquantel; no known or reported history of significant chronic
illness; no known recent history of anthelminthic treatment (within past 4 weeks). Figure 1 depicts the study timeline.

At baseline, mid- and endpoint, height and weight was measured (midpoint data not shown); children removed their shoes and wore light clothing. Weight was recorded to the nearest 0.1 kg (Skyline, Soehnle-Waagen GmbH & Co. KG; Murrhardt, Germany), and height to the nearest 0.5 cm (Person check®, KaWe; Asperg, Germany). The age of eligible children was obtained from the school registers based on birth certificates. Where age was reported by the teachers to be unlikely to reflect the child’s true age, age was omitted.

Ethical clearance
Ethical approval for the study was obtained from the ethics review board of the Swiss Federal Institute of Zurich, Switzerland (2006-23), the ethics committee of the University and the State of Basel (EKBB), Switzerland (224/06), and the ethical committee of the Ministry of Health in Côte d’Ivoire (Comité National d’Ethique des Sciences de la Vie et de la Santé, number 5782/MSHP/CAB/CNESVS/06). An independent data safety and monitoring board (DSMB) was established to monitor the trial and to review safety issues throughout. Ivorian health insurance was contracted to cover study participants (NSIA assurances, Côte d’Ivoire, number 5100581060007H). The study is registered with controlled-trials.com (number ISRCTN21782274).

Study design and interventions
The study was a double-blind, individually-randomised, placebo-controlled trial, using a 2x2x2 factorial design with a duration of 6 months. Eligible children were assigned to 1 of 8 groups, representing any possible combination of three interventions, namely (i) iron fortification, (ii) IPT of malaria, and (iii) anthelminthic treatment or the respective placebo (see Table 1).

Half of the children were assigned to iron fortification; hence they received two iron fortified biscuits (electrolytic Fe [A-131; Dr. Lohmann GmbH, Emmerthal, Germany], 20 mg Fe/d/child) during the morning break at each school day (4 times/week). Placebo recipients received the same biscuits but unfortified. The “petit beurre” type biscuits were produced in two batches on an industrial-scale production line by Midor (Meilen, Switzerland), and two-colour labelled (blue for iron fortified,
yellow for placebo) by a team of researchers not otherwise involved in the trial. The colour coding system was used to facilitate biscuit distribution by the teachers and thereby reducing administration errors. Iron content of the biscuits was evaluated for both batches in triplicate using atomic absorption spectrometry (Spectra AA-50; Varian, Palo Alto, CA). Biscuits were shipped to Côte d'Ivoire and stored under climate-controlled conditions. The two types of biscuits were compared in a triangle test and could not be distinguished by local adults (n=24; data not shown).

With regard to IPT of malaria, half of the children received SP (sulfadoxine, 500 mg plus pyrimethamine, 25 mg), administered twice; after baseline screening and 3 months later. The other half of the children received a matching placebo.

Half of the children were given albendazole (single oral dose of 400 mg) plus praziquantel (single oral dose of 40 mg/kg) after the baseline screening and 3 months later. Drug administration was spaced by 1 day to avoid potential drug interactions. Placebo recipients were given matching placebo at the same schedule.

Trial medication and matching placebo were provided by Dafra Pharma (Turnhout, Belgium); a certificate of analysis was provided by the manufacturer for each type of tablets. Trial medications were coded by eight letters, each coding for one of the eight treatment combinations as shown in Table 1, with no other indications on the packaging relating to whether they contained active ingredients. Children were individually randomised to one of the randomisation letters, in blocks of eight. The randomisation list was prepared and codes held by a member of the DSMB until the study database was locked. Drugs were administered in an empty class room at the schools by the medical team. Children remained under direct medical supervision for 3 hours after drug administration for monitoring of unsolicited adverse events. Children and teachers were encouraged to report any suspicion of adverse events that occurred within 24 hours after drug administration. Children reporting adverse events were examined by the study physician and, if need be, referred to the nearby health centre for further follow-up.

At pre-screening, a photograph of each child was taken and eligible children received a unique identification badge with a personalised identification number, photograph, name, age and treatment group (letter for drugs, colours for biscuits). Children were invited to wear this badge at every visit.

For the ease of detection and to increase specificity of clinical malaria cases, the local nurse and the medical field team were provided rapid malaria diagnostic kits
Children found to suffer from clinical malaria were treated with an artemisinin-based combination therapy (ACT, i.e. artesunate-amodiaquine; Co-arinate from Dafra Pharma, Turnhout, Belgium). ACTs have recently been adapted as the first-line malaria treatment in Côte d'Ivoire. During a clinical malaria episode, other study-related treatments were discontinued. Upon trial completion, all children received a single dose of mebendazole (500 mg), and children with a Hb value at endpoint between 80 and 100 g/L received adequate medical treatment (ACT or iron supplements). Children with Hb < 80 g/L were referred to the hospital.

Nutritional assessment was performed in 24 households after midpoint assessment as described elsewhere. Percentage of the absolute iron requirements were calculated using the recommended nutrient intake tables.

**Laboratory methods**

Biomedical parameters were assessed at baseline and endpoint. Blood measures: venous blood (7.5 ml) was drawn by experienced technicians by venipuncture using the Vacutainer® system (Sarstedt; Sevelen, Switzerland). The blood was transported on ice to the nearby field laboratory. From whole blood, Hb, hematocrit (HCT) and mean corpuscular volume (MCV) were measured using an AcT8 Counter (Beckman Coulter; Krefeld, Germany) on the day of blood sampling. Subsequently, a thick and a thin blood smear were prepared, stained by Giemsa and examined under a microscope for Plasmodium by experienced laboratory technicians. Parasites were counted against 200 leukocytes (if less than 10 parasites were identified, counting was continued up to 500 leukocytes). Counts were converted to the number of parasites per µL of blood, assuming a leukocyte count of 8000/µL. Infection intensities were defined according to the following cut-off values: (i) 0, (ii) 1-999, (iii) 1000-1999, (iv) 2000-4999 and (v) ≥5000 parasites/µL. At baseline, Hb typing was done by electrophoresis in a subsample of 112 randomly selected subjects.

The remaining blood was centrifuged and plasma was aliquoted and transported frozen and stored at −25°C for pending analysis of plasma ferritin (PF), soluble transferrin receptor (TfR), C-reactive protein (CRP) and α-1-acid glycoprotein (AGP). Zinc protoporphyrin (ZPP) was measured on the remaining washed red blood cells using a haematofluorometer (Aviv Biomedical; Lakewood, USA) within 7 days after sampling. PF and CRP were measured using an automated chemiluminescent
immunoassay system (IMMULITE®, Diagnostic Products Corporation; Los Angeles, USA). TfR was measured using an automated immunonephelometric assay (Cobas Integra 800, Roche Diagnostics; Rotkreuz, Switzerland); recent literature indicated a systematic bias between this and the standard manual method (RAMCO; Houston, USA), and thus, a method comparison on 76 random samples was performed, yielding good agreement ($y=1.01; r^2=0.84$). AGP was measured by immunoturbidimetry (Cobas Mira, Roche Diagnostics; Rotkreuz, Switzerland). For all of the above methods, quality controls as provided by the manufacturer were used; for PF analysis, quality controls from WHO were additionally used. Normal reference values are as follows: ZPP, <40 μmol/mol heme; TfR, >8.5 mg/L; PF, >30 μg/L; AGP <1.2 g/L and CRP <10 mg/L. ID was defined as [PF <15 μg/L or (TfR >8.5 mg/L and ZPP >40 μmol/mol haeme)]; body iron values were calculated according the following equation: (mg/kg)= -[lg(TfR*1000/PF)-2.8229]/0.1207. Inflammation as a binary variable was defined as AGP>1.2g/L and CRP>10 mg/L.

Urinary measures: Schistosoma haematobium infection intensities were assessed upon filtration of 10 mL of urine (collected between 10 am and 2 pm), filtered with Nucleopore filters (Sefar AG, Filtration Division; Heiden, Switzerland), using the syringe filtration technique. A drop of Lugol (Merck; Darmstadt, Germany) was placed on the filter prior to counting the number of S. haematobium eggs under a microscope. Light infections were defined as 1-49 eggs/10 mL of urine, heavy infections as >50 eggs/10 ml of urine.

Stool measures: Two Kato-Katz thick smears (42 mg) were prepared from each stool sample. The slides were examined immediately after preparation under a microscope and all hookworm eggs were counted. Subsequently, the slides were re-examined and the number of eggs of A. lumbricoides, Schistosoma mansoni and T. trichiura were counted and recorded separately. For the conversion to eggs per gram faeces (EPG), a multiplication factor of 24 was employed. Approximately 10% of the slides were re-examined, and the Spearman rank correlation coefficient for hookworms was 0.6. Criteria for light infections with A. lumbricoides (<5000 EPG), T. trichiura (<1000 EPG), hookworms (<2000 EPG) and S. mansoni (<100 EPG) were defined according to WHO guidelines.
**Statistical analysis**

Assuming a mean Hb of 117±12 g/L\(^{27}\) and that an increase of 8 g/L in Hb would be clinically relevant, and allowing for a dropout rate of 20%, we calculated that 80 children per group were initially needed to achieve a power level of 90% at a 5% level of significance.

