

Title: Malaria is a cause of iron deficiency in African children

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Malaria and iron deficiency (ID) are common and interrelated public health problems in African children. Observational data suggest that interrupting malaria transmission reduces prevalence of ID¹. To test the hypothesis that malaria might cause ID, we used sickle cell trait (HbAS, rs334), a genetic variant that confers specific protection against malaria², as an instrumental variable in Mendelian randomization analyses. HbAS was associated with a 30% reduction in ID among children living in malaria-endemic countries in Africa (n=7453), but not among individuals living in malaria-free areas (n=3818). Genetically predicted malaria risk was associated with an odds ratio of 2.65 for ID per unit increase in log incidence rate of malaria. This suggests that an intervention that halves the risk of malaria episodes would reduce the prevalence of ID in African children by 49%.

Malaria and iron deficiency (ID) are important co-existing public health problems across sub-Saharan Africa, and their relationship is complex and incompletely understood. Malaria transmission is persistent and widespread across sub-Saharan Africa with an estimated 24% parasite prevalence³ and 213 million cases in 2018⁴. Similarly, ID is also common and accounts for over 60% of anemia cases⁵. ID is associated with impaired cognitive development⁶ and is a leading cause of years lived with disability in African children⁷. ID may be caused by a number of factors, including diets low in available iron, dietary iron inhibitors such as polyphenols and phytates, and helminth infections. Iron supplementation, via tablets or syrups and micronutrient powders, are the primary interventions to manage ID and anemia in children. However, there are long-standing concerns regarding the safety and efficacy of iron supplements in malaria-endemic countries where it may predispose individuals to malaria and other infections⁸ and be poorly absorbed^{9,10}. New strategies are needed for the management of ID.

Malaria is known to cause anemia via various mechanisms including the destruction of parasitized and non-parasitized erythrocytes. This process affects hemoglobin concentrations, but the iron released from destroyed erythrocytes is not lost from the body and can be recycled. Little is known about whether malaria might be a cause of ID in African children¹¹. A study in the Kenyan highlands showed that interrupting malaria transmission reduced the prevalence of ID¹ and studies in Gambian and Kenyan children showed that the prevalence of ID increased over the malaria season¹². Moreover, stable isotope studies show that treatment of afebrile malaria increased dietary iron absorption and reduced the prevalence of ID in 17 Ivorian children and 23 Beninese women^{9,10}. These studies suggest that malaria-control could be an effective strategy for managing ID in sub-Saharan Africa. However, observational studies are susceptible to reverse causation and confounding, making causal inferences harder to obtain. Here, we have used a Mendelian randomization approach¹³ which reduces these biases and offers a method to test for causality by studying prevalence of ID in children whose risk of malaria is reduced by a known genetic variant (termed an instrumental variable) compared to children lacking such protection.

We utilized sickle cell trait (HbAS, rs334), a genetic variant which confers specific protection against all forms of clinical *P. falciparum* malaria², as an instrumental variable in Mendelian randomization analyses. To do this, we used data and stored samples from 7453 healthy children aged 0-8 years (median age 2.08, interquartile range 1.67-3.25) from 10 community-based cohorts in malaria-endemic areas in sub-Saharan Africa (Fig. 1a). We also tested whether HbAS might influence risk of ID via a mechanism that is independent of its effect on malaria (horizontal pleiotropy) in malaria-free populations including 3207 African Americans (21-93

years old) and 611 life-long Nairobi residents (10-16 years old) using no-relevance point sensitivity analyses¹⁴. We were unable to test for pleiotropy among children living in South Africa, another malaria-free area, since like most populations without historical exposure to malaria, few had HbAS (n = 11/845). These studies are described in detail in the methods section. We also considered using other polymorphisms, including G6PD A- deficiency and α -thalassemia, as instrumental variables, but in meta-analyses of published literature (Extended Data Fig. 1 and Supplementary Table 1) the protective effects of these polymorphisms are largely confined to severe malaria, an outcome of limited relevance as a cause of ID at a population-level. ID was defined according to World Health Organization guidelines as ferritin <12 μ g/L or <30 μ g/L in the presence of inflammation (defined as C-reactive protein (CRP) >5mg/L or α_1 -antichymotrypsin (ACT) >0.6g/L or α_1 -acid glycoprotein (AGP) >1g/L) in children <5 years old or <15 μ g/L in children $\geq$ 5 years old¹⁵ and as ferritin <30 μ g/L in African-American adults and Nairobi school children¹⁶. Anemia was defined as hemoglobin <11 g/dL in children aged <5 years, <11.5 g/dL in children $\geq$ 5 years, <12 g/dL in women, and <13 g/dL in men, and IDA as presence of ID and anemia¹⁷. The characteristics of the study populations are summarized in Table 1.

We first evaluated the effect of HbAS on the risk of ID. Prevalence of malaria parasitemia, HbAS, and ID varied across study sites (Fig. 1b). Overall prevalence of ID and HbAS was 26.93% and 13.42%, respectively. In a meta-analysis of 10 community-based studies of children living in malaria-endemic areas, HbAS was associated with 30% protection from ID (OR 0.70; 0.58, 0.82; P <0.001; Fig. 1c) and 31% protection against IDA (OR 0.69; 0.53, 0.85; P <0.001; Extended Data Fig. 2) and this protective effect on ID was consistent across cohorts (I^2 = 0.0%,

Fig. 1c). We found no evidence of an association between HbAS and potential confounders such as age, sex, or underweight (Fig. 1d). Since HbAS and α -thalassemia show negative epistasis in their malaria-protective effects¹⁸, we further adjusted for the effect of α -thalassemia, but this did not alter the effect of HbAS on risk of ID (Supplementary Table 2). HbAS was similarly associated with protection against ID in pooled analyses (OR 0.79 (0.68, 0.93); $P = 0.003$) and after ferritin levels were adjusted for inflammation (OR 0.77; 0.65, 0.89; $P < 0.001$) (Extended Data Fig. 3) using a regression-correction approach¹⁹. Prevalence of ID and IDA were similarly lower in African children carrying HbAS compared to those carrying wild-type normal hemoglobin (HbAA) (Fig. 2a, 2b). In populations not exposed to malaria (i.e. African-Americans and life-long Nairobi residents), HbAS was not associated with ID (OR 0.93; 0.55, 1.31; $P = 0.63$; Figs. 1c and 2a) or IDA (OR 1.17; 0.56, 1.78; $P = 0.84$; Fig. 2b and Extended Data Fig. 2), suggesting that HbAS is protective against ID only in malaria-endemic populations.

We then estimated the causal effect of malaria on ID using HbAS as an instrumental variable for malaria in a two-sample Mendelian randomization analysis¹³. First, to determine the effect of HbAS on uncomplicated malaria, we conducted a systematic review and meta-analysis of all published papers that assessed the effect of HbAS on the incidence of uncomplicated malaria in African populations to obtain an overall incidence rate ratio (Extended Data Fig. 4 and Supplementary Table 1). Overall, HbAS was associated with 31% protection from episodes of uncomplicated malaria (IRR = 0.69; 0.64, 0.74, Extended Data Fig. 4) and the F-statistic was 85.56. We estimated the causal log odds as the ratio of the log odds of HbAS on ID (Fig. 1c) to log incidence rate of HbAS on uncomplicated malaria (Extended Data Fig. 4) with a Mendelian randomization approach using the Wald ratio¹³. Estimates and standard errors are shown in Table

2. The causal odds ratio was calculated by exponentiating the causal log odds ratio (0.97). We observed a causal odds ratio of 2.65 (95% CI 1.64, 4.26; $P < 0.001$) suggesting that genetically predicted risk of uncomplicated malaria is associated with 2.65-fold higher odds of ID per unit increase in log incidence rate of malaria. We then calculated the effect of reducing malaria incidence by half on iron deficiency. We obtained this by multiplying the causal log odds (0.97) by natural-logarithm of 0.5 then exponentiated the result²⁰. We observed an odds ratio of 0.51 suggesting that reducing the incidence of uncomplicated malaria by half would reduce ID by 49%. Taken together, our findings suggest that malaria is an important cause of ID and that malaria-control may be an effective strategy for addressing ID among children living in sub-Saharan Africa.

