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Anemia Offers Stronger Protection Than Sickle Cell Trait Against the Erythrocytic Stage of Falciparum Malaria and This Protection Is Reversed by Iron Supplementation

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ABSTRACT

Background: Iron deficiency causes long-term adverse consequences for children and is the most common nutritional deficiency worldwide. Observational studies suggest that iron deficiency anemia protects against *Plasmodium falciparum* malaria and several intervention trials have indicated that iron supplementation increases malaria risk through unknown mechanism(s). This poses a major challenge for health policy. We investigated how anemia inhibits blood stage malaria infection and how iron supplementation abrogates this protection.
Methods: This observational cohort study occurred in a malaria-endemic region where sickle-cell trait is also common. We studied fresh RBCs from anemic children (135 children; age 6–24 months; hemoglobin <11 g/dl) participating in an iron supplementation trial (ISRCTN registry, number ISRCTN07210906) in which they received iron (12 mg/day) as part of a micronutrient powder for 84 days. Children donated RBCs at baseline, Day 49, and Day 84 for use in flow cytometry-based *in vitro* growth and invasion assays with *P. falciparum* laboratory and field strains. *In vitro* parasite growth in subject RBCs was the primary endpoint.
Findings: Anemia substantially reduced the invasion and growth of both laboratory and field strains of *P. falciparum in vitro* (~10% growth reduction per standard deviation shift in hemoglobin). The population level impact against erythrocytic stage malaria was 15.9% from anemia compared to 3.5% for sickle-cell trait. Parasite growth was 2.4 fold higher after 49 days of iron supplementation relative to baseline ($p < 0.001$), paralleling increases in erythropoiesis.
Interpretation: These results confirm and quantify a plausible mechanism by which anemia protects African children against *falciparum* malaria, an effect that is substantially greater than the protection offered by sickle-cell trait. Iron supplementation completely reversed the observed protection and hence should be accompanied by malaria prophylaxis. Lower hemoglobin levels typically seen in populations of African descent may reflect past genetic selection by malaria.
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1. Introduction

Malaria and iron deficiency anemia (IDA) impact the same geographic and demographic groups and the pathophysiological relationship between the two is complex. Acute malaria can cause severe anemia due to hemolysis of infected and uninfected RBCs, and chronic or subclinical malaria can induce anemia of inflammation (Clark et al., 2014a). There is clear epidemiological evidence in both children (Gwamaka et al., 2012; Jonker et al., 2012; Nyakeriga et al., 2004) and pregnant women (Kabyemela et al., 2008; Senga et al., 2011) that, once established, IDA is protective against malaria infection. In fact, in pregnant women, iron deficiency has been shown to reduce risk of

Abbreviations: AA, normal β-globin genotype; AC, heterozygous hemoglobin C β-globin genotype; AS, heterozygous sickle-cell trait β-globin genotype; CI, confidence interval; CRP, C reactive protein; G6PD, glucose-6-phosphate dehydrogenase; GPA, glycophorin A; GR, growth rate; Hgb, hemoglobin; IDA, iron deficiency anemia; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MFI, mean fluorescent intensity; MPV, mean platelet volume; Pf, *Plasmodium falciparum*; pp, population prevlance; RBC, red blood cell; RDT, rapid diagnostic test; RDW, red cell distribution width; RG, relative growth; SC, heterozygous sickle-cell trait and hemoglobin C β-globin genotype; SD, standard deviation; SI, susceptibility index; SS, homozygous sickle-cell anemia β-globin genotype; sTfR, soluble transferrin receptor; Tf, transferrin; TIBC, total iron binding capacity; Tsat, transferrin saturation; UIBC, unbound iron binding capacity; WBC, white blood cell.

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placental malaria to a greater extent than multiparity (Kabyemela et al., 2008).