Data entry was done in the Epi Info 3.2 or Excel (for laboratory parameters). Data were double entered and cross-checked. Data analysis was conducted in STATA v.9, SPSS v.15 and Excel v.2003. All analyses were done on a per-protocol basis. Due to the high percentage of missing ages, they were imputed by calculating the mean age per school grade (ninputed=77; 13.9%); no other parameters were imputed. Between-group comparisons were performed for baseline values using one-way ANOVA for normally distributed and Kruskall-Wallis-test for skewed continuous data; \(\chi^2\)-test or Fisher’s exact test as appropriate for binary data; and negative binomial regression for over-dispersed egg or parasite counts. A linear model was applied to assess the effects of treatment groups on differences in Hb (as primary outcome), iron stores, and anthropometrical measures (height-for-age z-score (HAZ), body mass index z-score (BMIZ), and after transformation, PF, Tfr and ZPP between the endpoint and baseline, including 95% confidence interval (CI). For outcomes which were not normally distributed, Boxcox transformations were applied. Logistic regressions were done to compare group effects for the binary variables of anaemia, ID, malaria and hookworm prevalence (odds ratio (OR), including 95% CI). Significance was assessed at 5% significance level using the likelihood ratio test. HAZ and BMIZ were calculated using the new WHO reference data.\(^{28}\)

**Role of the funding source**

This study was funded through a donation to the Swiss Federal Institute of Technology Zurich by a private Foundation, from the Swiss Foundation for Research in Nutrition (SFEFS), and the Hochstrasser Foundation (Zurich, Switzerland). Trial medications were provided free of charge by Dafra Pharma, (Turnhout, Belgium); electrolytic iron by Dr. Lohmann GmbH (Emmerthal, Germany); trial biscuits were produced by Midor (Meilen, Switzerland) at factory costs. The funding agencies cited above played no role in the design or implementation of the study reported, analysis of the data, or the preparation and submission of the manuscript. The first author has
full access to all the data in the study and the corresponding author and the last author had the final responsibility for the decision to submit for publication.

**Results**

At pre-screening, Hb was measured in 1034 children using the Haemocue® method; 63.7% were mild-to-moderately anaemic (Hb 80 to 115 g/L or 120 g/L depending on age), 35.7% of children were non-anaemic and 0.6% were severely anaemic. The mean Hb was 105.0 ± 7.7 g/L, mean age was 9.8 ± 2.5 years; and 44.0% were females. Children with mild-to-moderate anaemia were invited to participate in the baseline clinical exam. From the 659 eligible children, 591 were enrolled in the study, and 554 completed the study (Figure 2). Prior to group assignment, 41 children were absent, 1 refused to participate and 26 were excluded due to medical reasons. During the main study, most drop-outs were children leaving school due to a prolonged nationwide teacher's strike. In-trial reasons for exclusion were: PPP: splenomegaly (1); FPP: endocrinological disease (1); non-compliance to biscuits (1); PMP: adenopathy (1); FPH: adenopathy (1), pregnancy (1); FMH: massive haematuria (1), pneumonia/respiratory disease (1).

Table 2 summarises the baseline characteristics of the study population on per-protocol basis; there were no differences between groups for any of the assessed parameters. For the study cohort, mean Hb was 111.1 ± 9.8 g/L (using an automated cell counter) and anaemia prevalence was 68.4%, in contrast to the pre-screening results, where mean Hb (using the HemoCue™) of the eligible children was 105.0 ± 7.7 g/L, with 100% anaemia (by definition). Only 9.1% were ID, and 28.3% had an elevated CRP and/or AGP. The PF levels for subjects without inflammation are shown in Table 2; the high values of PF are similar to those found in earlier studies in Côte d'Ivoire.\(^{17}\)

Of the children in the intervention trial, 54.4% were infected with any species of intestinal helminths, with hookworm being predominant: the number of children infected with hookworm, T. trichiura and A. lumbricoides was 285, 14 and 7, respectively. The geometric mean infection intensity of helminth-infected children was 105 EPG (95% CI: 89, 123) for hookworm, 46 EPG (95% CI: 25, 83) for T. trichiura, and 797 EPG (95% CI: 307, 2075) for A. lumbricoides. Overall, infection intensity was low: only 5 children (1.8%) had hookworm infection intensity > 2000
EPG, and no child exceeded the WHO cut-off values for light infection intensities in the case of A. lumbricoides and T. trichiura. Due to the low number of infections with the latter two parasites, they were omitted in the subsequent regression analyses. Prevalence and intensity of Schistosoma spp. was very low, and hence not considered any further.

More than half of the children (58.0%, n=312) were infected with Plasmodium. The geometric mean of Plasmodium-positive children was 404.1 parasites/μL blood (95% CI: 346, 472). Infection intensity was low; among the infected children 68.9% had <1000 parasites/μL blood, 18.2% between 1000 and 1999, 8.8% between 2000 and 4999, and only 4.1% (n=13) had a heavy infection (≥5000).

Haemoglobinopathies were performed at baseline on 112 randomly selected children; 85% had normal genotype (HbAA), followed by HbAS (6%), HbAC (4%), HbAF (2%), HbCC (2%) and HbSC (1%). Daily mean iron intakes in children <10 years of age, boys >10 years, and girls >10 years were 12.2 ± 3.8 mg (n=16), 14.5 ± 3.5 mg (n=9) and 13.7 ± 2.9 mg (n=8), respectively. Assuming a dietary iron bioavailability of 10%, this translates into 138%, 94% and 44% of the absorbed iron requirements (95th percentile).

During the trial, mean ± SD biscuit consumption was 163.7 ± 22.5 out of the 200 potentially consumed biscuits (82% overall compliance), and there were no significant differences between the groups (data not shown; p=0.76). Less than 2% of the children consumed fewer than 50% of the designated biscuits, and 22.1% consumed less than 75%. The iron content of the biscuits was 9.6 ± 0.3 mg Fe/biscuit. Thus, total iron consumption of the children receiving the fortified biscuits was 1567.0 ± 215.4 mg of electrolytic iron. This corresponds to a daily intake of 8.7 ± 1.2 mg, more than three times the daily dose anticipated from the planned Ivorian flour fortification program for this age group (personal communication, R. Hurrell). Since data were analysed on per-protocol basis, compliance to anthelmintics and IPT is 100%; however, three children were administered the wrong drugs. Post-trial, two out of these three could be re-assigned to a new, matching treatment group; one child had to be excluded.

In the placebo group (PPP) during the study, there was a significant decrease in Hb of 4.6 g/L (95%CI: -6.1, -1.9); anaemia prevalence increased, TfR increased by 1.9 mg/L (95%CI: 1.0, 3.1) and as a consequence, body iron decreased despite PF concentrations remaining largely unchanged. Malaria prevalence and density...
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decreased, whereas hookworm prevalence and egg burden increased \( \left( p<0.05 \right) \). Relative to the placebo group (Table 3), all groups not receiving anthelminthics did not show improvements in Hb or the odds for anaemia. In contrast, in all groups receiving anthelminthics, compared to the placebo group, Hb increased or the odds for anaemia decreased. For groups PMH and FPH these Hb changes were statistically significant, for groups PPH and FMH, they were borderline significant \( \left( p=0.054 \text{ and } 0.056, \text{ respectively} \right) \). Anthelminthic treatment was highly effective in reducing the odds for hookworm infection in all four groups receiving this intervention. In contrast, IPT did not significantly reduce malaria parasitaemia; in one of the IPT groups (FMP), malaria density risk ratio was actually significantly elevated. Iron status, as measured by PF, Tfr, and body iron did not improve in any group and ID prevalence did not change in any group. One iron status indicator, ZPP, was significantly improved in three of the four groups receiving anthelminthics.

Regression analysis with variables adjusted for treatment, respective baseline parameter, inflammation, sex, age and biscuit compliance weakened the positive effects of anthelminthic treatment on Hb; the odds for anaemia remained significantly decreased by treatment only in the PPH and FMH groups (Table 4).

**Discussion**

Our aim was to investigate the interactions of three commonly recommended interventions — iron fortification, IPT for malaria and anthelminthic treatment — to reduce anaemia among school-aged children in sub-Saharan Africa. However, of these three interventions, only the administration of anthelminthic drugs (albendazole plus praziquantel) showed a significant effect on children's Hb level and anaemia. Since iron fortification and IPT for malaria showed no effect on Hb and anaemia, we could not examine the interactions of these three interventions.

Hb and iron status of the placebo group — assessed by, PF, Tfr, body iron — deteriorated during the study period. At the same time, malaria parasitaemia decreased. A possible explanation of these findings is an unusual delay in the onset of the rainy season during the study. Rains typically start in this region in March, but in the year 2007, they were delayed nearly 3 months and did not begin until June, at the end of our study. The prolonged dry season reduced dietary variety and may
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have decreased dietary iron bioavailability due to reduced availability of meat/fish/poultry and increased reliance on cereals high in phytic acid. However, this may have been balanced somewhat by greater consumption of fruits and vegetables containing ascorbic acid, an enhancer of iron absorption. The dietary assessment, done in the second half of the intervention period may thus not have represented iron intake over the entire study period. The prolonged dry season went hand-in-hand with the disappearance of small ponds, which might have reduced malaria transmission.29

Despite the high prevalence of anaemia, only 9% of the children were ID. Previous studies of rural Ivorian children at this age have reported anaemia prevalences of 36 to 50%, lower than in the present study, and ID prevalences of 23 to 35%, higher than in the present study17,20 These differences may be due to variations in diet, rates of infectious disease and/or the difficulty of accurately defining iron deficiency in sub-Saharan African children.23

