IRON DEFICIENCY ANEMIA AND THE PATHOGENESIS OF FALCIPARUM MALARIA

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ABSTRACT

Morgan McFarland Goheen: Iron Deficiency Anemia and the Pathogenesis of Falciparum Malaria
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Anemia, primarily iron deficiency anemia (IDA), affects up to 50% of pregnant women and 40% of preschool children in the developing world, significantly impacting perinatal and developmental health. However, clinical studies have found (1) iron deficiency protects from malaria, and (2) administration of iron to iron deficient individuals may increase the risk of malaria, thus complicating universal iron supplementation recommendations in malaria-endemic areas.

Our lab developed an *in vitro* model, obtaining red blood cells (RBCs) from IDA or healthy donors at UNC, to study mechanisms of malaria-associated IDA protection and iron treatment risk. We demonstrated decreased *P.falciparum* invasion and growth in IDA RBCs and increased infection susceptibility in young RBCs and reticulocytes. Given iron is essential for the parasite, it was previously thought iron deficiency inhibited malaria through starvation. However, IDA also limits erythropoiesis and induces physiologic RBC changes. Our UNC-based studies thus generated a novel and paradigm-shifting hypothesis – namely that changes in RBC properties and the RBC population structure drive IDA resistance to and iron supplementation risk for malaria.

Our next objective was to evaluate this hypothesis in pregnant women and children from a malaria-endemic area, via comprehensive longitudinal examination of *P.falciparum* pathogenesis in RBCs drawn from iron deficient Gambian children and pregnant women before, during, and after iron supplementation. RBCs were collected from 135 children and 165 pregnant women throughout 12 weeks of iron supplementation and used in flow cytometry-based *in vitro* assays. Our results demonstrate *P.falciparum* erythrocytic stage growth *in vitro* is low at baseline, correlating with hemoglobin levels and mean corpuscular volume. We also determined parasite growth increases during supplementation, using RBCs from both children and pregnant women. Additionally, we found reduced parasite invasion in RBCs from iron deficient Gambian children, which increases during iron supplementation. The elevated growth

rates following iron supplementation paralleled increases in circulating reticulocytes and other markers of young RBCs, kinetics of which correlate with overall increased erythropoiesis. We conclude malaria growth *in vitro* corresponds with elevated erythropoiesis, an inevitable consequence of iron supplementation. Our findings imply iron supplementation in malarious regions should be accompanied by effective preventative measures against falciparum malaria.

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It is difficult to describe the amount of gratitude I feel towards so many people in my life and to adequately thank everyone for helping me reach this point. I would like to start off by thanking my family and friends for their never-ending love and support, without which I would not ever have reached the point where I am today. I fully recognize just how fortunate I am to have such an incredible support system that has been with me for so many years. Knowing I always have my family believing in me has made all the difference when it comes to believing in myself and aiming for the next goal, and it is my family who has also demonstrated to me the importance of hard work and striving to be a good citizen. I am always inspired by them. I am similarly constantly amazed when I look back at the wonderful friendships I have been so fortunate to develop during each stage of my life, the core of which began in pre-school and have remained strong to this day. I would also like to thank my newfound Gambian friends, who have offered me a completely fresh perspective on life and supported me in countless ways regarding my research efforts as well as welcoming me into a new culture; I can truly say I feel like I have found a home away from home with them. It also goes without saying that I owe the ability to have done much of the work in this dissertation to the dedication and hard work of countless MRC staff, not to mention the people who were willing to participate in our research. I would certainly be remiss without mentioning my lab colleagues who have been incredibly rewarding to work with over the past several years, as well as the amazing support constantly available from the MD/PhD program leadership. Nor do I want to forget to thank all of institutions which contributed to the research funding, fellowship awards, and travel grants which I am so lucky to have benefited from, as well as my dissertation committee. Finally, I wish to sincerely thank my research mentors for the amazing opportunities they have provided me and the vast amounts of effort they have put in towards helping me develop as a scientist. I feel I could never come close to repaying them, but I will do my best to continue contributing to science in a meaningful way as an attempt to honor their dedication.

