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

Severe anaemia in Malawian children
A descriptive profile

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Submitted
ABSTRACT

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

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

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

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

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

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

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

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

PATIENTS, MATERIALS, AND METHODS

Population

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

Procedures
This study formed part of large severe anaemia research programme. Admission procedure and management have been described in detail elsewhere\textsuperscript{10}. In summary, on enrolment a standardized study questionnaire and physical examination were completed, and samples of blood, urine and stool were collected. In cases only, if the clinical condition permitted, a fine needle bone marrow aspirate was obtained under anaesthesia from the posterior superior iliac spine. Children requiring admission were treated in a study ward and all conditions were managed according to standard protocols.

Laboratory measurements
Laboratory tests crucial to patient care were performed within 24 hours, and sample aliquots were stored at -80°C for later analysis. Whole blood haemoglobin and plasma haemoglobin concentrations were measured using the HemoCue systems\textsuperscript{®} (Angelholm, Sweden). Plasma haemoglobin levels were assessed in all Cases and in a smaller, randomly selected, sub-group of 92 Control children. A full blood count, including absolute reticulocyte count, was performed by Coulter counter analyzer\textsuperscript{®} (Beckman Coulter, Durban, South Africa). Bone marrow aspirates were used for microscopy and for the cytokine assays. Wedged spread films were air dried, fixed in methanol and stained with May-Grüwald-Giemsa and iron stain (HematoGnost Fe, Darmstadt, Germany). Bone marrow differential count was conducted by a laboratory technician with more than 10 years experience (RV). Iron stained bone marrow slides were graded according to Gale’s criteria for iron content in stroma, macrophages and red cell precursors\textsuperscript{11}.

C-reactive protein, erythropoietin, haptoglobin, transferrin, iron, ferritin, folate and vitamin B12 concentrations were analyzed in heparin plasma on a Roche p800 system (Roche, Switzerland). Inflammatory cytokine profiles were measured by Cytometric Bead Array on a FACS-Calibur flow-cytometer (Becton-Dickinson, South Africa), which was also used to measure the red cell precursor fractions in the bone marrow aspirate (details have been described elsewhere\textsuperscript{10}). Serum vitamin A (retinol) and soluble transferrin receptor (sTfR) were measured using high performance liquid chromatography and enzyme linked immunosorbant assay (Ramco Laboratories, TX).
respectively. *Plasmodium falciparum* asexual parasites were counted against 200 white blood cells. Stool samples were examined for helminths using the Kato-Katz method and polymerase chain reaction (PCR). Urine specimens were examined for *Schistosoma haematobium* using a semi-quantitative concentration method.

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

**Definitions used**

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

**Malaria infection:** was defined as the presence of asexual *Plasmodium falciparum* parasites on a blood film; Parasitaemia in excess of 10,000 asexual parasites/μL was referred to as 'Pf>10^4/ul'.

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

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

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

**Iron deficiency:** In our study population, an sTfR/log ferritin-index of more than 5.6 has been shown to be the best predictor of bone marrow iron status irrespective of the presence of infection, and was used to define iron deficiency (sensitivity 70%, specificity 75%).
Statistical Methods
This is primarily a study of the diversity of mechanisms of severe anaemia, the identification of which requires an assessment of bone marrow appearances. Since bone marrow could only be examined in Cases, the principal statistical comparisons are of categories within the group of severe anaemia cases. The prevalence of each of the three anaemia mechanisms was assessed in the Cases group, and important aetiological factors were compared across the various mechanism sub-groups. More complex associations between factors and with the main mechanisms were explored using Structural Equation Modelling. Reported p-values are always two-sided. Data were analyzed using STATA 9 (Stata Corporation, TX), SPSS 14 and AMOS 6.0 (SPSS, IL).

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

Table 1. Pathophysiological mechanisms in Malawian children with severe anaemia

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

IV = intravascular, EV = extra vascular, GI = Gastro-Intestinal, UT = Urine Tract.
# Includes patients with a complete dataset only.
* Gastro-intestinal (GI) blood loss was measured indirectly, using a ‘high hookworm load’ as a substitute marker.
† If reticulocyte cut off <150,000/uL is used (3% of erythrocytes in a non anaemic child): RCPF = 86.4% (203/235) with 23.0% (54/235) overlap with haemolysis and blood loss.
Control population (HC & CC combined): 1.8% (10/566) and 1.1% (8/705) fulfilled the definition for haemolysis and blood loss, respectively.
Mechanisms

Red cell production failure (RCPF) was identified in 113 (48.1%) of whom 24 (10.2%) were overlapping with the other mechanisms (figure 1). When exploring the RCPF group in more detail, it was found that in these children 11.4% (inter quartile range 4.3-36.9) of the mononuclear fraction was defined as red cell precursors (nucleated red cells [NRC]). Dyserythropoietic features in the NRC were found in 61.3% of the children with RCPF with a mean of 3.6 ±2.8 % of cells affected (table 2).

Table 2: Laboratory markers associated with severe anaemia mechanisms

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

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

Haptoglobin: Low if <0.2mMol/L.

NRC fraction: Percentage of nucleated red cell (red cell precursors) of the mononuclear bone marrow cells.

Non Defined = not fulfilling the definitions for red cell production failure, haemolysis or blood loss.