The low ID prevalence may have reduced the effect of the iron fortification, as fractional absorption of dietary iron is inversely related to body iron stores. However, on average, over 1.5 g of electrolytic iron was consumed during the study period in the groups receiving the iron-fortified biscuits (8.7 mg/d). Even if only a small fraction of this iron dose was absorbed, it would have been detected in an increase in storage iron (PF); iron stores can be increased by iron fortification in Ivorian children who have an adequate iron status.17 The cause of the very low absorption of the electrolytic iron used in this study is unclear. Because the biscuits used in the trial were made from low extraction flour and were given at the morning breaks at schools, and hence there was no other food intake for at least another 2 hours, dietary inhibitors of iron absorption, such as phytic acid and/or polyphenols, are unlikely to have played a role. Three controlled trials using electrolytic iron at varying doses in infants or children in sub-Saharan Africa have not shown efficacy7,8,30, while in one trial it was efficacious31 Using a similar design in an 8 month feeding trial, 10 mg electrolytic iron per day fed to Thai women with low iron stores showed good efficacy.32 These differences are likely due to a higher frequency of infections and inflammation in our study population, despite the concurrent treatment of malaria and hookworm. In an efficacy study in younger Kenyan children, maize fortified with sodium iron EDTA improved iron status and reduced IDA prevalence, while electrolytic iron did not.7
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For IPT in pregnant women in stable transmission areas, current recommendations are for two courses during pregnancy; for infants, three courses over 9 months are recommended (at age of 2, 3 and 9 months)\textsuperscript{33}, but other schedules appear to be efficacious as well.\textsuperscript{34,35} At present there is no established treatment regimen for school-aged children. The two doses of SP administered with a 3-month interval in the present study may have been too low to achieve efficacy against \textit{P. falciparum}. Alternatively, it is possible that resistance to SP in Côte d'Ivoire has become as widespread as to reduce treatment efficacy, although information on resistance progression is scarce. Interestingly, the overall decrease in malaria parasitaemia in the study population had no measurable beneficial impact on Hb status or anaemia. Clinical malaria causes anaemia, but for asymptomatic malaria, this relationship is debated.\textsuperscript{36,37}

Hookworm was by far the most frequent helminth infection encountered in the present study, with more than half of the children screened at baseline found hookworm-positive; intensities of infection though were mainly light. The two treatment courses with albendazole, administered at a 3-month interval, were highly efficacious in reducing the prevalence and intensity of hookworm infection, as expected.\textsuperscript{12} With regard to praziquantel, because the number of children infected with \textit{Schistosoma} spp. was very low, this part of the anthelminthic intervention is unlikely to have had any clinical impact. Administration of anthelminthic drugs improved Hb and decreased the odds for anaemia relative to placebo. The beneficial effect of anthelminthics on the prevalence of anaemia is thought to be mediated through improved iron status, due to a combination of reduced intestinal bleeding and thus, reduced iron loss, along with a decrease in systemic inflammation,\textsuperscript{38} and a subsequent improvement in iron absorption and mobilization to the marrow.\textsuperscript{39} These effects were reflected in the modest improvement in ZPP, a measure of iron supply for erythropoiesis. However, TfR and PF were unchanged by treatment. Thus, other mechanisms, such as changes in eating behaviour\textsuperscript{40} and/or improved absorption of other haematopoietic nutrients, such as vitamin A and riboflavin, may also have played a role.

The findings of this study emphasise that strategies to control anaemia in sub-Saharan Africa children should be chosen based on identification of the local aetiology of anaemia. In rural central Côte d'Ivoire, because of the low prevalence of ID, iron fortification is unlikely to be an effective anaemia control strategy. Similarly,
IPT of malaria with SP in this setting also appears to be of little value. In contrast, antithelminthics may be a simple-to-implement and effective public health strategy to help control anaemia in this age group.

**Contributors**

FR, MBZ, JU, PV and RFH designed the study; FR, RJA, ABT, EKN, MC, MDT, HA and DES conducted the study; FR and CN performed dietary assessment; FR, MBZ, JU and PV did the statistical analysis; FR, MBZ, JU and RFH edited the first draft of this publication, with all authors contributing to it.

**Conflict of interest statement**

None of the authors have any conflict of interest to declare.

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infrastructure and William Moses for assistance in AGP-analysis; University Hospital Zurich for Tfr-analysis; Sarah Anderegg, Katche P Etchien, Kouadio K Olivier, Hervé K Kouassi, and Valentine K N'Da for assistance in the dietary assessment; Isabelle Aeberli, Ralf Biebinger and Sabine Renggli for back office assistance.

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Table 1: Description of the eight intervention groups. F codes for iron-fortified biscuits (Fe), M for intermittent preventive treatment (Malaria), H for anthelmintic treatment (Helminths), and P stands for placebo.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Treatments</th>
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<tr>
<td>PPP</td>
<td>Placebo  Placebo</td>
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<tr>
<td>FPP</td>
<td>Iron     Placebo</td>
</tr>
<tr>
<td>PMP</td>
<td>Placebo  IPT   Placebo</td>
</tr>
<tr>
<td>PPH</td>
<td>Placebo  Placebo  Anthelmintics</td>
</tr>
<tr>
<td>FMP</td>
<td>Iron     IPT   Placebo</td>
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<tr>
<td>FPH</td>
<td>Iron     Placebo  Anthelmintics</td>
</tr>
<tr>
<td>PMH</td>
<td>Placebo  IPT   Anthelmintics</td>
</tr>
<tr>
<td>FMH</td>
<td>Iron     IPT   Anthelmintics</td>
</tr>
</tbody>
</table>
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Figure 1: Study timeline with study-related activities and approximate schedule. Clinic: clinical assessment; blood/stool/urine: sampling; anthro: anthropometry; Alben/Plac.: albendazole or placebo administration; Prazi/Plac.: praziquantel or placebo administration; SP/Plac.: IPT or placebo administration; Iron: administration of iron fortified biscuits.
Figure 2: Trial profile. Aban.: school-abandon or long-term absence; excl.: excluded due to medical reasons or non-compliance; excl.*: exclusion after uncoding due to erroneous drug administration; refuse: child refused endpoint blood drawing.
### Table 2: baseline characteristics as analysed per-protocol

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<thead>
<tr>
<th></th>
<th>PPP</th>
<th>FPP</th>
<th>PPH</th>
<th>PMP</th>
<th>FPH</th>
<th>PMH</th>
<th>FMP</th>
<th>FMH</th>
<th>p</th>
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</tr>
<tr>
<td>N</td>
<td>70</td>
<td>69</td>
<td>65</td>
<td>70</td>
<td>70</td>
<td>72</td>
<td>76</td>
<td>62</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>9.1 (2.2)</td>
<td>9.5 (2.3)</td>
<td>9.9 (2.1)</td>
<td>10 (2.4)</td>
<td>9.9 (2.5)</td>
<td>9.8 (2.1)</td>
<td>10 (2.5)</td>
<td>9.9 (2.3)</td>
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<td>(n=58)</td>
<td>(n=57)</td>
<td>(n=57)</td>
<td>(n=63)</td>
<td>(n=62)</td>
<td>(n=60)</td>
<td>(n=69)</td>
<td>(n=53)</td>
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</tr>
<tr>
<td>Sex (n; % fem)</td>
<td>30 (43%)</td>
<td>29 (42%)</td>
<td>27 (42%)</td>
<td>29 (41%)</td>
<td>27 (39%)</td>
<td>34 (47%)</td>
<td>34 (45%)</td>
<td>23 (37%)</td>
<td>0.96</td>
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<td>HAZ (-)</td>
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<td>-0.9 (1.5)</td>
<td>-0.9 (1.4)</td>
<td>-1.0 (1.5)</td>
<td>-0.9 (1.5)</td>
<td>-1.0 (1.2)</td>
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<td>-0.8 (1.3)</td>
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<tr>
<td>BMIZ (-)</td>
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<td>-0.7 (0.8)</td>
<td>-0.7 (0.8)</td>
<td>-0.8 (0.9)</td>
<td>-0.7 (0.8)</td>
<td>-0.8 (0.8)</td>
<td>-0.6 (0.8)</td>
<td>-0.6 (0.9)</td>
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<td><strong>Biomedical: blood</strong></td>
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<tr>
<td>Hb (g/L)</td>
<td>111 (9.4)</td>
<td>111 (10.5)</td>
<td>111 (8.6)</td>
<td>113 (10.3)</td>
<td>111 (10.7)</td>
<td>111 (9.0)</td>
<td>113 (10.4)</td>
<td>111 (10.3)</td>
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<tr>
<td>(n; [%])</td>
<td>50 (71%)</td>
<td>52 (75%)</td>
<td>42 (65%)</td>
<td>46 (66%)</td>
<td>51 (77%)</td>
<td>51 (71)</td>
<td>49 (65%)</td>
<td>46 (74%)</td>
<td>0.73</td>
</tr>
<tr>
<td>PF no inflam.</td>
<td>76</td>
<td>65</td>
<td>82</td>
<td>72</td>
<td>66</td>
<td>76</td>
<td>60</td>
<td>62</td>
<td></td>
</tr>
<tr>
<td>(μg/L)*</td>
<td>(53, 122)</td>
<td>(45, 88)</td>
<td>(50, 118)</td>
<td>(46, 109)</td>
<td>(49, 98)</td>
<td>(50, 110)</td>
<td>(42, 79)</td>
<td>(45, 97)</td>
<td>0.26</td>
</tr>
<tr>
<td>Tfr (mg/L)*</td>
<td>4.2</td>
<td>3.9</td>
<td>3.8</td>
<td>3.9</td>
<td>4.2</td>
<td>4.2</td>
<td>4.0</td>
<td>3.9</td>
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<tr>
<td>(3.3, 5.1)</td>
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<td>(3.2, 4.8)</td>
<td>(3.0, 4.5)</td>
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<td>(3.1, 4.8)</td>
<td>(3.0, 5.1)</td>
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<tr>
<td>ZPP (μmol/mol heme)*</td>
<td>57 (45, 68)</td>
<td>55 (41, 80)</td>
<td>50 (39, 70)</td>
<td>49 (39, 58)</td>
<td>54 (42, 68)</td>
<td>55 (44, 69)</td>
<td>54 (42, 71)</td>
<td>48 (42, 71)</td>
<td>0.54</td>
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<tr>
<td>Body iron no inflam. (mg/kg)</td>
<td>9.1 (2.7)</td>
<td>8.8 (2.3)</td>
<td>9.9 (2.6)</td>
<td>9.1 (2.4)</td>
<td>9.1 (2.2)</td>
<td>9.0 (3.3)</td>
<td>9.1 (2.7)</td>
<td>8.9 (2.7)</td>
<td>0.66</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>ID (n; [%])</th>
<th>10 (15%)</th>
<th>6 (8.7%)</th>
<th>2 (3.2%)</th>
<th>8 (11%)</th>
<th>3 (4.3%)</th>
<th>7 (9.9%)</th>
<th>8 (11%)</th>
<th>7 (11%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inflammation (n; [%])</td>
<td>19 (28%)</td>
<td>19 (28%)</td>
<td>19 (29%)</td>
<td>23 (33%)</td>
<td>19 (28%)</td>
<td>16 (24%)</td>
<td>17 (23%)</td>
<td>19 (31%)</td>
</tr>
<tr>
<td>Malaria prev. (n; [%])</td>
<td>43 (61%)</td>
<td>37 (54%)</td>
<td>38 (59%)</td>
<td>41 (59%)</td>
<td>41 (59%)</td>
<td>43 (60%)</td>
<td>39 (51%)</td>
<td>36 (58%)</td>
</tr>
<tr>
<td>Malaria parasite density (parasites/µL)</td>
<td>394 (246, 631)</td>
<td>482 (316, 737)</td>
<td>358 (225, 569)</td>
<td>348 (230, 527)</td>
<td>378 (245, 582)</td>
<td>516 (319, 835)</td>
<td>277 (182, 421)</td>
<td>467 (287, 761)</td>
</tr>
<tr>
<td>Biomedical: stool/urine</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hookworm prev. (n; [%])</td>
<td>35 (51%)</td>
<td>37 (54%)</td>
<td>35 (54%)</td>
<td>42 (60%)</td>
<td>37 (53%)</td>
<td>36 (50%)</td>
<td>44 (58%)</td>
<td>27 (44%)</td>
</tr>
<tr>
<td>Hookworm intensity (epg)</td>
<td>123 (81, 189)</td>
<td>97 (64, 147)</td>
<td>59 (39, 88)</td>
<td>122 (78, 192)</td>
<td>120 (74, 194)</td>
<td>126 (79, 199)</td>
<td>105 (71, 155)</td>
<td>131 (69, 249)</td>
</tr>
<tr>
<td>A. lumbricoides intensity (epg)</td>
<td>0 (n=0)</td>
<td>1078 (n=2)</td>
<td>816 (n=1)</td>
<td>0 (n=0)</td>
<td>356 (n=2)</td>
<td>1824 (n=1)</td>
<td>0 (n=0)</td>
<td>0 (n=0)</td>
</tr>
<tr>
<td>T. trichiura intensity (epg)</td>
<td>38 (n=3)</td>
<td>21 (n=2)</td>
<td>91 (n=2)</td>
<td>59 (n=3)</td>
<td>71 (n=3)</td>
<td>48 (n=1)</td>
<td>34 (n=2)</td>
<td>0 (n=0)</td>
</tr>
<tr>
<td>S. haematobium (e/10 mL)</td>
<td>34 (n=1)</td>
<td>18 (n=4)</td>
<td>6.2 (n=2)</td>
<td>43 (n=2)</td>
<td>28 (n=2)</td>
<td>0 (n=0)</td>
<td>2.0 (n=1)</td>
<td>1.0 (n=1)</td>
</tr>
<tr>
<td>S. mansonii (epg)</td>
<td>0 (n=0)</td>
<td>24 (n=1)</td>
<td>0 (n=0)</td>
<td>0 (n=0)</td>
<td>120 (n=1)</td>
<td>12 (n=1)</td>
<td>0 (n=0)</td>
<td>12 (n=1)</td>
</tr>
</tbody>
</table>
Mean (SD); *median (IQT); ‡geometric mean (95% CI); §defined AGP>1.2 g/L AND CRP>10 mg/L; †defined as P<30 µg/L or ZPP>40 (µmol/mol heme) AND TfR>8.5 mg/L; 'geometric mean (n): p indicates the level of significance with a suitable test; values ≥10 have been rounded to the nearest whole number.
Table 3: unadjusted differences, odds ratios or incidence risk ratios of endpoint-baseline (95% CI), and biscuit compliance; as assessed by regression analysis, only entering baseline status and treatment as confounders