So how might malaria cause ID? A number of pathways might be involved, including urinary iron loss from hemolysis and the induction of inflammation. We found that children with afebrile and severe malaria had higher geometric mean CRP levels (4.62 mg/L and 100.28 mg/L, respectively) compared to those without malaria (1.15 mg/L). Carriage of HbAS was associated with a 25% reduction in inflammation (OR 0.75; 0.65, 0.87; $P < 0.001$; Figs. 1d and 2d) in African children, but not in malaria-free populations (OR 0.84; 0.65, 1.09; $P = 0.20$; Fig. 2d). The iron-hormone, hepcidin, is up-regulated by inflammation and down-regulated by ID and increased erythropoietic drive in African children¹². Hepcidin causes ID by blocking duodenal iron absorption and macrophage iron recycling via degradation of the iron transporter, ferroportin²¹. Malaria is known to strongly up-regulate the production of hepcidin by the liver^{22,23} and we similarly found that hepcidin concentrations were markedly increased in afebrile and severe malaria, above a threshold associated with reduced iron absorption^{24,25} (Fig. 2c). How this

might lead to ID is illustrated in Fig. 2. Malaria might also increase hepcidin concentrations via non-inflammatory pathways (Extended Data Fig. 5). We found that children with malaria parasitemia had higher concentrations of hepcidin, above a threshold blocking iron absorption, at almost every decile of CRP compared to children without malaria (Fig. 2e), and this effect was observed regardless of the presence of inflammation (Extended Data Fig. 6). Thus, HbAS carriers would avoid malaria-induced hepcidin-mediated blockades of iron absorption leading to improved iron status. Taken together, our findings suggest that malaria may drive a hepcidin-mediated block in iron absorption leading to ID, in agreement with previous studies^{9,10,26}.

Our study had a number of strengths and limitations. A strength of the study was that we used large-scale datasets from populations across sub-Saharan Africa. We also used an instrumental variable, HbAS, known to confer specific and strong protection from malaria, and can therefore proxy health benefits of malaria control²⁷. One limitation of our study was the use of African-American adults and Nairobi school children, who might have different and age-related causes of ID, to test whether HbAS might influence ID through a non-malaria related pathway. Further limitations were that we did not have data on other potential causes of ID in the various populations, such as differences in diet, risk of hookworm infection, or other infections such as HIV. Studies also differed in the assays used to determine ferritin levels and inflammation, and in their study design; for example, HbAS might be less likely to protect from ID in longitudinal studies where children are regularly monitored and treated for malaria. Nevertheless, despite these differences HbAS remained consistently associated with protection from ID across all of the studies in malaria-endemic areas. Another potential limitation is the uncertainty in estimating ID in the context of a high burden of infectious disease and we therefore also defined ID using

ferritin levels regression-corrected for inflammation¹⁹ and found similar results. HbAS might have been expected to have a larger protective effect against ID in populations with higher exposure to malaria²⁸, however we were unable to test for this as we did not have data on incidence of clinical malaria in most of the studies. It is also likely that our analyses may have underestimated the effect of malaria on ID since HbAS is not known to protect against afebrile malaria parasitemia². Since afebrile parasitemia increases hepcidin concentrations (Fig. 2c), is highly prevalent³, and is less likely to be treated, it may significantly impair iron absorption^{9,10}.

In summary, using large-scale data (n = 11,333) from children across the African continent and from African-Americans we provide evidence that malaria increases risk of ID in African children (OR = 2.65; 1.64, 4.26). The public health benefits of malaria control in terms of reducing ID depend on the efficacy of the specific interventions used. Our data suggest that an intervention that halves the incidence of malaria would reduce ID by 49%. In a small study of Ivorian children, the prevalence of ID was reduced by half following treatment for afebrile malaria⁹ suggesting that the predicted benefits from our analysis may be translatable to the real world. Studies in malaria endemic areas have focused on the potential for iron status to influence the risk of malaria infection. These studies have led to questioning whether iron supplementation can be safely given in the management of ID in malaria endemic areas^{8,29}. However, our findings suggest that malaria itself may be causing ID in African children. Other infections may similarly cause ID, for example respiratory infections were associated with hepcidin-mediated ID in Gambian children³⁰. The management of ID has traditionally involved iron supplementation. However, in addition to long-standing concerns regarding the safety of iron supplementation in Africa, there are also concerns regarding effects on the gut microbiome³¹ and lack of efficacy in

areas of high infectious burden where chronically raised hepcidin may inhibit iron absorption^{9,10}. Interventions that reduce malaria would allow iron to be more effectively absorbed from dietary sources. We recommend that strategies to prevent and treat malaria, and other infections, should be an integral part of programs to control ID in African children. Future research should confirm these findings through conducting trials of malaria control; for example, intermittent preventative treatment trials of malaria to evaluate the effect of malaria control on iron status.

Methods

Study Populations and Laboratory Methods

This study included 10 cohorts of healthy children (in Malawi, Ghana, Burkina Faso, Democratic Republic of Congo (DRC), Kenya (Kilifi and Western), Tanzania, The Gambia, Cameroon, and Uganda) living in malaria endemic countries. Four of these studies (Malawi, Ghana, DRC, and The Gambia) were cross-sectional studies, two were longitudinal (Tanzania and Kilifi Kenya), two were randomized controlled trials (RCT, Burkina Faso and Uganda), one was a cluster RCT (Western Kenya) and one a cluster survey (Cameroon). The study also included three cohorts living in countries with no malaria exposure, African-American adults from the longitudinal Jackson Heart Study (JHS), children from Soweto, South Africa and a survey of school children in Nairobi, Kenya. These studies are described below.

Malaria-endemic study sites

Malawi: The 2015-2016 Malawi Micronutrient Survey (MMS) that was conducted as part of the Malawi Demographic and Health Survey. This cross-sectional survey aimed to determine the prevalence of anemia, micronutrient deficiencies (iron and vitamin A), infections and

hemoglobinopathies³². The MMS included all children aged 6-59 months from randomly selected clusters and households. Details of the study design are available elsewhere³². Briefly, whole blood collected in EDTA tubes was used to test for malaria using rapid diagnostic test (RDT) (SD BIOLINE Malaria *Plasmodium falciparum* [HRP2], Alere, Inc., Waltham, MA) and to measure hemoglobin concentrations using the HemoCue 301 (Hemocue America, Brea, CA). Serum ferritin, C-reactive protein (CRP) and α 1-acid glycoprotein (AGP) were measured using sandwich enzyme-linked immunoassay (ELISA, VitMin Laboratory, Germany)³³. Genotyping of sickle cell trait and α -thalassemia was performed using polymerase chain reaction (PCR) as previously described³².

Ghana: This study was part of the 2017 Ghana Micronutrient Survey. Details of the cross-sectional study design and ethical approvals are presented in³⁴. Children aged 6 - 59 months were recruited from three strata (Southern Belt, Middle Belt, and Northern Belt) in Ghana and random selection was performed in each stratum. Blood sampling and anthropometry were conducted during the survey. Malaria testing was performed using RDT (SD BIOLINE Malaria Ag P.f/Pan RDT kit (Standard Diagnostics Inc, Gyeonggi-do, Republic of Korea). Hemoglobin concentrations were measured using HemoCue 301 AB, Ängelholm, Sweden). Sandwich ELISA³³ was used to measure serum ferritin, CRP, and AGP concentrations. DNA was extracted from blood pellets and used for typing sickle cell trait and α -thalassemia using PCR^{35,36}.

Burkina Faso: The VAC050 ME-TRAP Malaria Vaccine Trial: This was part of the *VaccGene* study that aimed to identify genetic variants associated with differential response to vaccination

in infancy but with ethical approval to undertake analyses to examine the effect of iron status on infection susceptibility. Details of the study design are described elsewhere³⁷. Infants between the ages of 6 and 18 months living in the Banfora region of Burkina Faso were recruited into a Phase 1/2b clinical trial to test the safety, immunogenicity and efficacy of an experimental heterologous viral-vectored prime-boost liver-stage malaria vaccine³⁷. Serum ferritin (Chemiluminescent Microparticle Immunoassay, Abbott Architect, USA), hepcidin (DRG Hepcidin 25 [bioactive] high sensitive ELISA Kit (DRG International, USA)), CRP (MULTIGENT CRP Vario assay, Abbott Architect, USA), hemoglobin (Coulter analyser, Beckman Coulter, USA), and malaria parasitemia (Giemsa stained thick and thin blood films) were measured at a single time point. Genotyping of sickle cell trait in the *VaccGene* study is described below.