PREFACE

The material included in Chapter 2 is a replication of our paper published in Nature Communications (2014) and is included here with permission from Nature Publishing Group. The authors of this paper are, in order: Clark MA, Goheen MM, Fulford A, Prentice AM, Elnagheeb MA, Patel J, Fisher N, Taylor SM, Kasthuri RS, Cerami C. As second author on this paper, I was involved in much of the experimental work in terms of data collection and analysis, as well as discussion of interpretations and ideas and editing of the manuscript. As first author, Martha Clark was unquestionably the one who spearheaded the research and development of this paper.

The material included in Chapter 3 is a replication of our paper published in the British Journal of Haematology (2016) and is included here with permission from John Wiley and Sons. The authors of this paper are, in order: Goheen MM, Clark MA, Kasthuri RS, Cerami C. As first author on this paper, I was centrally involved in experimental design, data analysis and results interpretation, and writing of the manuscript.

The material included in Chapter 4 is a replication of a manuscript conditionally accepted to EBioMedicine following minor formatting revisions. The authors of this paper are, in order: Goheen MM, Wegmuller R, Bah A, Darboe B, Danso E, Affara M, Gardner D, Patel JC, Prentice AM, Cerami C. As first author on this paper, I was centrally involved in experimental design, data analysis and results interpretation, and writing of the manuscript. I have also worked extensively with Dr. Meshnick and his epidemiology Ph.D. student Jordan Cates to include additional analyses and discussion of analytical approaches beyond the scope of our accepted manuscript at the end of this chapter.

The material included in Chapter 5 is a draft of a manuscript we plan to submit in the near future. The anticipated authorship of this paper would be, in order: Goheen MM, Bah A, Wegmuller R, Darboe B, Danso E, Patel JC, Prentice AM, Cerami C. As fist author on this planned manuscript, I have been centrally involved in experimental design, data analysis and results interpretation, and writing of the manuscript.

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AA – normal β-globin genotype

ABO – blood group consisting of A, B, and O antigens

AC – heterozygous hemoglobin C β-globin genotype

ANCOVA – analysis of covariance

ANOVA – analysis of variance

ARDS – acute respiratory distress syndrome

CC – homozygous hemoglobin C β-globin genotype

CR1 – complement receptor 1

AE – heterozygous hemoglobin E β-globin genotype

ACD – acid citrate dextrose

ACM – asexual culture media

AS – heterozygous sickle-cell trait β-globin genotype

ATP – adenosine triphosphate

CD – cluster of differentiation

CD71+ – reticulocyte

CI – confidence interval

CPDA – citrate phosphate dextrose adenine

CRP – C reactive protein

DARC – Duffy antigen receptor for chemokines

DPG – diphosphoglycerate

EBA – family of *Plasmodium falciparum* erythrocyte binding-like antigens

EPCR – endothelial protein C receptor

Fe – iron

G6PD – glucose-6-phosphate dehydrogenase

GPA – glycophorin A

GR – growth rate

GWAS – genome wide association study

EE – homozygous hemoglobin E β-globin genotype

Hb – hemoglobin (in the context of the β-globin genotype)

