Iron Deficiency Is Associated With Reduced Levels of Plasmodium falciparum-specific Antibodies in African Children

Caroline K. Bundi,1,2 Angela Nalwoga,3 Lawrence Lubyayi,1 John Muthii Muriuki,1 Reagan M Mogire,1 Herbert Opi,1 Alexander J. Mentzer,3,4 Cleopatra K. Muggenyi,4,5 Jedida Mwacharo,1 Emily L. Webb,6 Philip Bejon,1,8 Thomas N. Williams,1,8,9 Joseph K. Gikunju,2 James G. Beeson,4,10,11 Alison M. Elliott,3,12 Francis M. Ndungu,1 and Sarah H. Atkinson1,8,13

1Kenya Medical Research Institute (KEMRI) Centre for Geographic Medicine Coast, KEMRI-Wellcome Trust Research Programme, Kilifi, Kenya, 2Department of Medical Laboratory Science, Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya, 3Medical Research Council/Uganda Virus Research Institute and London School of Hygiene & Tropical Medicine Uganda Research Unit, Entebbe, Uganda, 4Burnet Institute, Melbourne, Australia, 5Wellcome Centre for Human Genetics, Nuffield Department of Medicine, University of Oxford, Oxford, United Kingdom, 6Big Data Institute, Li Ka Shing Centre for Health Information and Discovery, University of Oxford, Oxford, United Kingdom, 7MRC Tropical Epidemiology Group, Department of Infectious Disease Epidemiology, London School of Hygiene & Tropical Medicine, London, United Kingdom, 8Centre for Tropical Medicine and Global Health, Nuffield Department of Medicine, University of Oxford, Oxford, United Kingdom, 9Department of Medicine, Imperial College, London, United Kingdom, 10Department of Microbiology, and Central Clinical School, Monash University, Melbourne, Australia, 11Department of Medicine, University of Melbourne, Victoria, Australia, 12Department of Clinical Research, London School of Hygiene & Tropical Medicine, London, United Kingdom, and 13Department of Paediatrics, University of Oxford, Oxford, United Kingdom.

Background. Iron deficiency (ID) and malaria are common causes of ill-health and disability among children living in sub-Saharan Africa. Although iron is critical for the acquisition of humoral immunity, little is known about the effects of ID on antibody responses to Plasmodium falciparum malaria.

Methods. The study included 1794 Kenyan and Ugandan children aged 0–7 years. We measured biomarkers of iron and inflammation, and antibodies to P. falciparum antigens including apical merozoite antigen 1 (anti-AMA-1) and merozoite surface antigen 1 (anti-MSP-1) in cross-sectional and longitudinal studies.

Results. The overall prevalence of ID was 31%. ID was associated with lower anti-AMA-1 and anti-MSP-1 antibody levels in pooled analyses adjusted for age, sex, study site, inflammation, and P. falciparum parasitemia (adjusted mean difference on a log-transformed scale (β) −0.46; 95 confidence interval [CI], −.66, −.25 P < .0001; β −0.33; 95 CI, −.50, −.16 P < .0001, respectively). Additional covariates for malaria exposure index, previous malaria episodes, and time since last malaria episode were available for individual cohorts. Meta-analysis was used to allow for these adjustments giving β −0.34; −0.52, −0.16 for anti-AMA-1 antibodies and β −0.26; −0.41, −0.11 for anti-MSP-1 antibodies. Low transferrin saturation was similarly associated with reduced anti-AMA-1 antibody levels. Lower AMA-1 and MSP-1-specific antibody levels persisted over time in iron-deficient children.

Conclusions. Reduced levels of P. falciparum-specific antibodies in iron-deficient children might reflect impaired acquisition of immunity to malaria and/or reduced malaria exposure. Strategies to prevent and treat ID may influence antibody responses to malaria for children living in sub-Saharan Africa.

Keywords. iron deficiency; immunity; children; malaria; Africa.

Iron deficiency (ID) is highly prevalent among young children living in sub-Saharan Africa [1], and iron deficiency anemia (IDA) is the leading cause of years lived with disability among African children [2] due to its negative effects on child development [3]. Malaria is also a major public health problem causing approximately 405 000 deaths in 2018, of which 85% occurred in sub-Saharan Africa, mainly among young children [4]. Children acquire immunity to malaria over time and antibodies to merozoite antigens are important mediators of naturally-acquired immunity [5, 6], in addition to other responses.