<table>
<thead>
<tr>
<th></th>
<th>FPP</th>
<th>PPH</th>
<th>PMP</th>
<th>FPH</th>
<th>PMH</th>
<th>FMP</th>
<th>FMH</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \Delta ) Hb (g/L)</td>
<td>0.5</td>
<td>2.7</td>
<td>0.0</td>
<td>2.9</td>
<td>3.0</td>
<td>-0.2</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td>(-2.2, 3.2)</td>
<td>(-0.0, 5.5)</td>
<td>(-2.7, 2.7)</td>
<td>(0.2, 5.6)</td>
<td>(0.3, 5.7)</td>
<td>(-2.9, 2.4)</td>
<td>(-0.1, 5.5)</td>
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<tr>
<td>( \Delta ) anaemia odds ratio #</td>
<td>1.0 (0.5, 3.0)</td>
<td>0.3 (0.1, 0.8)</td>
<td>0.8 (0.3, 2.1)</td>
<td>0.4 (0.2, 1.1)</td>
<td>0.4 (0.2, 1.0)</td>
<td>1.0 (0.4, 2.7)</td>
<td>0.3 (0.1, 0.8)</td>
</tr>
<tr>
<td>( \Delta ) PF % change*</td>
<td>-1.1 (-21, 23)</td>
<td>0.1 (-20, 26)</td>
<td>1.3 (-18, 26)</td>
<td>5.7 (-15, 31)</td>
<td>4.5 (-16, 30)</td>
<td>-6.0 (-24, 16)</td>
<td>12 (-11, 41)</td>
</tr>
<tr>
<td>( \Delta ) TfR % change*</td>
<td>6.5 (-3.9, 18)</td>
<td>1.8 (-8.3, 13)</td>
<td>3.9 (-6.3, 15)</td>
<td>4.3 (-5.9, 15)</td>
<td>-2.5 (-12, 8.0)</td>
<td>-1.3 (-11, 9.3)</td>
<td>4.7 (-5.8, 17)</td>
</tr>
<tr>
<td>( \Delta ) ZPP % change*</td>
<td>-5.4 (-14, 4.4)</td>
<td>-10 (-19, -0.0)</td>
<td>-5.8 (-15, 3.9)</td>
<td>-8.1 (-17, 1.3)</td>
<td>-16 (-24, -7.5)</td>
<td>-11 (-20, -2.4)</td>
<td>-17 (-25, -7.8)</td>
</tr>
<tr>
<td>( \Delta ) body iron (mg/kg)</td>
<td>-0.5 (-1.4, 0.4)</td>
<td>-0.3 (-1.3, 0.6)</td>
<td>0.2 (-0.7, 1.1)</td>
<td>0.2 (-0.7, 1.2)</td>
<td>0.3 (-0.6, 1.2)</td>
<td>-0.2 (-1.1, 0.6)</td>
<td>0.3 (-0.6, 1.2)</td>
</tr>
<tr>
<td>( \Delta ) ID odds ratio #</td>
<td>1.6 (0.6, 4.0)</td>
<td>1.1 (0.4, 3.1)</td>
<td>1.4 (0.5, 3.5)</td>
<td>2.0 (0.8, 5.0)</td>
<td>0.8 (0.3, 2.2)</td>
<td>1.6 (0.6, 3.8)</td>
<td>1.0 (0.4, 2.8)</td>
</tr>
<tr>
<td>( \Delta ) inflammation odds ratio#</td>
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<td>1.0 (0.4, 2.2)</td>
<td>0.7 (0.3, 1.7)</td>
<td>1.1 (0.5, 2.5)</td>
<td>0.9 (0.4, 2.0)</td>
<td>0.9 (0.4, 2.0)</td>
<td>0.9 (0.4, 2.1)</td>
</tr>
<tr>
<td>( \Delta ) malaria density risk ratio|</td>
<td>1.9 (0.4, 8.3)</td>
<td>1.3 (0.3, 5.5)</td>
<td>1.7 (0.4, 7.7)</td>
<td>0.8 (0.2, 3.4)</td>
<td>1.2 (0.3, 5.3)</td>
<td>4.7 (1.1, 20)</td>
<td>1.3 (0.3, 6.0)</td>
</tr>
<tr>
<td>( \Delta ) malaria odds ratio#</td>
<td>1.0 (0.5, 1.9)</td>
<td>0.7 (0.3, 1.4)</td>
<td>1.1 (0.6, 2.3)</td>
<td>0.6 (0.3, 1.2)</td>
<td>0.7 (0.3, 1.3)</td>
<td>0.9 (0.5, 1.7)</td>
<td>0.8 (0.4, 1.7)</td>
</tr>
<tr>
<td>( \Delta ) Hookworm risk ratio|</td>
<td>0.8 (0.3, 2.3)</td>
<td>0.0 (0.0, 0.1)</td>
<td>0.7 (0.3, 1.9)</td>
<td>0.0 (0.0, 0.1)</td>
<td>0.0 (0.0, 0.1)</td>
<td>0.7 (0.3, 1.7)</td>
<td>0.0 (0.0, 0.1)</td>
</tr>
<tr>
<td>( \Delta ) Hookworms odds ratio#</td>
<td>1.5 (0.7, 3.5)</td>
<td>0.1 (0.1, 0.3)</td>
<td>1.3 (0.6, 2.8)</td>
<td>0.1 (0.0, 0.2)</td>
<td>0.0 (0.0, 0.1)</td>
<td>1.4 (0.6, 3.1)</td>
<td>0.1 (0.1, 0.3)</td>
</tr>
</tbody>
</table>

Mean difference (95% CI); *median difference (IQR); \#odds ratios (95% CI); \|incidence risk ratio (95% CI); **significantly higher at endpoint (p<0.05); significantly lower at endpoint (p<0.05); values ≥10 have been rounded to the nearest whole number.
Table 4: adjusted differences, odds ratios or incidence risk ratios of endpoint-baseline (95% CI), adjusted for treatment, baseline of respective parameter, inflammation, sex, age, biscuit compliance