Western Kenya: Children were recruited from rural villages in Bungoma, Kakamega, and Vihiga Counties in western Kenya using a cluster study design as part of the water, sanitation, and handwashing benefits randomized-controlled trial (WASH Benefits Trial). Details of the study design have been published elsewhere³⁸. We used samples collected from the environmental enteropathy endline survey. Venous blood samples were used to test for malaria parasitemia using RDT (SD BIOLINE Malaria *P. falciparum* [HRP2], Alere, Inc., Waltham, MA) and hemoglobin concentrations (Hemocue Hb 301). Serum ferritin and CRP were assayed using sandwich ELISA³³. Serum hepcidin-25 was quantified by using a competitive ELISA kit (PenLabs). Genotyping of sickle hemoglobin types and α -thalassemia was conducted using PCR^{35,36}.

Sud Kivu and Kongo Central, Democratic Republic of Congo (DRC): This study used data collected during a nutrition cross-sectional survey of mothers and their children aged 6–59 months in rural Sud Kivu and Kongo Central provinces in the DRC as described elsewhere³⁹. Venous blood samples were used to test for malaria parasitemia using RDT (CareStart Malaria Screen, AccessBio, Inc.). Serum ferritin, CRP and AGP were assayed using sandwich ELISA³³. Hemoglobin typing for sickle cell trait was conducted using Pyrosequencing while PCR was used to detect α -thalassemia as described elsewhere³⁹.

Kilifi, Kenya: This was an ongoing rolling longitudinal study designed to evaluate malaria immunity in children and is described elsewhere⁴⁰. Within this cohort, children were followed-up to 8 years of age with weekly follow-ups and annual cross-sectional surveys during which anthropometry measurements were made and blood samples were collected. Serum ferritin (Chemiluminescent Microparticle Immunoassay, Abbott Architect, USA), hepcidin (DRG Hepcidin 25 [bioactive] high sensitive ELISA Kit (DRG International, USA)), CRP (MULTIGENT CRP Vario assay, Abbott Architect, USA), hemoglobin (Coulter analyser, Beckman Coulter, USA), and malaria parasitemia (Giemsa stained thick and thin blood films) were measured from blood samples collected at a single cross-sectional survey based on the availability of plasma samples archived at -80°C. Genotyping of hemoglobin types and α -thalassemia was conducted by PCR^{35,36} using DNA extracted by Qiagen DNA Blood Mini Kit (Qiagen, West Sussex, United Kingdom).

Muheza, Tanzania: Children were enrolled at delivery into the Mother–Offspring Malaria Studies (MOMS) Project longitudinal birth cohort at Muheza District Hospital in northeastern Tanzania between 2002–2006. Children were assessed for malaria parasitemia every 2 weeks during infancy and monthly thereafter, as well as at the time of any illness. Singleton children without evidence of HIV in themselves or their mothers during follow up were included in studies of iron status and risk of malaria infection as described elsewhere⁴¹. Blood samples were collected at 3, 6, and 12 months of age then once every 6 months in years 2 and 3⁴¹. In this study, we used data from samples collected at a single timepoint, each child’s oldest timepoint, since older children were likely to have experienced more malaria episodes. *P. falciparum* parasitemia was determined using Giemsa-stained thick blood smears while hemoglobin was measured using an impedance-based analyzer (Abbott Cell Dyn 1200). Plasma ferritin and CRP were assayed using a multiplex bead-based platform (BioRad) and custom assay kits, and hemoglobin was typed by electrophoresis (Helena Laboratories, Beaumont, Texas, USA)⁴¹. Genotyping for α -thalassemia was conducted as described by Chong *et al*³⁶.

The Gambia: West Kiang study: All children aged 2 to 6 years were recruited from 10 rural villages in the West Kiang region of The Gambia during the malaria season (July to August 2001)⁴². We used cross-sectional data collected at the start of the malaria season. All children had a clinical examination, anthropometric measurements, and a 3-day course of mebendazole for possible hookworm infection. A blood sample was collected for complete blood count (CBC), malaria slide, ferritin (Microparticle Enzyme Immunoassay (Abbott Architect, USA)), hepcidin (Hepcidin-25 [human] EIA Kit (Bachem, Switzerland), α -1-antichymotrypsin (ACT, Immunoturbidimetry, Cobas Mira Plus Bio-analyser, Roche), and DNA extraction. Children with

a temperature $>37.5^{\circ}\text{C}$ had a malaria blood film, appropriate clinical treatment, and a blood sample 2 weeks later after recovery from illness. Genotyping of sickle cell was performed on amplified DNA as detailed elsewhere⁴². The Gambian Bachem hepcidin values were harmonized by converting to the old DRG hepcidin assay values $[(0.266 \times \text{Bachem values}) + 1.633]$ and then to the new high sensitive DRG hepcidin assay values $[(1.989 \times \text{old DRG values}) - 3.24]$ as previously validated⁴³.

Yaoundé and Douala, Cameroon: Children 12-59 months of age were recruited to a cluster survey that aimed to determine the prevalence of inherited hemoglobin disorders in Yaoundé and Douala, Cameroon, as described elsewhere⁴⁴. Venous blood samples were collected for malaria testing using RDT (SD Bioline Malaria Ag Pf/Pan, Standard Diagnostics; Gyeonggi-do, Republic of Korea) and hemoglobin measurement using a photometer (Hemocue, Ängelholm, Sweden). Plasma ferritin, CRP and AGP were assayed using ELISA³³. Hemoglobin genotypes were determined by High Performance Liquid Chromatography (HPLC) using an ultra-Resolution Variants Analyzer (Trinity Biotech, Bray, Ireland) while α -thalassemia type was determined by PCR⁴⁴.

Uganda: The Entebbe Mother and Baby Study (EMaBS): EMaBS is a prospective birth cohort that was originally designed as a randomized controlled trial to test whether anthelmintic treatment during pregnancy and early childhood was associated with differential response to vaccination or incidence of infections such as pneumonia, diarrhea or malaria (<http://emabs.lshtm.ac.uk/>)⁴⁵. This cohort was part of the *VaccGene* study as described

elsewhere⁴⁶. Blood samples were collected in vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA) at birth and at subsequent birthdays up to five years of age. Anthropometry and iron / inflammatory biomarkers were measured from a single annual visit based on the availability of stored samples. Serum ferritin (Chemiluminescent Microparticle Immunoassay, Abbott Architect, USA), CRP (MULTIGENT CRP Vario assay, Abbott Architect, USA), hepcidin (DRG Hepcidin 25 [bioactive] high sensitive ELISA Kit (DRG International, USA)), hemoglobin (Coulter analyser, Beckman Coulter, Nyon, Switzerland), and malaria parasitemia (Giemsa stained thick and thin blood films) were measured. Genotyping of sickle cell trait in the *VaccGene* study is described below.

Malaria-free study sites

South Africa: The Soweto Vaccine Response Study: Infants born in Chris Hani Baragwanath Hospital living in the Soweto region of Johannesburg, South Africa were recruited from vaccine trials⁴⁷ coordinated by the Respiratory and Meningeal Pathogens Unit (<http://www.rmpru.com/>). Mothers of the infants were approached if the infants had received all of their EPI vaccines up to six months of age. The infants were sampled prospectively at 12 months after receipt of measles vaccine at 9 months. Single whole blood samples were collected in EDTA vacutainer tubes for measurement of iron and inflammatory markers and DNA extraction. Serum ferritin (Chemiluminescent Microparticle Immunoassay, Abbott Architect, USA), CRP (MULTIGENT CRP Vario assay, Abbott Architect, USA) and hepcidin (DRG Hepcidin 25 [bioactive] high sensitive ELISA Kit (DRG International, USA)) were measured. This cohort was part of the *VaccGene* study and genotyping is described below.