Iron is important for the development of humoral immunity and antibody production. ID impairs B-cell proliferation and antibody production [7], and a mutation in transferrin receptor 1 (TfR1), which causes insufficient cellular iron uptake, leads to defective B- and T-cell activation and combined-immunodeficiency [8]. ID is associated with reduced antibody levels in children [9–11] and in rat models [12], as well as with weakened vaccine responses [7, 13], although other studies have found little association with antibody levels [14–16] or vaccine responses [17, 18]. ID has also been associated with reduced frequencies of B and T cells and cytokines, necessary for antibody production [8–10, 19].

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Although ID is highly prevalent among African children and is known to influence immune responses little is known about the effect of ID on the acquisition of immunity to malaria. We previously observed that ID was associated with decreased total immunoglobulin G (IgG) and immunoglobulin E (IgE) levels to \textit{P. falciparum} schizont extract [20] and that hepcidin, the master iron-hormone, was associated with increased levels of antibodies to anti-AMA-1 and anti-MSP-2 antigens [21], in small studies. In the current study, we investigated the relationship between iron status and antibody levels to specific \textit{P. falciparum} antigens in 1794 Kenyan and Ugandan children. We evaluated antibodies to 2 major merozoite antigens, anti-AMA-1 and anti-MSP-1, which are targets of acquired immunity, and antibodies to these antigens have previously been associated with protective immunity to malaria in our study population [5, 22].

**MATERIALS AND METHODS**

**Ethical Approval**

Ethical approval was provided by the Scientific Ethics Review Unit of the Kenya Medical Research Institute (KEMRI/SERU/CGMR-C/046/3257/2983), by the Uganda Virus Research Institute (reference GC/127/12/07/32), the Uganda National Council for Science and Technology (MV625), and in the United Kingdom by the London School of Hygiene & Tropical Medicine Ethics Committee (A340) and the Oxford Tropical Research Ethics Committee (OXTREC, 39-12 and 42-14 and 37-15).

**Study Population**

We used data from community-based cohorts of children in Kilifi, Kenya, and Entebbe, Uganda.

**Kenya:** The Kenyan children included two community-based cohorts exposed to varying levels of malaria transmission, Junju and RTS,S. Junju is a surveillance cohort evaluating immunity to malaria as described elsewhere [23]. The RTS,S cohort is an extension of the RTS,S/AS01E vaccine trial against malaria conducted between 2007 and 2008 [24]. Both cohorts are under active weekly surveillance to assess for fever, and a malaria blood film is taken if the temperature is > 37.5°C. Additionally, annual cross-sectional blood samples are taken for immunology and parasitology during the dry period before the main annual malaria transmission season. Iron biomarkers and malaria antibodies were measured on the same plasma sample from a single annual cross-sectional bleed based on the availability of a sample archived at −80°C.

**Uganda:** The Entebbe Mother and Baby Study (EMaBS) is a prospective birth cohort that was originally designed as a randomized double-blind placebo-controlled trial to determine whether anthelmintic treatment during pregnancy and early childhood was associated with differential responses to vaccination or incidence of infections such as malaria, pneumonia and diarrhea [25]. Children had active surveillance for malaria and other infections during fortnightly home visits and quarterly clinic visits, and an annual blood sample was collected. Malaria antibodies were measured from a sample taken at 5 years of age, and iron biomarkers were measured from a single annual bleed taken between 1 and 4 years of age based on the availability of plasma samples archived at −80°C.

**Laboratory Procedures**

**Iron and Inflammation Biomarkers**

The measured biomarkers of iron status and inflammation were iron (MULTIGENT iron calorimetric assay, Abbott Architect, USA), ferritin, transferrin (chemiluminescent microparticle immunoassay [CMI], Abbott Architect), soluble transferrin receptor (sTfR, Human sTfR ELISA, BioVendor), hepcidin (DRG Hepcidin 25 [bioactive] high sensitive ELISA Kit, DRG Diagnostics), transferrin (CMI, Abbott Architect) hemoglobin (Coulter analyzer, Beckman Coulter), and C-reactive protein (CRP, MULTIGENT CRP Vario assay, Abbott Architect). In Uganda, hemoglobin concentrations were adjusted for an altitude of > 1000 m above sea level (by subtracting 0.2 g/dL) [26]. \textit{P. falciparum} parasitemia was determined at the time of malaria antibody measurement using Giemsa-stained thick and thin blood smears.