<table>
<thead>
<tr>
<th></th>
<th>FPP</th>
<th>PPH</th>
<th>PMP</th>
<th>FPH</th>
<th>PMH</th>
<th>FMP</th>
<th>FMH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δ Hb (g/L)</td>
<td>0.2</td>
<td>2.2</td>
<td>-0.7</td>
<td>2.3</td>
<td>2.1</td>
<td>-1.0</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>(-2.4, 2.8)</td>
<td>(-0.5, 4.8)</td>
<td>(-3.3, 2.0)</td>
<td>(-0.4, 4.9)</td>
<td>(-0.6, 4.7)</td>
<td>(-3.6, 1.6)</td>
<td>(-0.4, 5.0)</td>
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<tr>
<td>Δ anaemia odds ratio*</td>
<td>1.1</td>
<td>(0.4, 3.1)</td>
<td>0.3</td>
<td>(0.1, 0.9)</td>
<td>0.9</td>
<td>(0.3, 2.5)</td>
<td>0.5</td>
</tr>
<tr>
<td>Δ PF % change*</td>
<td>-0.6</td>
<td>(-20, 24)</td>
<td>4.8</td>
<td>(-16, 31)</td>
<td>3.8</td>
<td>(-16, 29)</td>
<td>9.0</td>
</tr>
<tr>
<td>Δ Tfr % change*</td>
<td>7.0</td>
<td>(-3.5, 18)</td>
<td>2.3</td>
<td>(-7.9, 13)</td>
<td>4.7</td>
<td>(-5.7, 16)</td>
<td>5.1</td>
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<tr>
<td>Δ ZPP % change*</td>
<td>-4.3</td>
<td>(-13.3, 5.6)</td>
<td>-8.7</td>
<td>(-17.3, 0.1)</td>
<td>-4.1</td>
<td>(-13.1, 5.9)</td>
<td>-6.3</td>
</tr>
<tr>
<td>Δ body iron (mg/kg)</td>
<td>-0.5</td>
<td>(-1.4, 0.4)</td>
<td>-0.1</td>
<td>(-1.1, 0.8)</td>
<td>0.3</td>
<td>(-0.6, 1.3)</td>
<td>0.4</td>
</tr>
<tr>
<td>Δ ID odds ratio#</td>
<td>1.6</td>
<td>(0.6, 4.0)</td>
<td>1.0</td>
<td>(0.4, 2.9)</td>
<td>1.4</td>
<td>(0.5, 3.5)</td>
<td>1.9</td>
</tr>
<tr>
<td>Δ inflammation odds ratio#</td>
<td>1.0</td>
<td>(0.4, 2.3)</td>
<td>1.1</td>
<td>(0.5, 2.5)</td>
<td>0.9</td>
<td>(0.4, 2.0)</td>
<td>1.3</td>
</tr>
<tr>
<td>Δ malaria density risk ratio</td>
<td>2.1</td>
<td>(0.5, 9.7)</td>
<td>1.0</td>
<td>(0.2, 4.2)</td>
<td>2.3</td>
<td>(0.5, 9.9)</td>
<td>1.2</td>
</tr>
<tr>
<td>Δ malaria odds ratio#</td>
<td>1.0</td>
<td>(0.5, 2.0)</td>
<td>0.8</td>
<td>(0.4, 1.6)</td>
<td>1.4</td>
<td>(0.7, 2.7)</td>
<td>0.7</td>
</tr>
<tr>
<td>Δ Hookworm risk ratio</td>
<td>0.9</td>
<td>(0.3, 2.4)</td>
<td>0.0</td>
<td>(0.0, 0.1)</td>
<td>0.7</td>
<td>(0.3, 2.0)</td>
<td>0.0</td>
</tr>
<tr>
<td>Δ Hookworms odds ratio#</td>
<td>1.6</td>
<td>(0.7, 3.6)</td>
<td>0.1</td>
<td>(0.1, 0.3)</td>
<td>1.3</td>
<td>(0.6, 2.8)</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Mean difference (95% CI); *median difference (IQT); # odds ratios (95% CI); † incidence risk ratio (95% CI); significantly higher at endpoint (p<0.05); significantly lower at endpoint (p<0.05); values ≥10 have been rounded to the nearest whole number.
5 SYNTHESIS, CHARACTERIZATION, AND BIOAVAILABILITY IN RATS OF FERRIC PHOSPHATE NANOPARTICLES

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Abstract

Particle size is a determinant of iron (Fe) absorption from poorly soluble Fe compounds. Decreasing the particle size of metallic Fe and ferric pyrophosphate added to foods increases Fe absorption. The aim of this study was to develop and characterize nanoparticles of FePO₄ and determine their bioavailability and potential toxicity in rats. Amorphous FePO₄ nanopowders with spherical structure were synthesized by flame spray pyrolysis (FSP). The nanopowders were characterized and compared with commercially available FePO₄ and FeSO₄, including measurements of specific surface area (SSA), structure by transmission electron microscopy, in vitro solubility at pH 1 and 2, and relative bioavailability value (RBV) to FeSO₄ in rats using the hemoglobin repletion method. In the latter, the potential toxicity after Fe repletion was assessed by histological examination and measurement of thiobarbituric acid reactive substances (TBARS). The commercial FePO₄ and the 2 FePO₄ produced by FSP (mean particle sizes, 30.5 and 10.7 nm) had the following characteristics: SSA: 32.6, 68.6, 194.7 m²/g; in vitro solubility after 30 min at pH 1: 73, 79, and 85% of FeSO₄; and RBV: 61, 70, and 96%, respectively. In the histological examinations and TBARS analysis, there were no indications of toxicity. In conclusion, nanoparticles of FePO₄ have a solubility and RBV not significantly different from FeSO₄. Reducing poorly soluble Fe compounds to nanoscale may increase their value for human nutrition.

Abbreviations used: AAS, atomic absorption spectrometry; BET, Brunauer-Emmett-Teller method; d_BET, particle diameter as calculated from SSA measurements; d_LS, particle diameter as measured by light scattering; Fe-def, Fe-deficient; FSP, flame spray pyrolysis; Hb, hemoglobin; ICP-MS, inductively coupled plasma mass spectrometry; RBV, relative bioavailability value; MPS, mean particle size; SAED, selected area electron diffraction; SSA, specific surface area; TBARS, thiobarbituric acid reactive substance; TEM, transmission electron microscopy.
Introduction

Iron deficiency anemia is a major global public health problem (1). Food fortification with iron (Fe) can be an effective strategy to control iron deficiency anemia, but adding Fe to foods can be problematic (2). Water-soluble, highly bioavailable Fe compounds often cause adverse organoleptic changes, whereas low solubility Fe compounds, although more stable in foods, tend to have low bioavailability. Particle size can be an important determinant of Fe absorption from poorly soluble Fe compounds in foods. Decreasing the particle size of metallic Fe powders by 50–60% to a mean particle size (MPS)\(^6\) of 7–10 \(\mu m\), as determined from dynamic light scattering (dLS), has been reported to increase Fe absorption by 50% in rats (3,4). In humans, Fe absorption from hydrogen-reduced Fe with an MPS of 5 and 10 \(\mu m\) was comparable to that of ferrous sulfate (5). Reducing the particle size of ferric pyrophosphate by conventional grinding from a MPS of \(\geq 20 \mu m\) to 2–3 \(\mu m\) resulted in a nonsignificant increase in its relative bioavailability value (RBV) to ferrous sulfate (6). However, smaller ferric pyrophosphate particles, with an MPS of 0.3–0.5 \(\mu m\) in aqueous solution with emulsifiers preventing agglomeration, have an RBV of \(\sim 95\%\) (7,8). These studies suggest reducing the particle size of low solubility Fe compounds to submicron size may be promising for food fortification, because they may cause fewer sensory changes yet be highly bioavailable.

Nanotechnology deals with materials and systems of characteristic scale below 100 nm to exploit novel properties and phenomena (9). Potential medical/nutritional applications for nanomaterials include new systems that may allow targeted delivery of substances, as well as enhanced permeability and increased retention (10,11). Recent research on the gastrointestinal absorption of nanoparticles has focused on enhancing the absorption of drugs, vaccines, and nutrients that are either degraded by the digestive process or poorly absorbed (12,13). However, there are toxicity concerns with nanoparticles. As most studies in this area have focused on airborne nanoparticles, there are only limited data on orally ingested trace element nanoparticles (14,15) focusing on selenium, copper, and zinc (16–18). Nanoparticles may be taken up by persorption and/or absorbed by gut-associated lymphoid tissue and pass through the mesenteric lymphatics to the venous circulation (19).
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Ferric phosphate (FePO₄) is a white-colored, poorly soluble Fe compound of little nutritional value due to its low bioavailability (20). In this study, we used flame technology to produce FePO₄ nanoparticles of various sizes. This is a fast, dry, and versatile process for synthesis of nanostructured commodities such as carbon black, fumed silica, alumina, etc. (21). Flame spray pyrolysis (FSP) is a scalable process (22) allowing for production of tailor-made particles with high specific surface area (SSA) and well-defined chemical composition (23). We aimed to develop and characterize nanoparticles of FePO₄ and determine the effects of particle size reduction into the nano-range on bioavailability and safety of these particles in rats.

Materials and Methods

Commercially available compounds

In this study, 4 Fe compounds were analyzed. Two of them were commercially available FePO₄ and ferrous sulfate hydrate (FeSO₄·H₂O), obtained from Dr. Paul Lohmann GmbH KG, Emmerthal, Germany (Art. nos. 3043355 and 3590548, respectively).