Jackson Heart Study: This is a population-based longitudinal study of African-Americans aged ≥ 21 years living in the Jackson, Mississippi metropolitan area in the USA⁴⁸. This study was designed to evaluate risks of cardiovascular disease as described elsewhere^{48,49}. Serum ferritin (Roche immunoturbidimetric assay), CRP (ELISA) and hemoglobin (Coulter analyser, Beckman Coulter, USA) were measured from blood samples collected at a single clinic visit. Whole blood was used to extract DNA using Puregene reagents (Gentra System, Minneapolis, USA). Genetic studies were conducted as described elsewhere⁴⁹ and rs334 genotypes were extracted from exome sequencing datasets as described in Peloso *et al*⁵⁰.

Nairobi, Kenya: Children aged 10-16 years were recruited from the Nairobi Urban Health and Demographic Surveillance System⁵¹ as part of studies investigating the relationship between sickle cell trait, malaria and blood pressure^{52,53}. Nairobi is located at high altitude (1,800 m above sea level) and there is no evidence of malaria transmission there⁵⁴. Population wide censuses are conducted four times a year within the study area⁵¹. Using census data, we selected all school children aged 10-16 years who had a continuous record of residence since birth and had therefore had minimal exposure to malaria. In order to increase our efficiency in recruiting participants with sickle cell trait, we limited our recruitment to those who identified themselves as genetically descended from ethnic groups whose ancestral residence was in regions endemic for malaria (e.g., Luhya, Luo, Teso, Mijikenda). The frequency of sickle cell trait is much higher in these ethnic groups⁵⁵. We measured ferritin, CRP (immunoturbidimetry ILab[®] systems immunoturbidimetric assay, Instrumentation Laboratory, Spain) and hemoglobin (Coulter analyser, Beckman Coulter, USA) from stored blood samples. Sickle cell trait was typed by

PCR^{35,36} using DNA extracted by Qiagen DNA Blood Mini Kit (Qiagen, West Sussex, United Kingdom).

Study of hospitalized children

Kilifi hospital-based study: We measured hepcidin in children with severe malaria admitted to Kilifi County Hospital⁵⁶. 62 samples were randomly selected. Hepcidin was measured using the Hepcidin-25 [human] EIA Bachem Kit and harmonized to DRG hepcidin values⁴³. Severe malaria was diagnosed as *P. falciparum* parasites in the blood film plus clinical features of severe malaria including hemoglobin <5g/dL, hematocrit level of <15% (for severe malarial anaemia) or Blantyre coma score of <3 (for cerebral malaria).

Genotyping of sickle cell trait in the *VaccGene* Study

Sickle cell trait (rs334) single nucleotide polymorphisms (SNP) was directly genotyped in the *VaccGene* populations (in Uganda, Burkina Faso and South Africa) using the Illumina HumanOmni 2.5M-8 ('octo') BeadChip array version 1.1 (Illumina Inc., San Diego, USA) (n = 648) and the Illumina Multi-Ethnic Global Array (n=197) performed by the Genotyping Core facilities at the Wellcome Trust Sanger Institute (WTSI). Genomic DNA underwent whole genome amplification and fragmentation before hybridization to locus specific oligonucleotides bound to 3µm diameter silica beads. Fragments were extended by single base extension to interrogate the variant by incorporating a labelled nucleotide enabling a two-color detection (Illumina, 2013). Genotypes were called from intensities using two clustering algorithms

(Illuminus and GenCall) in GenomeStudio (Illumina Inc., San Diego, USA) incorporating data from proprietary pre-determined genotypes. Details of genotyping and quality control are described elsewhere⁴⁶. The variant rs334 was retained in all datasets following stringent quality control processes⁴⁶.

Definitions

Iron deficiency (ID) was defined according to World Health Organization (WHO) recommendation as ferritin <12µg/L or <30µg/L in the presence of inflammation (defined as CRP >5mg/L, ACT >0.6g/L or AGP >1g/L) in children <5 years or <15µg/L in children ≥5 years¹⁵. Anemia was defined as hemoglobin <11 g/dL in children aged <5 years, or hemoglobin <11.5 g/dL in children ≥5 years¹⁷. In the JHS and Nairobi, ID was defined as ferritin <30µg/L. In the JHS, anemia was defined as hemoglobin <12 g/dL in women or <13 g/dL in men^{16,17}. Iron deficiency anemia was defined as presence of ID and anemia¹⁷. Malaria parasitemia was defined as a blood slide positive for asexual *P. falciparum* parasites. Underweight was defined as weight-for-age z-score < -2 using WHO Growth Standards⁵⁷.

Regression-correction

Since ferritin is an acute phase reactant and correlates positively with inflammatory markers^{58,59}, we further defined ID after regression-correction for the effect of inflammation on ferritin levels as proposed by the Biomarkers Reflecting Inflammation and Nutritional Determinants of Anemia (BRINDA) project^{19,60}. This approach predicts what the ferritin level would have been in the absence of inflammation or infection and then applies the corrected values to estimate the prevalence of ID. The regression-correction approach followed a three-step process. In the first

step, internal reference values for inflammatory markers (CRP or ACT) were defined as the 10th percentile. CRP levels were measured in all studies except in The Gambia where ACT was measured. In addition to CRP, AGP was measured in Malawi, Ghana, DRC, and Cameroon, however, since CRP was positively correlated with AGP, and for consistency, we only corrected for CRP in these studies. To avoid overcorrection for very low levels of inflammatory markers only participants with CRP or ACT values above the 10th percentile had their ferritin values subtracted from observed values in equation (1) below¹⁹. In the second step, univariable linear regression models were applied to each study, with ferritin as the dependent variable, to estimate regression coefficients for the crude association between inflammatory marker and ferritin (β). In the third step, the regression coefficients estimated in step 2 were used to calculate adjusted ferritin values using equation (1). Ferritin and inflammatory markers were applied in the equations after ln-transformation.

$$Ferritin_{adjusted} = Ferritin_{unadjusted} - \beta(CRP \vee ACT_{obs} - CRP \vee ACT_{ref}) \quad (1)$$

where ‘obs’ is the observed value and ‘ref’ is the reference value.

We then defined ID using the regression-corrected unlogged ferritin (i.e. adjusted for the effects of inflammation) using the same thresholds that were applied to the uncorrected ferritin levels in the WHO recommendations (i.e. ferritin <12µg/L in children <5 years or <15µg/L in children aged ≥5 years¹⁵).

Systematic review and meta-analysis of common genetic polymorphisms associated with malaria risk

Mendelian randomization is an instrumental variable analysis that reduces biases from confounding and reverse causation by using genetic variants to proxy the exposure (e.g, malaria) and estimate a causal effect of that exposure on the outcome (e.g, ID). To perform Mendelian randomization analyses, valid instrumental variables are required. The instrumental variables must be associated with the exposure of interest and the effect size can be obtained from association studies. We therefore performed a systematic review and meta-analysis of published studies in African populations to determine the overall effect of common genetic polymorphisms that are associated with uncomplicated malaria. These polymorphisms included sickle cell trait, α -thalassemia, and G6PD. We performed the search in PubMed database for papers published before 20th March 2020. For sickle cell trait the search terms included (“malaria” [Title/Abstract] OR “malaria/blood” [MAJR] OR “malaria/genetics” [MeSH Terms] OR "Malaria"[Mesh] OR "Malaria, Falciparum"[Mesh] OR "Plasmodium falciparum"[Mesh]) AND (“HbAS” [Title/Abstract] OR “sickle cell trait” [Title/Abstract] OR “sickle cell trait/genetics” [MeSH Terms] OR “sickle cell trait/blood” [MeSH Terms] OR "Hemoglobin, Sickle"[Mesh] OR "Sickle Cell Trait"[Mesh] AND "Africa" [MeSH Terms] giving 594 articles. For α -thalassemia, the search terms included (“malaria” [Title/Abstract] OR “malaria/blood” [MAJR] OR “malaria/genetics” [MeSH Terms] OR "Malaria"[Mesh] OR "Malaria, Falciparum"[Mesh] OR "Plasmodium falciparum"[Mesh]) AND (“alpha-thalassemia” [Title/Abstract] OR “alpha-thalassemia /genetics” [MeSH Terms] OR “alpha-thalassemia/blood” [MeSH Terms] OR " α -thalassemia "[Mesh]) AND "Africa" [MeSH Terms] and yielded 65 articles. The search terms for G6PD included (“malaria” [Title/Abstract] OR “malaria/blood” [MAJR] OR “malaria/genetics”

[MeSH Terms] OR "Malaria"[Mesh] OR "Malaria, Falciparum"[Mesh] OR "Plasmodium falciparum"[Mesh]) AND ("G6PD" [Title/Abstract] OR "glucose-6-phosphate-dehydrogenase" [Title/Abstract] OR "glucose-6-phosphate-dehydrogenase/genetics" [MeSH Terms] OR "glucose-6-phosphate-dehydrogenase/blood" [MeSH Terms]) AND "Africa" [MeSH Terms] and yielded 187 articles.