Hgb – hemoglobin

HLA – human leukocyte antigen

IDA – iron deficiency anemia

ICAM – intracellular adhesion molecule

IL – interleukin

IRB – institutional review board

IPT – intermittent preventative treatment of malaria

IPTp – intermittent preventative treatment of malaria in pregnancy

IR – iron replete

MACS – magnetic activated cell sorting

MCH – mean corpuscular hemoglobin

MCHC – mean corpuscular hemoglobin concentration

MCV – mean corpuscular volume

MFI – mean fluorescent intensity

MHC – major histocompatibility complex

MNP – micronutrient powder

MPV – mean platelet volume

MRC – Medical Research Council

MRCG – Medical Research Council The Gambia Unit

NADPH – nicotinamide adenine dinucleotide phosphate

NHANES – National Health and Nutrition Examination Survey

NOS – nitric oxide synthase

PEMR – parasite erythrocyte multiplication rate

Pf – *Plasmodium falciparum*

PfEMP1 – *Plasmodium falciparum* erythrocyte membrane protein 1

PfRh – family of *Plasmodium falciparum* reticulocyte binding-like homologs

PK – pyruvate kinase

pRBC – parasitized red blood cell

RBC – red blood cell

RDW – red cell distribution width

Rh – rhesus factor for human blood types

RDT – rapid diagnostic test

RDW – red cell distribution width

ROS – reactive oxygen species

SAO – Southeast Asian ovalocytosis

SC – heterozygous sickle-cell trait and hemoglobin C β-globin genotype

SCC – scientific coordinating committee

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

TNF – tumor necrosis factor

Tsat – transferrin saturation

UIBC – unbound iron binding capacity

UNC – University of North Carolina at Chapel Hill

UNIMMAP – UNICEF international multiple micronutrient preparation

WBC – white blood cell

WHO – World Health Organization

CHAPTER ONE: INTRODUCTION TO ERYTHROCYTE ALTERATIONS WHICH IMPACT FALCIPARUM MALARIA INFECTION

1.1 INTRODUCTION

Malaria has been a threat to humankind for centuries, impacting vast swaths of societal function from geographical settlement to war and colonial infiltration, to economic productivity. Still, today an estimated 438,000 people die annually (primarily children under 5 succumbing to *Plasmodium falciparum* in Sub-Saharan Africa) (1). The lost economic cost is substantial, and correlates directly with per capita GDP in the developing world (2). This highlights how much more work needs to be done if we are to win the battle against this formidable infectious disease.

The life cycle of malaria parasites is complex, involving the mammalian host and the mosquito vector. The mosquito injects the sporozoite form of the parasite into the human host. Sporozoites quickly home to the liver and after a brief asymptomatic hepatic stage, merozoites burst out of hepatocytes to enter the bloodstream and begin the intra-erythrocytic infection cycle. During this cycle (which lasts 48hrs for *P.falciparum*), the parasite develops from the ring stage to the metabolically active trophozoites, which then mature into schizonts. When they are completely mature, the schizonts (up to 36 per RBC) burst out of the RBC and again become merozoites. Merozoites remain extracellular for a very brief time before invading new red blood cells (RBCs) and starting the 48 hour cycle again. The most severe clinical symptoms of malaria are associated with this burst of RBCs when the parasites leave one host RBC and move to the next.

There are five species of Plasmodium that infect humans*, P.falciparum, P.vivax, P.malariae, P.ovale*, and *P.knowlesi*. All of these species pass from mosquitos to humans, where they infect first hepatocytes and then RBCs; however they differ in their abilities to invade RBCs. For example, *P.vivax* and *P.knowlesi* infect only young RBCs (reticulocytes) and *P.falciparum* is able to infect RBCs of all ages. In fact, it is this ability to invade RBCs of all ages that makes *P.falciparum* more pathogenic. *P.falciparum*

is endemic throughout parts of Asia, Africa, and Central and South America, however, its highest infection burden falls on Sub-Saharan African children under 5.

There is much to be learned about malaria pathogenesis from uncovering the relationship between the human RBC and the malaria parasite. With drug resistance now arising to even the most important first line artemisinin-based antimalarials (3), and disappointing recent large-scale vaccine trials (the most promising vaccine candidate has been found to only offer roughly 30% reduction in severe malaria in infants and children with limited cross-strain protection and waning efficacy over time (4–7)), we clearly are still in need of new strategies to combat malaria. Turning our attention towards naturally derived alternations in the RBC that confer protection against malaria may provide much needed insight into possible drug development strategies as well as better understanding of malaria pathogenesis and transmission. Of course the immune system, endothelium, and liver also impact malaria infection; however in this introductory chapter, I will focus attention on changes in RBC physiology that impact malaria pathogenesis. One such example is sickle-cell trait, which is so well-known that the average high school student knows it as a biological example of human evolution and selective pressure. I will discuss recent findings on the cellular and molecular mechanisms that help explain how sickle-cell trait and other hemoglobinopathies impact malaria pathogenesis. In addition, I will discuss other common genetic mutations that effect RBC physiology (via RBC enzyme deficiencies and abnormalities in RBC membranes) and thereby impact malaria pathogenesis. Finally, in my own work presented in Chapters 2-5, I will discuss how changes in RBCs secondary to anemia decrease malaria parasite growth and development. My work also raises the interesting possibility that the high prevalence of anemia in people of African descent may not only be secondary to nutritional iron deficiency, but may also be the result a genetic attempt to alter RBC physiology in an effort to adapt to malaria.