Nanoparticle synthesis

FePO₄ nanoparticles were made by FSP (24). The precursors, Fe(III)-acetylacetonate and tri-butylphosphate (reagent grade, Fluka), were dissolved in xylene (Riedel-de-Haen, >96%, dried over molecular sieves) at an Fe and phosphorus ion concentration of 0.2 mol/L each. This solution was metered into the reactor nozzle by a syringe pump (Inotech R232) at a rate of 3–7 mL/min and dispersed by 3–8 L/min O₂ (PanGas, purity 99.95%) into fine droplets by a gas-assist nozzle; the pressure drop at the nozzle tip was 1.5 bar. The spray was ignited by a premixed methane/oxygen flame ring surrounding the spray capillary at a radius of 6 mm with a spacing of 0.15 mm (25). The premixed flame was fed by 1.13 L/min CH₄ (PanGas, purity 99.5%) and 2.40 L/min O₂ (PanGas, purity 99.95%). An additional oxygen sheath flow of 5 L/min was fed through a sinter metal ring of 8-mm width and 9-mm i.d., surrounding the supporting flame to ensure complete conversion of the reactants. The rates of the liquid feed and dispersion oxygen flow were varied to
select the desired particle characteristics (24). The gas flows were monitored by calibrated mass flow controllers (Bronkhorst). Precursor evaporation within the liquid feed lines and nozzle overheating was prevented by water cooling the reactor. Using a vacuum pump, product particles were collected on a Teflon-supported Teflon membrane filter (BHA Technologies, 1TMTF700WHT, Muemliswil) placed in a water-cooled holder 50–65 cm above the nozzle, keeping the off-gas temperature below 200°C.

**Powder characterization**

The powder SSA was determined by the Brunauer-Emmett-Teller (BET) method from a 5-point nitrogen adsorption isotherm at 77 K in the relative pressure range \((p/p_0) = 0.05\) to 0.25 (Tristar, Micromeritics Instruments). Prior to analysis, samples were outgassed at 150°C for 1 h. Assuming dense spherical particles, the particle diameter as calculated from SSA measurements (\(d_{\text{BET}}\)) was calculated according to \(d_{\text{BET}} = \frac{6}{(\text{powder SSA})}\). For transmission electron microscopy (TEM) investigation, particles were dispersed in ethanol and deposited onto a carbon foil supported on a copper grid. TEM investigations were performed on a CM30ST microscope (FEI; LaB_6 cathode, operated at 300 kV, point resolution ~2Å). Selected area electron diffraction (SAED) was used to verify the amorphous state of the synthesized powder.

The stochiometric composition of the FePO_4 particles was analyzed using inductively coupled plasma mass spectrometry (ICP-MS). A total of 200 mg of sample was dissolved in 5% nitric acid and analyzed on a sector field ICP-MS (Element 2, Finigan-MAT). The instrument was equipped with a perfluoroalkoxy (PFA)-screw top (ST) microflow nebulizer operated at a sample uptake rate of 300 \(\mu\)L/min and a PFA spray chamber, both from Elemental Scientific. The mass spectrometer was operated in a medium resolution mode \((m/\Delta m = 4000)\) to separate molecular interferences \(^{40}\text{Ar}^{16}\text{O}^+\) and \(^{15}\text{N}^{16}\text{O}^+\) from the monitored ions, \(^{56}\text{Fe}\) and \(^{31}\text{P}\), respectively. The electric scan acquisition mode was used and 9 scans were performed per sample. The optimization was restricted to adjustments of sample (0.8 L/min), make-up (0.3 L/min), and auxiliary (0.6 L/min) gas flow rates to obtain a stable response at a maximum signal, and 1.3 kW rf-power was applied at a cool gas flow rate of 16 L/min. The obtained ratios were subsequently converted into molecular formulae assuming the existence of hydrates.
Raman spectroscopy was performed on FePO₄ particles using a Renishaw InVia Reflex Raman system equipped with a 514-nm diode (solid state, 25 mW) laser as an excitation source focused in a microscope (Leica, city magnification x50). Three spectra were recorded (60 s) on a charge-coupled device camera after diffraction (1200 lines \cdot mm⁻¹) using 0.25-mW laser energy to avoid thermal alteration. The spectra obtained were compared with those obtained from the commercially available FePO₄.

To characterize the Fe species (Fe²⁺/Fe³⁺) in the FePO₄ particles, they were dissolved in sulfuric acid and underwent reduction-oxidation using cerium as reducing agent and ferroin (1,10-phenanthroline) as indicator (26). This method allows visual detection of the occurrence of ferrous Fe >5% of the total Fe content. After dissolution of Fe powders in hydrochloric acid (32%), their Fe content was measured by atomic absorption spectrometry (SpectrAA-240FS; Varian).

The in vitro solubility of the Fe compounds was tested following Swain et al. (27). A compound containing 20 mg Fe was added to 250 mL aqueous solution of 0.1 and 0.01 mol/L hydrochloric acid, corresponding to pH 1 and 2, respectively, and mixed in an orbital shaker at 150 rpm and 37°C (Aqua shaker, Adolf Kühner). The percentage of dissolved Fe was assessed after 5, 15, 30, 45, and 60 min in a 1.5-mL solution aliquot. After centrifugation (11,600 \times g; 4 min), the Fe content of the supernatant solution was measured by atomic absorption spectroscopy (AAS) with external calibration.

**Rats and diets**

Ethical approval for the study was granted by the Veterinary Office of the Canton of Zurich's Department of Health. The bioavailability of the Fe compounds was determined by the hemoglobin (Hb) repletion method (20,28,29). Two levels of dietary Fe were used for each compound. Male Sprague-Dawley rats (\(n = 115\); Charles River) 21 ± 3 d old were housed individually in stainless steel cages with grated stainless steel floors. The rats were kept at 23.6 ± 0.5°C and a relative humidity of 51.3 ± 6.8%, with a 12-h-light/-dark cycle. All rats were handled daily to reduce stress at blood collection and killing. Body weight was measured 3 times/wk. Rats consumed Millipore water (Milli-Q UF Plus, Millipore) ad libitum. The diets were prepared by Dyets following the AIN-93G purified rodents guidelines (30). The rats
consumed an Fe-deficient (Fe-def) diet (Fe content, 2.5 mg/kg, as measured by AAS) ad libitum for 24 d. After this depletion period, rats were weighed and blood was collected by tail incision (31), with precautions taken to avoid hemodilution. To increase tail vein vasodilation, the tail was wrapped in a warming towel. The blood was collected into heparin-coated capillaries for immediate Hb determination and into EDTA-coated capillary tubes (Microvette 300, Sarstedt) for further analysis of plasma. Blood from these containers was centrifuged (3000 x g; 8 min at 4°C) and plasma for thiobarbituric acid reactive substances (TBARS) measurement was separated and stored at -80°C.

Rats with an Hb concentration of 35 ± 4 g/L (range 28–46 g/L) after depletion were randomly assigned to 9 groups of 9–12 rats. The rats of each group consumed the same Fe-def diet fortified with 1 of the 3 FePO₄ compounds (each at intended levels of 10 or 20 mg Fe/kg diet), ferrous sulfate (FeSO₄·H₂O; at 10 or 20 mg Fe/kg diet), or no added Fe (2.5 mg Fe/kg diet; Fe-def) for 15 d ad libitum. Other than their Fe concentrations, the diets were equivalent and conformed to the recommendations for AIN-93 purified diets (30). The Fe concentration of all diets was verified by AAS (SpecrAA-240Z with GTA-120 Graphite Tube Atomizer, Varian Techtron). To serve as Fe-sufficient controls, 3 rats received a regular rodent diet (Kliba) throughout the experimental period (Fe-sufficient). These rats were used for comparisons in the histological assessment. Individual food consumption was recorded daily throughout the repletion period. After the repletion period, rats were weighed, and blood was collected by tail incision and processed as described above, with plasma for TBARS measurement stored at -80°C. The rats were then killed using CO₂.

Laboratory analysis

Hb concentration was measured in triplicate in whole blood with a commercial kit (D5941; Sigma) using the cyanmethemoglobin method (32) and commercially available control material (Digitana AG). TBARS were measured in plasma in duplicate with a commercially available test (TBARS assay kit, ZeptoMetrix); normal plasma concentrations are <1.5 malondialdehyde units.

Histological examination
From 3 of the rats in each of the following 6 groups: control (Fe-sufficient), Fe-def, the 3 FePO₄ compounds fed at 20 mg Fe/kg, and the FeSO₄ fed at 20 mg Fe/kg (total n = 18), tissue samples of the stomach, duodenum, jejunum, ileum, colon, liver, spleen, kidney, pancreas, lymphatic tissue, and sternum were excised immediately after killing. For light microscopy, tissues were fixed by immersion in 4% buffered formaldehyde, dehydrated with xylene and a descending alcohol row (Tissue Tek VIP), paraffin embedded, and subsequently stained with hematoxylin-eosin, Prussian Blue for detection of Fe³⁺, and Turnbull Blue for detection of Fe²⁺. TEM (CM10 Philips) was used to examine sections of duodenal mucosa from a single rat from each group (n = 6). Tissues were fixed in 2.5% glutaraldehyde and embedded in epoxy before further processing into ultrathin sections. The veterinary pathologists and microscopists were unaware of the group assignment.