We restricted our analysis to studies conducted in Africa since our outcome of interest (iron deficiency) was measured in African populations. We also focused on studies reporting incidence rate ratio (IRR) since children are repeatedly infected with malaria and therefore an IRR provides a better estimate of the true malaria risk reduction attributable to genetic polymorphisms. Studies included in the meta-analysis are shown in Supplementary Table 2.

Statistical analysis

All statistical analyses were conducted using STATA 13.0 (StataCorp., College Station, TX). All measurements were taken from distinct samples. We conducted both cohort-specific and pooled analyses. Where appropriate, we computed percentages, geometric means, and deciles. We used two-tailed Student's *t*-tests to test for difference in means of log_e-transformed hepcidin concentrations between children with and without malaria parasitemia or between those with severe malaria and those without parasitemia. Where appropriate, we fitted adjusted logistic regression models to determine the effect of sickle cell trait on iron deficiency. The cohort-specific odds ratios were meta-analyzed assuming fixed-effects since there was little evidence of heterogeneity between studies. We used meta-analysis to determine the overall effect size, and also to identify potential heterogeneity that may have been introduced by different populations and laboratory facilities. A P-value <0.05 was considered statistically significant. All P-values

reflect two-tailed tests. Individuals with sickle cell disease (HbSS) were not included in the analyses since numbers were few and there is little evidence suggesting that HbSS protects against uncomplicated malaria.

We used the online <http://cnsgenomics.com/shiny/mRnd/> MR power calculator to calculate sample size. The sample size of 7453 had power above 80% given the observed odds ratio of the outcome variable per standard deviation of the exposure variable of 2.65, 2% variation of clinical malaria that is explained by sickle cell trait^{61,62}, 27% prevalence of ID and a type-I error rate of 0.05.

In order to investigate whether uncomplicated malaria is causally associated with ID, a two-sample Mendelian randomization⁶³ was conducted using the *mrrobust* software package⁶⁴ in STATA 13.0. This is a Wald ratio involving two estimates: the SNP-outcome effect divided by the SNP-exposure effect, in this case HbAS (rs334)-ID effect divided by HbAS (rs334)-malaria effect. The first estimate (SNP-outcome) for sickle cell trait (HbAS, rs334) on ID was from the above described community-based cohorts where the overall log-odds estimate was determined using the meta-analyzed cohort-specific estimates (Fig. 1c). The second estimate (SNP-exposure) was from the meta-analyzed overall log incidence rate ratio of sickle cell trait on uncomplicated malaria (Extended Data Fig. 4). A causal log-odds estimate (equation 2), which is the ratio of the first estimate and the second estimate was computed⁶³. The causal odds ratio was obtained by exponentiating the causal log-odds estimate and interpreted as change in ID per unit increase in log incidence rate of malaria. We further calculated the effect of reducing malaria incidence by

half on ID. This was obtained by multiplying the causal log-odds by natural-logarithm of 0.5 and then exponentiating the result²⁰.

$$\text{Causal log odds} = \frac{\log \text{odds of iron deficiency} \in \text{HbAS}}{\log \text{incidence rate ratio of malaria} \in \text{HbAS}} \quad (2)$$

To determine whether sickle cell trait influences iron deficiency independently of malaria, we conducted sensitivity or zero relevance point analyses in negative controls i.e. populations that are not exposed to malaria¹⁴. To do this we repeated our analyses in two separate populations that are not exposed to malaria including i) an African-American adult population (n = 3207) and ii) life-long adolescent residents of Nairobi (n = 611) where there is no evidence of malaria transmission⁵⁴. As this analysis suggested an effect is absent among populations that are not exposed to malaria, allowing us to interpret the effect amongst children exposed to malaria.

Ethical Approvals

For the *VaccGene* studies and Gambian study, individual study site ethical approvals were obtained. For Kilifi, Kenya (by the Scientific Ethics Review Unit of the Kenya Medical Research Institute (KEMRI/SERU/CGMR-C/046/3257/2983)); Entebbe, Uganda (locally by the Uganda Virus Research Institute (GC/127/12/07/32) and Uganda National Council for Science and Technology (MV625), and in the UK by the London School of Hygiene and Tropical Medicine (A340) and Oxford Tropical Research (OTR) (39-12, 42-14 and 37-15) Ethics Committees); Banfora, Burkina Faso (by Ministere de la Recherche Scientifique et de l’Innovation in Burkina Faso (2014-12-151) and the OTR Ethics Committees (41-12)); Soweto, South Africa (by the University of Witwatersrand Human Research (M130714) and the OTR Ethics Committees

(1042-13 and 42-14)); and West Kiang, The Gambia (by the Gambian Government / Medical Research Council Ethics Committee (874/830)). For the additional seven study sites, individual study data transfer agreements were signed with the responsible study/institution's principal investigator (PI) and the KEMRI-Wellcome Trust Research Programme study PI, Dr Sarah Atkinson. These studies had ethical approval to share de-identified data for further secondary analyses presented in this study. Informed written consent was obtained from all children's parents or guardians.

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Competing interests: The authors declare no competing interests.

Data and materials availability: All data are available in the main text or the Supplementary Materials. Primary individual level de-identified data for the Kilifi, Kenya; Entebbe, Uganda; Banfora, Burkina Faso; and West Kiang, The Gambia cohorts are available in Harvard Dataverse at <https://doi.org/10.7910/DVN/UKGRVJ>; applications for access can be made through the Data Governance Committee cgmrc@kemri-wellcome.org. Data from the 2015-16 Malawi Micronutrient Survey are available from the DHS Program at <https://dhsprogram.com/what-we-do/survey/survey-display-483.cfm>. The data underlying the results from the Ghana site are owned by UNICEF Ghana and the Ministry of Health Ghana and contain confidential, identifying information. Data are available from UNICEF Ghana (accra@unicef.org) for researchers who meet the criteria for access to confidential data. De-identified data from the western Kenya study are available on Open Science Framework at the following link: <https://osf.io/dsrv2/>. Data from the Sud Kivu and Kongo Central, DRC study are available at <https://doi.org/10.7910/DVN/RNWYR8>. All data used in the analysis of the MOMS Project cohort (Muheza, Tanzania) are available under human data transfer agreement for purposes of reproducing or extending the analysis. Data for the Cameroon study are available upon reasonable request to the survey representative Alex Ndjebayi (andjebayi@hki.org), Helen Keller International – Cameroon Office | Rue 1771, Bastos | BP 14227 | Yaounde.

Code availability: All statistical analyses were conducted using STATA 13.0 (StataCorp., College Station, TX). The STATA do file used to generate results that are reported in the paper is available in the KEMRI Wellcome Trust Research Programme Harvard Dataverse repository <https://doi.org/10.7910/DVN/UKGRVJ>.