Before delving further into discussion of these topics, however, it is important to review the range of clinical presentations of malaria seen in children and non-pregnant adults.

(1) Asymptomatic malaria. This is also sometimes referred to as asymptomatic parasitemia. In many malaria-endemic areas, often significant portions of the population have a relatively low level of parasitemia and they remain asymptomatic. The simplest explanation for this is that many of these people are immune to malaria and are able to fight it enough to keep the parasite

replication in check (8). However, the mechanisms responsible for this non-sterile immunity are unclear. There is also controversy about whether or not patients with asymptomatic malaria should be treated (9).

(2) Uncomplicated malaria. Malaria is considered uncomplicated when symptoms are present, but there are no clinical or laboratory signs of organ dysfunction. Symptoms of uncomplicated malaria are not specific and can include: fever, sweating, general weakness, enlarged spleen, mild jaundice, liver enlargement and increased respiratory rate.

(3) Severe malaria. Malaria is considered severe when it is complicated by serious organ failures or abnormalities in the patient's blood or metabolism. The manifestations of severe malaria can include cerebral malaria, severe anemia due to hemolysis, hemoglobinuria (hemoglobin in the urine) due to hemolysis, acute respiratory distress syndrome (ARDS), abnormalities in blood coagulation, acute kidney failure, metabolic acidosis, hypoglycemia, and hyperparasitemia, where more than 5% of the red blood cells are infected by malaria parasites.

Note that the term parasitemia is not a specific term: It is typically used to describe the simple presence of parasites or to refer to the level of parasite-infected RBCs, but it does not include any clinical information. Hence, people with either asymptomatic or uncomplicated malaria can be referred to as parasitemic.

Why some people develop severe malaria while others do not and how to predict progression to severe malaria remain highly important research topics. One reason children are at greatest risk for severe *P.falciparum* malaria infection certainly is because they have had less exposure and less immunity to malaria, although this does not tell the whole story and the role of acquired immunity can vary greatly depending on the degree of malaria endemicity in a population. Understanding RBC alterations that developed in the human genome in relationship to malaria pressure can shed additional light on the variation in human susceptibility to severe malaria. Much of the evidence for malaria protection due to RBC alterations will be presented below in the context of the aforementioned levels of malaria infection: severe malaria, uncomplicated malaria, and asymptomatic parasitemia. It is important to keep in mind that protection from severe malaria (the most important factor from an evolutionarily life-saving point of view) may not necessarily translate into protection from uncomplicated malaria or parasitemia.

1.2 HEMOGLOBINOPATHIES

1.2.1 Sickle-cell Trait

Human hemoglobin is comprised of two α-globin and two β-globin proteins; the sickle-cell mutation occurs in the gene encoding β-globin, causing a glutamic acid to valine switch at the 6th codon that leads to a sickling hemoglobin phenotype (HbAS in heterozygote form, HbSS for homozygotes) (10). This single nucleotide polymorphism is commonly found in populations of sub-Saharan African descent but is present globally, reaching a prevalence of 18% in some areas and affecting nearly 6 million neonates in 2010 (11). It was first hypothesized by Allison in the 1950s to relate to malaria protection, after he observed an increase in HbAS prevalence in the lowland populations where malaria was frequently transmitted in comparison to the prevalence in the highland areas of Kenya and Uganda where malaria was rare and he observed malaria was less common in people with sickle-cell trait versus those without (12, 13).