Figure 1: Pathophysiological mechanisms in Malawian children with severe anaemia syndrome.
Hemolysis was found in 51 out of 235 cases (21.7%) with 17 children (7.2%) also having criteria for RCPF and/or blood loss. Of all cases 19.3% had elevated plasma haemoglobin levels, indicative of intra-vascular hemolysis, and 5.0% had a raised unconjugated bilirubin level. In the haemolysis group 24.4% (IQR 16.2-40.9) of the mononuclear fraction were NRCs and dyserythropoiesis was found in 62.9% of children.

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

Aetiologies

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

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

Table 3. Pathophysiological mechanism in Malawian children with severe anaemia syndrome by main etiological factors present on hospital admission

<table>
<thead>
<tr>
<th>Mechanisms †</th>
<th>Aetiology factors</th>
<th>Infection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Malaria Positive</td>
<td>HIV Positive</td>
</tr>
<tr>
<td></td>
<td>all No other infection</td>
<td>all No other infection</td>
</tr>
<tr>
<td>RCPF</td>
<td>145 87 31 7</td>
<td>42.1 (61) 40.2 (35) 38.7 (12) 85.7 (6)</td>
</tr>
<tr>
<td>Haemolysis</td>
<td>17.9 (26) 17.2 (15) 19.4 (6) 28.6 (2)</td>
<td>25.7 (9) 38.5 (5)</td>
</tr>
<tr>
<td>Non defined</td>
<td>41.4 (60) 43.7 (38) 48.4 (15) 14.3 (1)</td>
<td>25.7 (9) 30.8 (4)</td>
</tr>
</tbody>
</table>

Cell content: % (n),
All = all children with the indicated infection as a single infection or part of multiple infections
No other infection = Children with the indicated infection only (as single infection)
† Folate deficiency was not found in the study population and therefore folic acid was not included in the table.
† ‘Blood loss’ was excluded from this table because of the small number (n = 17).

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

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

Aetiologies

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

In the children with a positive malaria slide, RCPF and haemolysis were found in 42.1% and 17.9%. In the sub-group with a malaria parasite count >10^4/uL, the equivalent proportions were 36.6% and 17.1%.

**Structural Equation Model**

In order to further explore the association between the aetiological factors and anaemia mechanisms and to correct for interaction between factors a structural equation model was developed. In the model, with a goodness of fit (RMSEA) of 0.053 (0.038-0.068), iron deficiency, vitamin B12 deficiency and malaria were directly and significantly associated with RCPF (standard regression coefficient of +0.14, -0.13, +0.15, respectively). Associations between RCPF and HIV, hookworm and vitamin A were indirect, through the previous 3 aetiologies and no significant associations were found with haemolysis (figure 2).
Severe anaemia in children in Africa has usually been investigated in the context of a condition that may contribute to it, such as malaria, malnutrition or helminth infection. We have studied the syndrome in its own right, by enrolling consecutive Malawian children presenting to hospital with severe anaemia, irrespective of its possible aetiology. This has allowed us to build up a picture of contributory causes in both an urban and a rural population in southern Malawi.

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

Our results confirm that in Malawian children, although severe anaemia is associated with many potential aetiological factors, one mechanistic pathway – failure of red cell
production (RCPF) – predominates as the final pathway leading to anaemia. This was true when using stringent criteria for RCPF (reticulocytes <50,000/uL and haemoglobin <5 g/dl) and became even more apparent when the cut-off for reticulocyte count was put at <150,000/uL (3% of erythrocytes in a non-anaemic child) when 86% of all severely anaemic children would fall into the RCPF category. The importance of RCPF may have been expected, since the study was conducted in an area with a high infection pressure, reflected in a high prevalence of single or mixed infections, and in the fact that the majority of cases had a raised plasma CRP concentration. In these circumstances, RCPF may be the result of pro-inflammatory cytokine activation giving rise to apoptosis of red cell precursors, dyserythropoiesis and down-regulation of erythropoietin production, with additional blunting of the effect of erythropoietin on the bone marrow. This interpretation – that severe anaemia is largely mediated by inflammatory mechanisms – is in part supported by the results listed in Table 2. However, inflammatory responses may not fully explain the RCPF, since nearly a quarter (23.6%) of children with a RCPF were not found to have an infectious aetiology, while nutritional deficiencies, which are on their own able to affect red cell production, were found in over 40% of RCPF cases.

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

The Structural Equation Model (Figure 2) underlines both the importance of RCPF and the complexity of the syndrome, and indicates the apparently minor role of haemolysis in the patients we studied. There were no significant associations between haemolysis and any of the aetiologies, including malaria. This was the case whether malaria was defined as any parasitaemia or a parasite density >10⁵/μl.

A limitation of a study of this kind is that data can be collected at one time point only, in a disease process that may have lasted for weeks with markers fluctuating from day to day. We have tried to limit this effect by using a large sample size. Another limitation is the difficulty of quantifying haemolysis and gastro-intestinal blood loss. For haemolysis, the kinetics of free haemoglobin in plasma are not well known, and 1g/dl of free haemoglobin is sufficient to saturate circulating haptoglobin, limiting its value as quantitative marker.

For gastro-intestinal blood loss we assumed that hookworm is the only likely cause and that blood loss is likely to accompany a heavy egg-load in the stool.
These limitations are unlikely to affect the main findings of our study and their implications for the control of severe anaemia in Malawi and similar settings. The syndromic approach that we have adopted allows a profile of paediatric severe anaemia to be developed, based a description of both aetiologies and mechanisms for a particular population. This provides a basis for the design of a locally relevant package of measures that can be deployed in the prevention and treatment of severe anaemia.
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