Multiple studies have raised concern that iron supplementation in malaria-endemic areas may put people at increased risk of acquiring malaria (Murray et al., 1978, 1975; Oppenheimer et al., 1986; Smith et al., 1989; Veenemans et al., 2011). Most importantly, a large childhood nutritional supplementation study in Zanzibar was halted due to increased morbidity and mortality in children receiving iron (Sazawal et al., 2006). Subsequently, WHO modified its recommendation for universal iron supplementation and now recommends that, in malarious regions, iron supplements be given where malaria management and prevention services are present (Neuberger et al., 2016; World Health Organization, 2016). This has severely disrupted iron supplementation campaigns in malaria endemic areas, despite IDA being the leading cause of years lived with disability among children and adolescents according to the 2013 Global Burden of Disease Study (Global Burden of Disease Pediatrics Collaboration et al., 2016). Reducing the prevalence of anemia is one of the six priorities of the WHO's Comprehensive Implementation Plan on Maternal, Infant, and Young Child Nutrition (World Health Organization, 2014). Further complicating research in this area, it is now difficult to ethically study the safety of iron supplementation in malarious areas. In most developing countries iron supplements cannot be withheld during a study and all children in iron supplementation studies must be provided malaria prevention services and monitored closely for illness. As a result, recent studies evaluating the safety of iron supplementation have done so in the context of providing malaria prevention services and extensive medical care (Mwangi et al., 2015; Zlotkin et al., 2013) – a scenario that would not necessarily exist in reality.

In an effort to assess the magnitude of protection from anemia and the safety of iron supplementation in a malaria endemic area where sickle-cell trait is common, we have systematically characterized *P. falciparum* growth *in vitro* in RBCs from anemic African children before, during, and after 12 weeks of iron supplementation.

2. Methods

2.1. Subject recruitment, study design, and blood samples for parasite assays

The blood samples for the parasite assays were taken from children enrolled in the control arm of a randomized trial testing the efficacy and safety of a hepcidin-guided screen-and-treat strategy for combatting anemia (see published protocol for full details) (Wegmüller et al., 2016). (Note we also assayed RBCs from children in the other two arms of this trial, but only for observation at baseline, pre-randomization/pre-intervention.) Study participants were recruited from 12 communities in Jarra West (Soma, Karantaba, Kani Kunda, Sankwia, Mansakonko, Pakalinding, Jenoi and Si Kunda) and Kiang East (Toniataba, Jiffin, Kaiaf and Genieri), in the Lower River Region of The Gambia. The study took place from May 2014 through December 2015 in five cohorts. In total 407 healthy young children, aged 6–23 months, were identified during child welfare clinics at the health facilities of Jarra West and Kiang East. After informed consent was obtained, children had to meet the inclusion/exclusion criteria to be enrolled. For inclusion children must have been apparently healthy, 6–23 months old, not severely malnourished (z-scores for Height-for-Age, Weight-for-Age, Weight-for-Height > −3 SD), 7 g/dl ≤ Hgb < 11 g/dl, free of malaria, resident in the study area, able and willing to comply with the study protocol, have had no congenital disorders or chronic disease, and must not have been taking regular medication nor participating in another study. Sample size was calculated based on the primary endpoint in the parent study (Wegmüller et al., 2016).

As per current WHO recommendations, children in the control arm received 12 mg/d iron as ferrous fumarate, given orally within a micronutrient powder (modified MixMe™ supplied by DSM Nutritional Products). Field workers visited children daily in order to supervise the micronutrient powder administration and check the children's health status. For baseline population characteristics, see Supplemental Table 1. Fresh RBCs were obtained from these anemic (Hgb < 11 g/dl) but otherwise healthy children (6–23 m) living in rural Gambia (Wegmüller et al., 2016). Blood was collected at Days 0 (baseline), 49, and 84 during 12 weeks of iron supplementation (Fig. 1) with the primary objective of evaluating *in vitro P. falciparum* growth characteristics to model malaria susceptibility in anemic subjects before and after iron supplementation. We compared subject characteristics of those whose blood was and was not able to be used for growth rate data to ensure no sampling bias occurred (Supplemental Table 2). For a full description of this embedded observational study, please see the published protocol (Wegmüller et al., 2016).