Since then, it has been unequivocally shown that the heterozygous HbAS genotype protects against risk of severe malaria by analysis and meta-analysis of several studies (discussed and analyzed in (14–16)). Specifically, studies in West Africa have found sickle-cell trait to afford significant reduction in severe malaria cases (with comparable protection from the sub-types of severe malaria) in the range of 90% (17–20). Meta-analysis of studies totaling over 10,000 patients revealed the odds ratio of severe malaria in HbAS populations to be 0.09, or again a roughly 90% reduction in risk of overall (14). This has been further confirmed by extensive genome wide association studies (21). The impact of HbAS genotype on uncomplicated malaria is also clear; studies have found lower parasite densities and uncomplicated malaria rates in those with sickle-cell trait (22–26). The same meta-analysis showed that HbAS genotype protects against uncomplicated malaria by approximately 30% (14). Less clear is the impact of HbAS genotype on the risk of parasitemia. Several conflicting cross-sectional studies report a range of parasitemia prevalence in HbAS vs HbAA genotype children, from lower prevalence in HbAS (12, 27, 28), to comparable (29–32) or higher (33, 34), which together has been taken to mean that there is insufficient evidence to show that HbAS genotype protects against parasitemia.

There are multiple cellular and molecular mechanisms that may impact the pathogenesis of falciparum malaria in people with the HbAS genotype and the picture is not yet complete despite decades

of research. (1) Effect of HbAS genotype on parasite growth and development: My own work (Chapters 4 and 5) and that of many others has shown that invasion and growth of *P.falciparum in vitro* within HbAS RBCs is reduced in low oxygen tension growth conditions (1%- 5% gas mixture versus candle jar oxygen tension of ~18%) (15, 35–37). Some have proposed that the increased sickling of infected HbAS RBCs may be a mechanism for impaired growth (35, 38, 39), however, more recent research suggests the story may not be that simple. LaMonte *et al.* discovered differential trafficking and effects of host miRNAs from HbAS and HbSS RBCs. Their research shows HbAS and HbSS RBCs have higher levels of certain host miRNAs (which can specifically inhibit translation of parasite enzymes important for growth and development) and treatment with antisense oligonucleotides partially rescued parasite growth (40) in HbAS RBCs. (2) Effect of HbAS genotype on adhesion of parasitized RBCs: Fairhurst and colleagues have shown that HbAS RBCs exhibit aberrant *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) expression on the RBC surface (41). This parasite protein is primarily responsible for cytoadhesion of infected RBCs to the endothelial surface and to other uninfected RBCs (termed "rosetting"), both phenomena being significant contributors to severe disease. Cytoadhesion was found to be reduced in HbAS RBCs *in vitro* (41–43). In fact, altered cytoskeletal properties have been described in HbS-containing RBCs, hypothesized to impact parasite protein trafficking to the RBC surface (44, 45). (3) Effect of HbAS genotype on the immune system: First, it is possible that the reduction in parasite invasion and growth in HbAS RBCs (discussed above) and the subsequently reduced levels of parasitemia would give the immune system time to better combat the infection which could then prevent severe forms of disease (discussed in (15)). Second, it is possible that HbAS genotype favorably alters the host immune response to malaria infection, by increasing the rate of clearance of HbAS infected RBCs or by altering the induction of inflammatory cytokines following endothelial activation (discussed in (15, 46)). Of note, as Mangano *et al.* find and discuss in relation to others' findings, HbS protection from parasitemia is often not evident by single cross-sectional observations but rather requires longitudinal analysis, and trends show enhanced protection with increasing age (46, 47). They suggest HbAS helps protect the host by increasing immunological development against malaria and further hypothesize that altered PfEMP1 display, which increases the number of infected RBCs in circulation (by reducing cytoadherence), might increase display to immune cells (46). In fact, others have found enhanced phagocytosis of HbAS

infected RBCs versus HbAA infected RBCs (48). In conclusion, the impact of HbAS genotype is multifactorial with several of the aforementioned mechanisms working together to alter the pathogenesis of falciparum malaria (discussed in (15)).

However, this benefit comes with a cost. Individuals with the HbAS genotype are usually asymptomatic, but can develop clinical symptoms similar to a sickle cell crisis during extreme circumstances such as dehydration. In contrast, individuals with the homozygous HbSS genotype suffer from the physiological effects of sickling RBC deformations: microvascular obstruction and hemolysis leading to many downstream negative health effects, frequent pain crises, and reduced lifespan (reviewed in (10)). The widespread prevalence of HbAS despite the cost of HbSS inheritance demonstrates the profound advantage given by HbAS in the face of malaria infection.