Statistical analysis

Data processing and analyses were done using SPLUS-2000 (Release 3, Insightful Corporation), SPSS (version 13.0; SPSS) and EXCEL (Enterprise Edition; Microsoft). Values in the text are means ± SD. Using the slope-ratio method, the bioavailability (RBV) of each Fe compound relative to FeSO₄ was calculated by comparing the change in Hb [g/(L·15 d)] with the measured Fe intake (µg/d) (33,34). The slope of the responses for each dietary Fe compound was calculated by using a common-intercept multiple linear regression model with the Fe-def group serving as the blank. Linearity of the regression curves was determined for each Fe compound separately and tests were conducted to determine whether the mean of the blank differed significantly from the common intercept for the 4 Fe compounds. Tukey's method was applied to test whether the slopes of the 3 FePO₄ compounds were significantly different from that of FeSO₄ and from each other. Using Fieller's method (35), [95% CI] for the RBV to ferrous sulfate were obtained. To compare means between the treatment groups, 1-way ANOVA was done with post-hoc t tests adjusted for multiple comparisons (Bonferroni). Independent sample t tests were used to compare the in vitro solubility results, and adjusted for multiple comparisons (Bonferroni). Percentages were compared using chi-square tests. Differences were considered significant at P < 0.05.
Results

Material characterization

The SSA of the 3 FePO₄ compounds and their calculated d_BET are shown in Table 1. The 2 FSP-made FePO₄, medium and small particles, were dense spherical particles and the d_BET matched well the observation from TEM (Fig. 1 B,C). The TEM image of the commercial powder, FePO₄ large particle (Fig. 1 A), shows irregular and highly porous particles. For all 3 FePO₄ compounds, the SAED images were characteristic of an amorphous substance (Fig. 1). Handling characteristics of the fine powders were similar to commercially available small particle size Fe compounds, such as ground micronized ferric pyrophosphate (Dr. Paul Lohmann GmbH KG, Emmerthal, Germany).
Table 1: Compound characteristics: SSA, calculated MPS (d_{BET}), physical structure, stochiometry (chemical composition), and in vitro solubility after 15 and 30 min in 0.1 mol/L HCl (SOL) and RBV in rats.

<table>
<thead>
<tr>
<th>Compound</th>
<th>SSA</th>
<th>Calculated MPS</th>
<th>Structure</th>
<th>Stochiometry</th>
<th>SOL 15</th>
<th>SOL 30</th>
<th>RBV</th>
</tr>
</thead>
<tbody>
<tr>
<td>FePO_{4}</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Large</td>
<td>32.6</td>
<td>(64.2)</td>
<td>A, IP</td>
<td></td>
<td>37.8\textsuperscript{a}</td>
<td>73.4\textsuperscript{a}</td>
<td>60.9 (52.6, 69.3)\textsuperscript{a*}</td>
</tr>
<tr>
<td>Medium</td>
<td>68.6</td>
<td>30.5</td>
<td>A, S</td>
<td>FePO_{4}\cdot2\text{H}_2\text{O}</td>
<td>56.8\textsuperscript{b}</td>
<td>79.2\textsuperscript{a,c}</td>
<td>69.5 (61.1, 78.4)\textsuperscript{a*}</td>
</tr>
<tr>
<td>Small</td>
<td>194.7</td>
<td>10.7</td>
<td>A, S</td>
<td>FePO_{4}\cdot2\text{H}_2\text{O}</td>
<td>82.5\textsuperscript{c,d}</td>
<td>84.7\textsuperscript{c,d}</td>
<td>95.7 (85.7, 107.0)\textsuperscript{b}</td>
</tr>
</tbody>
</table>

\textsuperscript{1} Percentages in a column without a common letter differ, P < 0.05. \textsuperscript{*}Different from the reference substance (100%), P < 0.05. \textsuperscript{2} Assuming dense spherical particles (not the case for FePO_{4} large). \textsuperscript{3} From TEM: A (amorphous), S (spherical), IP (irregular porous).
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Figure 1 TEM and SAED (insets) images of the 3 FePO$_4$ compounds: (A) FePO$_4$ large particle, (B) FePO$_4$ medium particle, and (C) FePO$_4$ small particle. The 2 compounds made by FSP, medium and small FePO$_4$ particles, were dense and spherical (Fig. 1B,C). The FePO$_4$ large particle (Fig. 1A) exhibited irregular and highly porous particles. For all 3 compounds, the SAED images were characteristic of an amorphous substance.

For the FSP-made FePO$_4$ medium and small particles, ICP-MS demonstrated a Fe:P ratio of 1.93 as opposed to the expected 1.80 for anhydrous FePO$_4$. Including hydrates in the ratio calculation, best fit with the expected ratio was obtained with FePO$_4$·2H$_2$O (ratio = 1.80). Raman spectroscopy confirmed the presence of Fe phosphate (data not shown). Using the Fe speciation assay, there were no detectable ferrous ions, indicating that >95% of the Fe was in the ferric state (data not shown). AAS analysis revealed Fe contents of the FePO$_4$ large, medium, and small particles of 25.6 ± 0.4%, 33.8 ± 0.8%, and 33.2 ± 0.5%, respectively.

**In vitro solubility**

In the tests of in vitro solubility, all 3 FePO$_4$ compounds were very poorly soluble at pH 2; solubility was <5% at all time points, with no significant differences among the compounds (data not shown). In contrast, at pH 1, a solubility dependence on SSA was observed during the first 30 min of the measurement (Table 1); the higher the SSA, the higher the dissolution rate. At pH 1, the FePO$_4$ small particle was more soluble than FeSO$_4$·H$_2$O ($P < 0.05$) after 5 min and there was no difference between the solubility of the FePO$_4$ small particle and FeSO$_4$·H$_2$O at the other time points (Fig. 2).
Figure 2. In vitro solubility test of the 3 FePO₄ compounds and FeSO₄ at pH 1. Values are means ± SD, n = 3. Values marked with *, **, *** are different from the reference compound, FeSO₄, P < 0.05. Values at each time point without a common number of asterisks differ, P < 0.05.

**RBV**

The results of the Hb repletion study, including diet fortification level, rat number per group, daily Fe intake, body weight gain, and Hb change over the repletion period are shown in Table 2. Dose-response curves were calculated based on daily Fe intake (Fig. 3). In this model, the regression lines for the 4 Fe compounds did not significantly deviate from linearity and the mean of the blank (circles) was not significantly different from the common intercept (data not shown). The RBV of FePO₄ large and FePO₄ medium particles did not differ from each other, but both were lower than the RBV of the FePO₄ small particle (P < 0.05) (Table 2). The RBV of the FePO₄ small particle did not differ from the reference, FeSO₄·H₂O.
Table 2 Fe fortification level, fortified Fe intake, body weight gain, change in Hb in Fe-depleted rats during the 15-d repletion period, and plasma TBARS at d 15

<table>
<thead>
<tr>
<th>Iron compound</th>
<th>Fortification level (mg/kg diet)</th>
<th>Number of animals/group</th>
<th>Daily iron intake (µg/d)</th>
<th>Body weight gain (g/15 d)</th>
<th>Hb change (g/L*15d)</th>
<th>TBARS (µmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe-deficient control</td>
<td>2.51 ± 0.42</td>
<td>12</td>
<td>34.2 ± 3.3</td>
<td>54.4 ± 10.9^a</td>
<td>-2.9 ± 3.2</td>
<td>1.05 ± 0.44</td>
</tr>
<tr>
<td>FeSO₄·H₂O</td>
<td>9.11 ± 1.10</td>
<td>10</td>
<td>170.5 ± 14.8^a</td>
<td>101.6 ± 10.6</td>
<td>27.9 ± 5.8^a</td>
<td>0.69 ± 0.16</td>
</tr>
<tr>
<td>FeSO₄·H₂O</td>
<td>20.01 ± 4.02</td>
<td>10</td>
<td>410.2 ± 30.9^b</td>
<td>112.9 ± 32.7</td>
<td>66.9 ± 7.1^b</td>
<td>0.96 ± 0.19</td>
</tr>
<tr>
<td>FePO₄ large</td>
<td>13.57 ± 0.24</td>
<td>9</td>
<td>223.6 ± 24.1^c</td>
<td>93.3 ± 11.1</td>
<td>17.6 ± 6.0^ad</td>
<td>0.68 ± 0.19</td>
</tr>
<tr>
<td>FePO₄ large</td>
<td>25.73 ± 0.39</td>
<td>10</td>
<td>483.9 ± 41.3^d</td>
<td>110.0 ± 14.5</td>
<td>49.1 ± 11.9^c</td>
<td>0.89 ± 0.12</td>
</tr>
<tr>
<td>FePO₄ medium</td>
<td>12.74 ± 0.51</td>
<td>10</td>
<td>232.2 ± 13.9^c</td>
<td>88.0 ± 14.3</td>
<td>16.3 ± 5.7^d</td>
<td>0.80 ± 0.11</td>
</tr>
<tr>
<td>FePO₄ medium</td>
<td>20.18 ± 0.15</td>
<td>10</td>
<td>367.7 ± 18.4^e</td>
<td>105.8 ± 11.7</td>
<td>43.8 ± 8.3^c</td>
<td>0.88 ± 0.29</td>
</tr>
<tr>
<td>FePO₄ small</td>
<td>11.63 ± 0.16</td>
<td>10</td>
<td>201.5 ± 9.7^ac</td>
<td>100.4 ± 9.3</td>
<td>20.3 ± 6.7^ad</td>
<td>0.84 ± 0.24</td>
</tr>
<tr>
<td>FePO₄ small</td>
<td>20.01 ± 0.78</td>
<td>10</td>
<td>379.5 ± 23.0^be</td>
<td>112.8 ± 10.9</td>
<td>51.2 ± 10.6^c</td>
<td>0.84 ± 0.19</td>
</tr>
</tbody>
</table>

^1 Values are means ± SD. Means in a column without a common letter differ, P < 0.05.
Figure 3  Dose-response curves for the Hb repletion assay in depleted rats consuming a Fe-def diet or the Fe-def diet fortified in graded concentrations with FeSO₄, FePO₄ small particle, FePO₄ medium particle, or FePO₄ large particle for 15 d. Regression lines were calculated on daily Fe intake (μg/d) and change in Hb concentration [g/(L·15 d)]. Values are shown individually, n = 9–12/group.

**Histology and TBARS**

On the hematoxylin-eosin stained sections, histologic changes consistent with hypoxic damage to the liver were found in 3 rats from the Fe-def group. There were no visible inflammatory changes or other adverse findings in the tissues sampled. No detectable Fe²⁺ was seen in the Turnbull Blue stains. In the Prussian Blue staining for Fe³⁺, only in 2 of the rats receiving the 20 mg Fe/kg diet as FeSO₄, the reference material, were small amounts of Fe detected in the liver, gastric mucosa, and kidney.