2.2. P. falciparum Culture

Parasite lines FCR3-FMG (MR4, MRA-736) and 3D7 (MR4, MRA-102) were routinely cultured in RBCs from healthy donors using standard methods (Clark et al., 2014a). Parasite strains 952, 998, and 1029 were isolated from patients presenting with symptomatic malaria infections at the Jammeh Foundation for Peace hospital in Serekunda and the

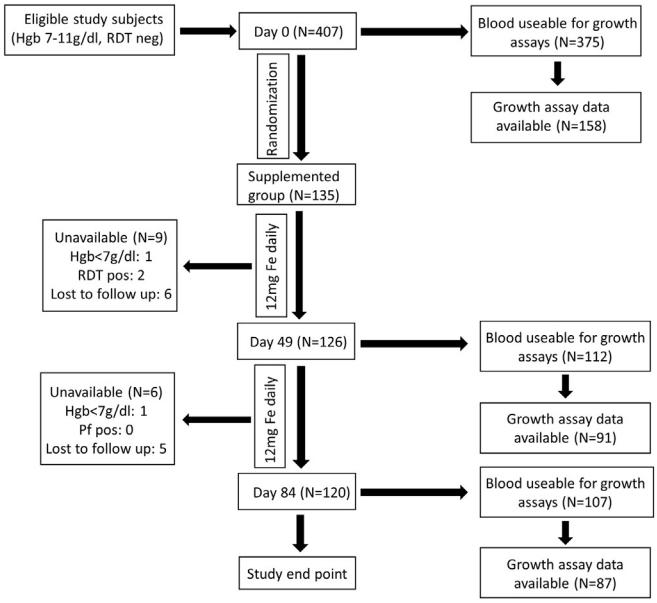

Fig. 1. Description of subjects and flow chart of sample collection and assays performed. Blood samples for hematological, biochemical, and parasite growth analyses were drawn at Day 0, as well as Day 49 and Day 84 for those taking iron. A full hematology panel was measured in EDTA-stabilized blood (Medonic M20M GP). We also assayed plasma ferritin, soluble transferrin receptor (sTfR), serum iron, transferrin saturation (TSAT), C-reactive protein (CRP), alpha 1-acid glycoprotein (AGP) (Cobas Integra 400 plus); and hepcidin (Hepcidin-25 (human) EIA Kit (Bachem)). Genotyping for hemoglobinopathies was performed using hemoglobin electrophoresis. Glucose-6-phosphate dehydrogenase (G6PD) enzyme activity was measured by commercial kit (R&D Diagnostics Ltd). For malaria assays, 2.5 ml of venous blood was drawn directly into microvette tubes containing CPDA-1 (Sarstedt, Germany). Unavailable donors include safety exclusion (Hgb < 7 g/dl or positive malaria test, RDT pos) or general loss to follow up (withdrawal and travel). Failure to collect blood from subjects (*e.g.* from phlebotomy failure, subject moved or withdrew, or became significantly ill) was 7.8% (32/407) at Day 0, 17.0% (23/135) at Day 49, and 20.7% (28/135) at Day 84. RBCs from study subjects were evaluated with *in vitro P. falciparum* growth assays (using strain FCR3-FMG) as a proxy measure for malaria susceptibility. In order to standardize the growth assays, control for inter-assay variability and variability between parasite preparations, assays on clinical samples were run in parallel with and reported relative to growth assays done using RBCs from non-anemic donors. Each available blood sample at every time point was subjected to growth assays but not all produced growth data, as some blood was unusable (*e.g.* clotted, hemolysed, contaminated). Further growth data exclusions (*e.g.* parasites died or control blood did not provide a readable output for comparison) do not represent population sampling bias, as subject characteristics are the same between those with and without corresponding growth data (Supplemental Table 2).

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