**Plasmodium falciparum Antibody Assays**

Antibodies against the AMA1 3D7 sequence and MSP1\textsubscript{42}, 3D7 sequence of \textit{P. falciparum} antigens were measured from plasma samples by enzyme-linked immunosorbent assays (ELISAs) according to standard protocols as previously described for the RTS,S, Junju [27] and EMaBS cohorts [28]. A pool of malaria hyperimmune sera was serially diluted on each plate, and the optical densities from these dilutions were used to generate a standard curve. From this standard curve, an arbitrary unit per milliliter (AU/mL) was calculated for each sample based on the relative optical density obtained. Different pools of malaria-hyperimmune sera and ELISA antigens were used in the Kenyan and Ugandan laboratories.

**Definitions**

Inflammation was defined as CRP > 5 mg/L. ID was defined as plasma ferritin < 12 µg/L or < 30 µg/L in the presence of inflammation in children < 5 years or < 15 µg/L in children ≥ 5 years as defined by the World Health Organization (WHO) [29]. Low transferrin saturation (TSAT) < 10% (calculated as iron in µmol/L/(transferrin in g/L × 25.1) × 100) [30] was considered as a secondary definition of ID. TSAT was calculated in Kenya only because Ugandan plasma samples were stored in EDTA, which chelates iron. We did not define ID by hepcidin or sTfR since there are no internationally established cutoffs. Anemia was defined as hemoglobin < 11 g/dL in children aged 0–4 years or hemoglobin < 11.5 g/dL in children >4 years. IDA
Iron Deficiency Earlier in Life May Influence Subsequent Antibody Levels

We tested the hypothesis that ID might influence subsequent malaria antibody levels for a prolonged period of time in the EMaBS birth cohort. ID was associated with lower anti-AMA-1 and anti-MSP-1 antibody levels up to 2 years after iron status measurements and with lower anti-AMA-1 antibody levels 2–4 years after iron measurements (Supplementary Figure 3).
DISCUSSION

We have investigated the association between iron status and anti-\textit{P. falciparum} antibodies in 1794 Kenyan and Ugandan children. We found that ID was associated with reduced levels of anti-AMA-1 and anti-MSP-1 antibodies, even after adjustment for potential confounders including previous malaria exposure. TSAT < 10% was similarly associated with reduced levels of anti-AMA-1 antibody levels. A range of individual iron markers, including ferritin, TSAT, hepcidin, and sTfR levels were positively associated with malaria antibody levels. TSAT, an indicator of low levels of circulating iron, may more accurately reflect what iron status would be in the absence of malaria and inflammation compared to ferritin circulating iron, which may differ between countries.

We further investigated the effects of a range of iron markers on malaria-specific antibody levels. TSAT, an indicator of low levels of circulating iron, may more accurately reflect what iron status would be in the absence of malaria and inflammation compared to ferritin circulating iron, which may differ between countries.

ID on immune development. IDA was similarly associated with reduced anti-AMA-1 and anti-MSP-1 antibody levels in Kenya but not Ugandan children, perhaps because few Ugandan children had IDA or because anemia has a multifactorial etiology that may differ between countries.

We found that ID was associated with lower anti-AMA-1 and anti-MSP-1 malaria antibody levels, even after adjustment for potential confounders including previous malaria exposure. The relationship between malaria, iron parameters, and antibody levels differed between study sites. The effect of ID on anti-\textit{P. falciparum} antibody levels was most marked among children with the highest malaria exposure and antibody levels, as seen in the Junju cohort, although little difference by ID was observed in children with very low levels of malaria exposure and antibody levels, as seen in the RTS,S cohort. In agreement with our findings, Nyakeriga et al reported that total IgG, IgG2, and IgE antibody levels were lower in iron-deficient compared to iron-replete Kenyan children [20]. We found that the effects of ID on anti-AMA-1 and anti-MSP-1 antibody levels persisted over time, perhaps due to continuing ID or a long-term effect of ID on immune development. IDA was similarly associated with reduced anti-AMA-1 and anti-MSP-1 antibody levels in Kenyan but not Ugandan children, perhaps because few Ugandan children had IDA or because anemia has a multifactorial etiology that may differ between countries.