In the duodenum sections examined by TEM, no particulate material consistent with Fe particles was seen.

Plasma TBARS did not differ among the groups at the end of the repletion period (Table 2) and all values were well within the reference range for the assay.
Discussion

To our knowledge, this is the first study investigating the potential nutritional value of Fe-containing nanoparticles. The FePO₄ small particles, with a \( d_{\text{BET}} \) of 10.7 nm, demonstrated in vitro solubility and in vivo RBV equivalent to commercial FeSO₄. Moreover, comparing the nanoparticulate FePO₄ to commercial FeSO₄, there was no evidence of potential toxicity in the histologic examinations or the TBARS analyses. This suggests that FePO₄, considered a poorly soluble compound with a low RBV, when reduced to nanoparticle size, shows performance characteristics similar to FeSO₄, the reference Fe compound for food fortification for humans. Although sensory aspects were not considered in this study, compared with FeSO₄, the FePO₄ nanoparticles may possibly produce fewer adverse organoleptic changes in food vehicles, particularly in color-sensitive foods, such as rice, salt, milk-based drinks, and highly refined cereal flours. However, the sensory characteristics of the Fe nanoparticles needs further investigation.

Few studies have investigated the potential of FePO₄ for food fortification (20,36,37). Despite good sensory characteristics (bright color, only minor off-flavors in foods), FePO₄ is generally considered to have little nutritional value due to its low bioavailability. In these studies, no consideration was given to whether the FePO₄ was in the crystalline or amorphous state, although this structural characteristic was shown to significantly affect FePO₄ bioavailability (38). Compared with previous studies, the higher relative bioavailability of commercial FePO₄ used in the present study may have been, at least partially, caused by its amorphous state, its irregular porous structure, and its high surface area. It was reported that the RBV of FePO₄ varies between 6–46% (2) and in a study comparing crystalline with amorphous FePO₄ in humans (36), higher bioavailability was found for the latter (20 vs. 28%).

Two previous studies have demonstrated that metallic Fe particles of \( \mu \text{m} \) (39) and nm (40) size may cross into the circulation through paracellular uptake through tight junctions. Inert nanoparticles may also be absorbed through Peyer’s patches of the small intestine, passing through the mesenteric lymphatics to the liver and spleen (41). Particles that are smaller, hydrophilic, and positively charged are more readily absorbed by this process (42,43).
This study does not suggest absorption of the FePO₄ nanoparticles via paracellular uptake or the lymphatic system, as there was no visible or stainable Fe in the mesenteric lymphatics in the histologic sections. Considering the high solubility of the FePO₄ nanoparticles, they were most likely dissolved during digestion in the intestinal lumen and absorbed through the usual receptor-mediated pathway of nonheme Fe absorption, i.e. uptake of Fe by the divalent metal transporter 1 on the duodenal brush border (44).

To assess potential oxidative stress in the rats during Fe repletion, we measured the production of TBARS, a by-product of reactive oxygen processes. Previous in vitro and in vivo studies on the toxicology of airborne nanoparticles demonstrated increased production of reactive oxygen species, which has been attributed to preferential mobilization of the nanoparticles to mitochondria and/or redox active organelles (15), their increased surface area (45), and/or the presence of free Fe (46). However, given orally in this study, the Fe nanoparticles did not appear to promote oxidation; there were no differences in TBARS production among the groups administered the various Fe compounds.

Data on the nutritional and/or toxicological effects of other nanoscale trace elements is limited. Nanosized particles of selenium (30–60 nm) fed to rats had a bioavailability comparable with sodium selenite (17) and lower subchronic toxicity (47). In contrast, in mice, the acute oral toxicity of nanoscale zinc (58 nm) and copper (24 nm) were higher than equivalent amounts of microscale zinc (16,18). Although this study was not specifically designed to investigate the toxicity of Fe compound nanoparticles, feeding 150–370 μg Fe/d for 15 d to weanling rats did not induce measurable histologic or biochemical adverse effects. Further studies are needed to determine the acute and subchronic toxicity of Fe-containing nanoparticles.

The SSA represents the accessible surface area. Thus, if the $d_{BET}$ derived from the SSA under the assumption of dense spheres is smaller than the diameter measured by light scattering, the particles are either porous or agglomerated. The FePO₄ large particles investigated in this study were porous and irregular (Fig. 1A) and the measured particle size ($d_{LS, 50} = 2.5 \mu m$), as well as the manufacturer’s specifications for particle size based on sieve analysis ($d_{50} = 4.4 \mu m$), did not reflect the actual SSA ($d_{BET} = 64.2 \text{ nm}$) of the material due to additional internal surface. Solubility is a
surface-dependent phenomenon and thus determined by the SSA rather than the overall particle size as, for example, measured by light scattering. This may explain earlier studies where no significant differences in RBV were found despite an apparent reduction in particle size ($d_{LS}$) of ferric pyrophosphate (3,4,6,48).

Flame technology is currently being used on a large scale to produce carbon black, fumed silica, and titania pigments (49). This established technology may thus be potentially competitive and cost effective for production of FePO$_4$ nanoparticles (or nanoparticles of other Fe compounds) for nutritional supplementation and/or food fortification. The results of this study suggest further research on the synthesis, efficacy, and safety of Fe-containing nanoparticles in nutrition would be valuable.

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References


6 CONCLUSIONS AND PERSPECTIVES

The aim of this thesis was to contribute knowledge for the development of strategies to reduce anaemia and iron deficiency in sub-Saharan Africa. Anaemia in this setting is regarded as multifactorial in cause, with nutritional iron deficiency, helminths and malaria thought to be the main contributing factors. However, other nutritional and environmental factors can contribute to its aetiology.

After methodological optimization of the measurement of serum ferritin, an innovative nano-sized iron compound with a high relative bioavailability was synthesized and characterised. The possible influence of riboflavin deficiency on iron metabolism was investigated, followed by a study looking at the relative contribution of iron fortification, malaria parasitaemia and helminth infection to anaemia.

Nutritional iron deficiency is thought to be an important factor in the aetiology of both iron deficiency and anaemia. In food fortification, the challenge is to find iron compounds that are well absorbed, yet do not cause noticeable sensory changes. Efforts to improve iron compounds in this respect are still in progress. The development of an iron phosphate nanopowder with high bioavailability shows good promise. In an animal model, the compound was as well absorbed as ferrous sulphate, a water-soluble compound and appeared to be safe in preliminary toxicity assessments. Further research should address: i) the organoleptic stability of the nanopowder in different food matrices and during food processing; ii) a thorough toxicity evaluation iii) its bioavailability in inhibitory meals; iv) investigation of food items that are suitable to be fortified with this compound; v) its cost; and vi) the potential of co-fortification with other minerals.

In children free of malaria parasites, an association between mild riboflavin deficiency and poor iron status was found in Ivorian school-children. However, riboflavin status did not correlate with haemoglobin concentration or anaemia, possibly due to the only mild severity of the riboflavin deficiency. Alternatively, inflammation due to infection may be more strongly related to iron metabolism than riboflavin status. These cross-sectional findings generally support previous studies reporting riboflavin
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deficiency impairs iron metabolism. Due to the mild severity of riboflavin deficiency in the investigated population and methodological challenges of determining riboflavin status, the relevance of these findings remains to be elucidated.

In several sub-Saharan African countries, including Côte d'Ivoire, Nigeria and South Africa, wheat flour fortification with electrolytic iron is mandatory (Andang'o et al., 2007; Flour Fortification Initiative, 2007). Electrolytic iron is recommended for wheat flour fortification by the World Health Organization (WHO, 2006) and a recent review confirmed its potential efficacy, although higher doses were proposed (Hurrell, personal communication). Efforts to reduce malaria prevalence in infants and pregnant women by means of intermittent preventive treatment or insecticide-treated bednets are ongoing in endemic countries. This may increase risk for malaria in school-age children who received intermittent preventive treatment in early infancy due to their lack of built up semi-immunity (Brooker et al., 2008). Therefore, one strategy that has been proposed to reduce malaria incidence in school-children is intermittent preventive treatment of malaria (Greenwood, 2006). Chemotherapy to reduce helminth infections, in particular hookworms and schistosomiasis, in school-children has been proposed and is being implemented on a large-scale in some countries (Utzinger & de Savigny, 2006).

The combination of universal iron fortification, a strategy that may be sustainable and controlled centrally, with the simultaneous administration of anthelmintics and intermittent preventive treatment for malaria could be a highly promising multi-prong approach to reduce disease burden and thereby, control anaemia. In particular with regard to the restricted health systems in some countries of sub-Saharan Africa, this approach could be of interest. Thus, the three above interventions were tested in school-age children in a well-controlled study and the impact of each intervention on its respective disease (iron deficiency, helminths, malaria), as well as on anaemia were investigated.

Surprisingly, only antihelminthic chemotherapy was efficacious in improving the anaemia prevalence compared to the placebo group. The two other interventions had no impact on their respective diseases, or anaemia. However, the low numbers of children with iron deficiency may have reduced the power of the study to detect a
potential positive effect of fortification. Alternatively, the relatively high prevalence of inflammation may have blunted the response to iron fortification. The treatment interval and dosage for the intermittent preventive treatment for malaria in school-age children were adopted from experiences in infants and pregnant women; the administration regimen and prevalence of drug resistance may need to be reassessed and optimized for this age group.

These findings show: i) a careful assessment of the aetiology of anaemia for each setting and population group is of high relevance prior to launching countrywide intervention programs; ii) the efficacy of an intervention needs to be proven for the given context prior to implementing it on a national scale; iii) iron fortification with electrolytic iron in sub-Saharan Africa may need to be reconsidered.


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