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Figure legends

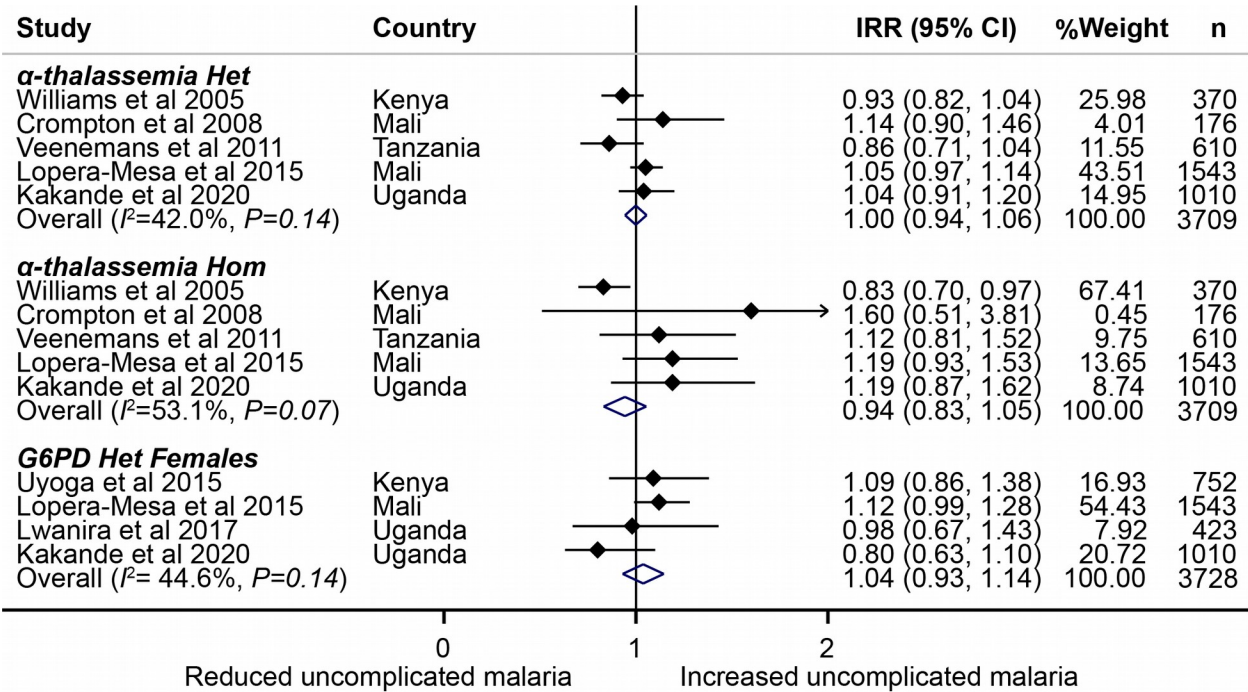
Fig. 1. Sick cell trait (HbAS) is associated with protection from iron deficiency (ID).

a) Africa malaria map showing the predicted posterior predictions of age-standardized *P. falciparum* prevalence (PfPR₂₋₁₀) taken from Snow *et al* 2017³ and the location of the current study sites with data on sickle cell trait (HbAS). Map was reproduced with permission. **b)** Prevalence of malaria parasitemia, HbAS, and iron deficiency (ID) for each study site. **c)** Summary results of the effect of HbAS on ID by study site. Overall represents a fixed-effect meta-analysis of study-specific odds ratios. **d)** Odds ratios for the associations between sickle cell trait and the variables age, sex, inflammation, and underweight. Inflammation was defined as CRP >5mg/L or ACT >0.6g/L or AGP >1g/L. Underweight was defined as a WHO 2006 reference weight-for-age z-score <-2. HbAA, normal hemoglobin, OR, odds ratio.

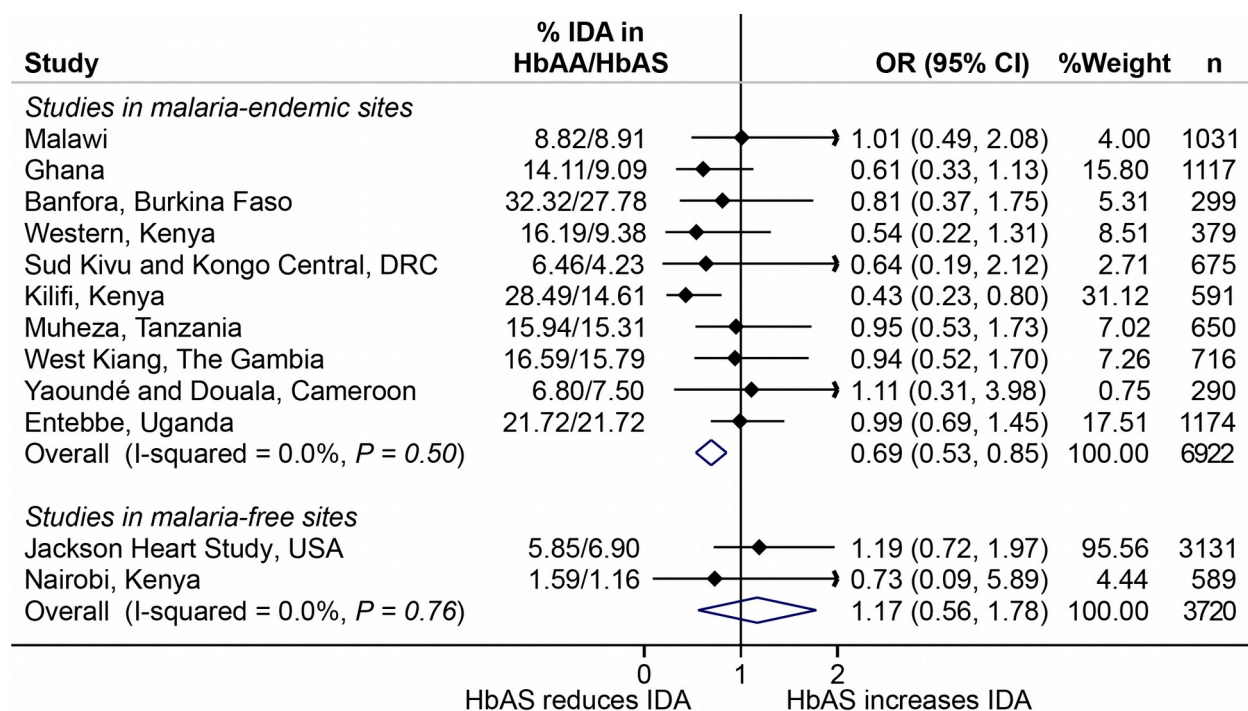
Fig. 2. How malaria might cause a hepcidin-mediated blockade of iron absorption and recycling leading to iron deficiency. **a)** Prevalence of ID and IDA **b)** is lower in African children carrying sickle cell trait (HbAS), a genetic variant that protects against malaria, than in those with normal hemoglobin (HbAA) in malaria-endemic sites but not in non-malaria sites. P values were derived from logistic regression analyses adjusted for age, sex, inflammation, and study site. **c)** Geometric means of hepcidin concentrations are higher in children with malaria parasitemia or severe malaria compared to those without. P-values were derived from two-tailed Student's *t*-tests. **d)** Prevalence of inflammation is

lower in HbAS than in HbAA individuals in malaria-endemic sites but not in non-malaria sites. P-values were derived from logistic regression analyses adjusted for age, sex, and study site. **e)** Geometric means of hepcidin concentrations increase with increasing CRP concentrations and remain above threshold for iron absorption at almost all deciles of CRP in children with malaria parasitemia. Brown line shows children with malaria parasitemia and blue line those without parasitemia. Yellow line shows point at which inflammation is clinically diagnosed. Red horizontal line indicates the threshold of hepcidin above which iron absorption is inhibited (5.5µg/L). Data were pooled for malaria-endemic sites and for non-malaria sites (Jackson Heart Study and Nairobi). Hepcidin was measured in the Burkina Faso, Western Kenya, Uganda, The Gambia, and Kilifi, Kenya cohorts. CRP was measured in all sites except The Gambia. Cp, Ceruloplasmin; DMT1, divalent metal transporter 1; FPN, ferroportin; GIT, gastrointestinal tract; Par, malaria parasitemia; Pf, *P. falciparum*; Tf, transferrin; TfR, transferrin receptor.