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and recycling of iron, and is regulated by iron stores, infection and erythropoietic drive [35]. We found that increased hepcidin levels were associated with increased anti-MSP-1 antibody levels in overall multivariable analyses. In a previous study of 324 Kenyan children we similarly found that hepcidin levels were positively associated with anti-AMA-1 and anti-MSP-2 antibody levels [21]. In contrast to the other iron markers, we found that increased sTfR levels, an indicator of both increased ID and erythropoietic drive, were associated with increased anti-AMA-1 antibody levels. This might be explained by the strong association between sTfR levels and malaria [36], thus increased sTfR could indicate recent malaria exposure rather than ID.

How might ID lead to reduced malaria-specific antibody levels? One explanation is that iron may play a critical role in humoral immunity and particularly in antigen-specific antibody production as suggested by recent studies [7, 8, 11]. A missense mutation in transferrin receptor 1, necessary for iron uptake by cells, was associated with defective B-cell proliferation and reduced IgG production in children and in mouse models [8]. ID is similarly associated with markedly reduced antigen-specific antibody responses, likely due to impaired iron-dependent histone 3 lysine 9 demethylation, critical for B cell proliferation [7]. There is sparse literature in humans with conflicting findings. ID has been associated with reduced IgG antibodies, including to pneumococcal antigen [8–11], although some studies report little association [14, 16]. ID has also been associated with weakened antibody responses to measles, diphtheria, whooping cough, and tetanus vaccines in some studies [7, 13] but not others [17, 18].

Reduced malaria-specific antibodies in iron deficient children may also be explained by the complex relationship between iron status and malaria. ID has some protective effect against malaria infection in children [37], and thus iron-deficient children may have fewer malaria episodes leading to reduced malaria-specific antibody levels. Another explanation is that malaria influences measures of iron status. Ferritin levels are elevated for a prolonged period after a malaria infection, even after CRP levels have normalized [34], so that children with low ferritin levels may be less likely to have had recent malaria and thus might have reduced antibody levels. Moreover, the malaria-specific antibodies, anti-AMA-1 and anti-MSP-1, are markers of malaria exposure [38, 39], as well as correlates of naturally acquired immunity against clinical malaria [5, 6, 22]. We adjusted for previous malaria exposure in meta-analyses; however, it is likely that not all previous malaria was fully accounted for.

Strengths of our study included its large sample size of 1794 children from cohorts of varying malaria intensity in Kenya and Uganda. We also measured specific malaria antibodies known to contribute to immunity to clinical malaria [6, 22], assayed a wide range of markers of iron status, and adjusted for known potential confounders in our models. There were also some important limitations to our study. First, apart from malaria parasitemia, we did not have standardized measures for malaria exposure available for

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all cohorts, however, we conducted meta-analyses that accounted for measures of previous malaria, including malaria exposure index, incidence of clinical malaria, and time since a malaria episode, as available for each study site. Another limitation was that ID was defined using WHO guidance [29], which adjusts ferritin levels for inflammation (CRP > 5 mg/dl), however since ferritin levels are elevated for a prolonged period after CRP levels have normalized following malaria infection [34], lower ferritin levels could also reflect less recent malaria exposure. In addition to adjusting for recent malaria, we also defined ID using TSAT, which is less influenced by inflammation and malaria [33, 34], although this marker was not available for all cohorts. A further limitation of our study is that elevated anti-AMA-1 and anti-MSP-1 antibody levels may not be mechanically related with clinical protection against malaria. However, even as correlates of exposure the responses may still be useful indicators of the host’s immunological response. Antibody levels were also measured using different pools of malaria-hyperimmune control sera in different laboratories in Kenya and Uganda, although protocols were similar between sites. Despite these differences, our findings were notably similar between the different study sites.

In summary, we found that ID was associated with lower levels of anti-AMA-1 and anti-MSP-1 malaria antibodies, known to be important in antibody-mediated immunity to clinical malaria in African children [5, 6, 22]. Our findings are supported by studies demonstrating that iron is critical for the development of humoral immunity [7, 8]. ID is highly prevalent among African children, and it is not known whether improving iron status might improve immune function and reduce disease burden. The current study supports WHO recommendations to offer iron supplementation coupled with malaria treatment in malaria endemic regions to prevent and treat iron deficiency [40]. Further research to infer causality between ID and malaria immunity, such as randomized controlled trials of the effects of iron supplementation on malaria antibody levels are needed, as well as further studies to assess associations between ID and malaria vaccine responses.

Supplementary Data
Supplementary materials are available at Clinical Infectious Diseases online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.
Notes

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