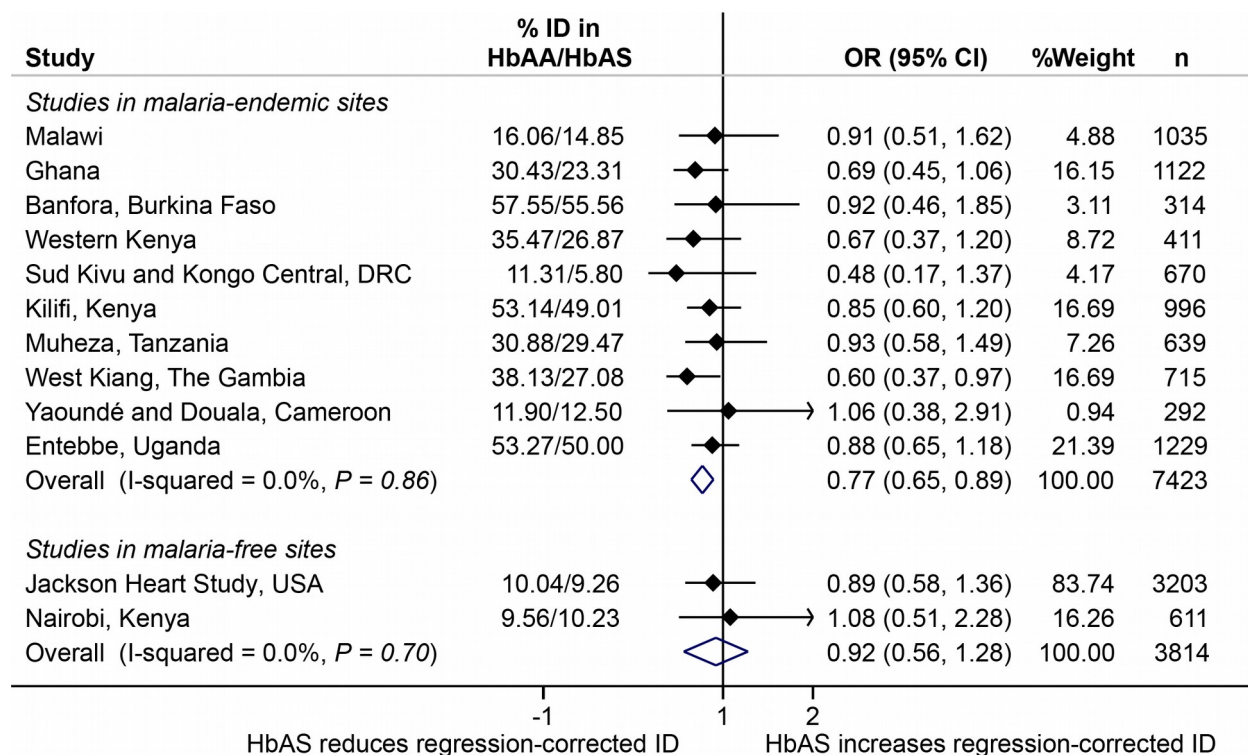
Extended Data Figures



Extended Data Fig. 1. A meta-analysis of previous African studies investigating the effect of α -thalassemia and G6PD A and A- polymorphisms on uncomplicated febrile malaria. Overall represents a fixed-effect meta-analysis of study-specific incidence rate ratio (IRR) by genetic polymorphism. n shows the number of individuals included in the analysis. Few studies included G6PD homozygous females and numbers were small (Table S2). Het, heterozygous; Hom, homozygous.

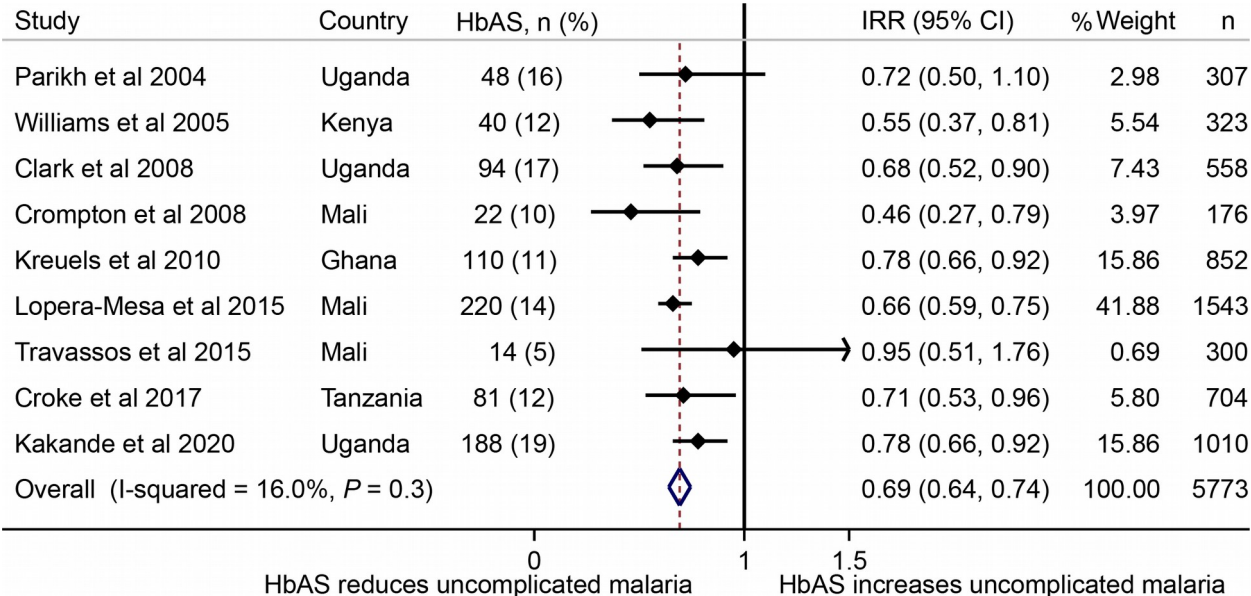


Extended Data Fig. 2. A meta-analysis of the effect of sickle cell trait on iron deficiency anemia (IDA). Overall represents a fixed-effect meta-analysis of cohort-specific odds ratios. n shows the number of individuals included in the analysis. Numbers for IDA are fewer compared to those for ID since not all children had hemoglobin concentrations measured.

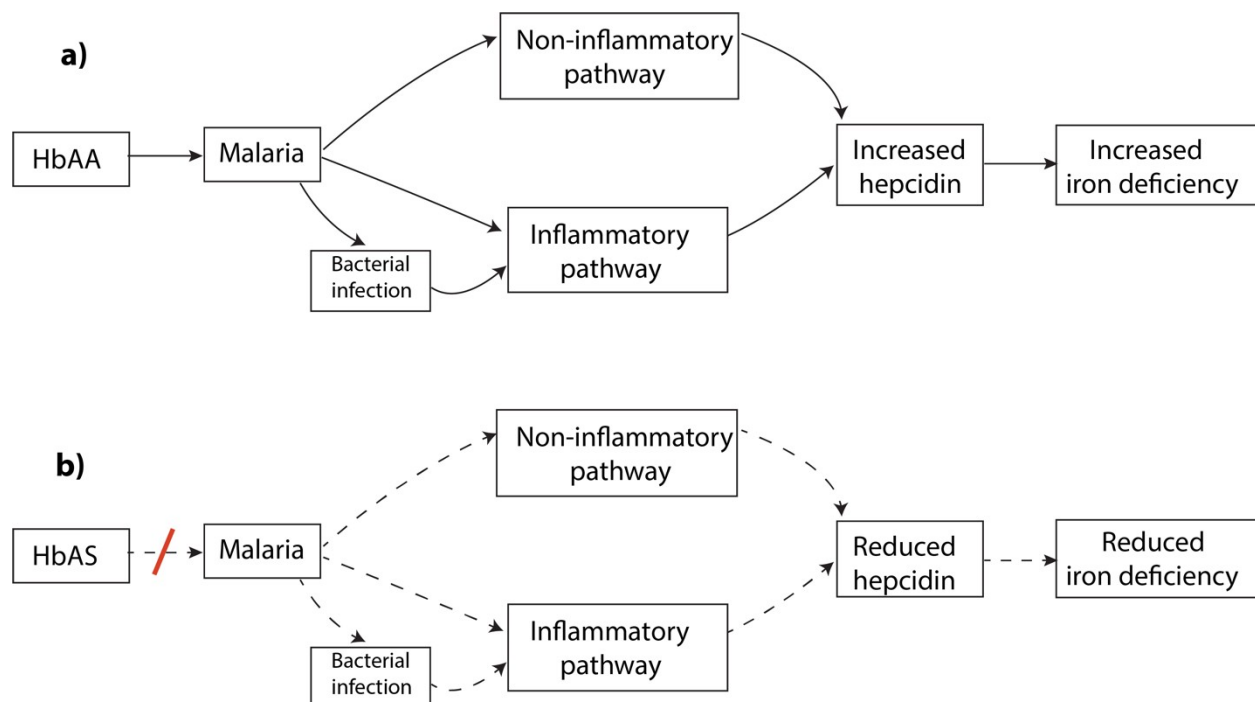


Extended Data Fig. 3. A meta-analysis of the effect of sickle cell trait on iron deficiency (ID) regression-corrected for inflammation. ID was defined using ferritin levels adjusted for the effects of inflammation using a regression-correction approach as developed by BRINDA. Overall represents a fixed-effect meta-analysis of cohort-specific odds ratios. n shows the number of individuals included in the analysis.

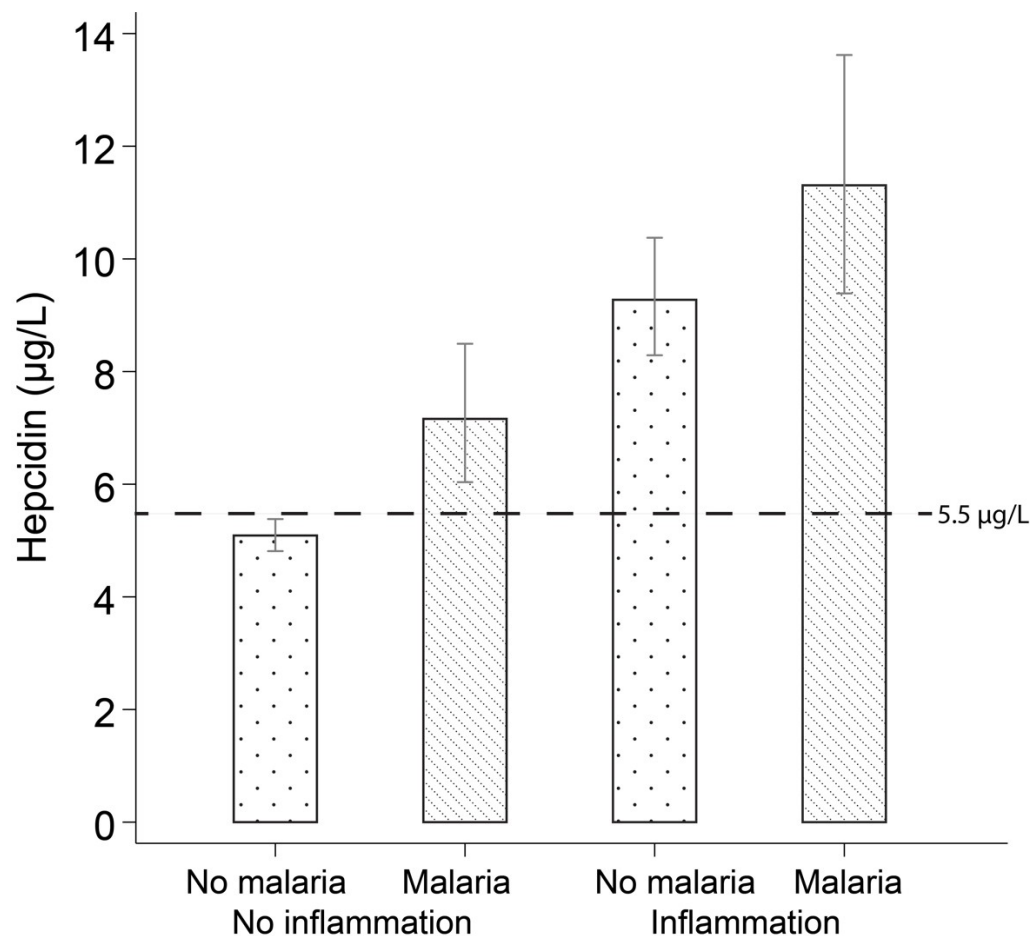
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Extended Data Fig. 4. A meta-analysis of the effect of sickle cell trait on uncomplicated febrile malaria. Overall represents a fixed-effect meta-analysis of study-specific incidence rate ratio (IRR). n shows the number of individuals included in the analysis.



Extended Data Fig. 5. How HbAS, a genetic proxy for malaria exposure, may protect children from iron deficiency. **a)** Individuals carrying normal beta hemoglobin gene (HbAA) are not protected from malaria. Malaria up-regulates production of hepcidin through inflammatory and non-inflammatory pathways and by increasing the prevalence of other infections. Hepcidin in turn blocks iron absorption. **b)** Sickle cell trait (HbAS) partially protects individuals from malaria infection, therefore inflammation is reduced leading to reduced hepcidin stimulation and increased iron absorption.



Extended Data Fig. 6. Relationship between geometric mean hepcidin concentrations, malaria parasitemia and inflammation. Error bars indicate 95% confidence intervals. Horizontal dotted line indicates the threshold of hepcidin above which iron absorption is inhibited (5.5µg/L). Inflammation was defined as CRP >5mg/L, ACT >0.6g/L or AGP >1g/L. Malaria was defined as a blood slide positive for asexual *P. falciparum* parasites. Hepcidin was measured in the Burkina Faso, Western Kenya, Uganda, The Gambia, and Kilifi, Kenya cohorts.

Tables

Table 1. Characteristics of study participants by site

	Total	Age (years) mean (sd)	Sex: females, n (%)	Inflammation*, n (%)	Underweight†, n (%)
<i>Studies in malaria-endemic sites</i>					
Malawi	1035	2.75 (1.24)	512 (49.47)	604 (58.36)	134 (13.00)
Ghana	1123	2.67 (1.28)	560 (49.96)	230 (20.48)	177 (16.06)
Banfora, Burkina Faso	314	1.86 (0.46)	153 (48.73)	106 (33.76)	57 (19.32)
Western Kenya	411	1.85 (0.15)	210 (51.09)	92 (22.38)	32 (7.82)
Sud Kivu and Kongo Central, DRC	678	2.41 (1.14)	338 (49.85)	462 (68.14)	141 (22.49)
Kilifi, Kenya	996	2.20 (1.47)	489 (49.10)	261 (26.20)	72 (26.67)
Muheza, Tanzania	652	1.64 (0.79)	313 (48.01)	370 (56.75)	49 (8.02)
West Kiang, The Gambia	723	3.91 (1.16)	333 (46.06)	107 (14.80)	145 (25.53)
Yaoundé and Douala, Cameroon	292	2.76 (1.05)	143 (49.48)	62 (21.23)	15 (5.21)
Entebbe, Uganda	1229	2.30 (0.82)	603 (49.06)	291 (23.68)	100 (8.16)
<i>Studies in malaria-free sites</i>					
Jackson Heart Study	3207	55.57 (12.83)	2000 (62.36)	940 (29.31)	12 (0.38)
Nairobi, Kenya	611	12.67 (1.17)	277 (45.34)	32 (5.24)	n/a
Soweto, South Africa	845	1.01 (0.10)	431 (51.01)	145 (17.16)	n/a
<i>Hospitalized children with malaria</i>					
Kilifi hospital-based study	62	1.86 (1.06)	28 (45.16)	62 (100.00)	26 (41.94)

DRC, Democratic Republic of Congo; RCT, randomized controlled trial; sd, standard deviation; n/a, not available

*Inflammation was defined as C-reactive protein (CRP) >5mg/L or α_1 -antichymotrypsin (ACT) >0.6g/L or α_1 -acid glycoprotein (AGP) >1g/L. CRP was measured in all sites except West Kiang, The Gambia where only ACT was measured. In addition to CRP, AGP was measured in Malawi, Ghana, DRC, and Cameroon.

†Underweight was defined as a WHO 2006 reference weight-for-age z-score <-2. Causes of underweight in African-American adults are likely to be different from those in African children.

Table 2: Estimates used in Mendelian randomization analyses

Relation	Estimate (SE)	P	Source
HbAS - ID	-0.36 (0.09)	<0.001	Meta-analyzed African studies (Fig. 1c)
HbAS - uncomplicated malaria	-0.37 (0.04)	<0.001	Meta-analysis of published studies (Extended Data Fig. 4)
MR causal estimate	0.97 (0.24)	<0.001	Calculated as HbAS-ID/HbAS-malaria

The first estimate, the HbAS-ID estimate, is the natural log-odds of the meta-analyzed cohort-specific odds ratio from Fig. 1c. The second estimate, the HbAS-uncomplicated malaria estimate, is the natural log-incidence rate ratio of the meta-analyzed study-specific incidence rate ratios from published studies, Extended Data Fig. 4. The MR causal estimate (the causal log-odds) is the ratio of the first and second estimates, the Wald ratio. SE, standard error; HbAS, sickle cell trait; ID, iron deficiency; MR, Mendelian randomization.