1.2.2 Hemoglobin C

The Hemoglobin C mutation (HbAC for heterozygotes; HbCC for homozygotes) is very similar to sickle-trait, in that it involves a point mutation at the same position in the gene encoding β-globin, however it causes a different amino acid substitution (glutamic acid to lysine) at the 6th codon. It is also most common in populations in West Africa, with prevalence reported as high as 15% in parts of Burkina Faso, though outside of that epicenter the prevalence diminishes accordingly (49). For severe falciparum malaria, evidence of protection by the hemoglobin C variant is clear. Homozygous HbCC individuals show 80% reduction in the risk of severe malaria and heterozyogous HbAC people show 30% reduction (18, 50–52). Meta-analysis of 4 case-control studies revealed odds ratio of severe malaria to be 0.27 in HbCC and 0.83 in HbAC individuals compared to normal genotype children (14) which is on par with these results. As further strong evidence for overall protection, a recent genome-wide association study (GWAS) concluded that for each copy of the HbC allele, risk for severe malaria was reduced by 29% (21).

For uncomplicated malaria, the picture is muddled: some studies show protection for HbAC and HbCC (24, 50, 53), but others do not (52). A meta-analysis of available studies concluded protection from uncomplicated malaria is as yet unknown in terms of HbC mutations (14). Similarly there is no conclusive evidence that HbAC or HbCC provides protection against parasitemia (14, 24, 32, 34, 54). The data on protection against parasitemia is particularly confusing and further research is needed. A recent study in Malian children actually found 15% increased risk of clinical malaria incidence in HbAC individuals (26).

However, another recent longitudinal cohort study in Malian children found reduced risk of both uncomplicated clinical malaria and lower parasitemia burden in HbAC individuals (55) and a recent cross-sectional study from Burkina Faso found significant protection from parasitemia in HbCC and HbSC individuals (odds ratio 0.04 versus HbAA), even more so than for HbAS (odds ratio 0.27), with no differences in parasitemia for HbAC versus HbAA individuals (46).

The molecular mechanisms of protection from malaria following HbC inheritance are again not fully elucidated, but, again, the same three areas of (1) parasite growth and development; (2) adhesion of parasitized RBCs to endothelial cells; and (3) immune system changes, have all been implicated. *In vitro* studies indicate reduced growth in HbCC RBCs (56–58), with some further evidence of normal invasion but reduced schizont lysis and merozoite egress (58), while growth in HbAC RBCs has been reported to be normal (56, 58). Similar to results with the HbAS genotype, both the HbAC and HbCC genotypes can result in altered PfEMP1 expression on the infected RBC surface (41, 42). Further microscopic analysis has revealed an altered cytoskeleton in RBCs from donors with HbAC genotype which was hypothesized to impair trafficking of parasite proteins to the RBC surface (44, 45). The impact of the HbC variants on the immune system are even less clear. Thus, exact mechanisms for protection provided by the HbCC and HbAC genotypes remain an open question (15, 59).

Clinically, the tradeoff of inheriting the most protective form, HbCC, is mild hemolysis and anemia, while those with the HbAC genotype are asymptomatic. More significant clinical issues tend to arise with HbC co-inheritance with HbS or β-thalassemia (60).

1.2.3 Hemoglobin E

The Hemoglobin E mutation encompasses a point mutation that results in a glutamic acid to lysine switch at position 26 of the β-globin gene. It is most commonly found in parts of Southeast Asia and India, even reaching prevalence of up to 60% in some areas (60).

Hemoglobin E protection against malaria is not well characterized and the available data is contradictory. One study examining parasitemia levels and clinical severity in malaria cases in Myanmar found no distinctive protective effect from HbAE (61), whereas another study looking at prevalence of HbAE in malaria cases from Thailand did find the HbAE genotype associated with a reduced odds of severe malaria by almost seven-fold (62). A further study in India examining malaria cases did find

reduced prevalence of HbAE and HbEE individuals compared to prevalence of these people in the population as a whole (63).

Little has been done to characterize the mechanisms by which HbE might contribute to reduced parasitemia or malaria protection. Again, three broad categories of effects come forward: reduced parasite growth and development, altered adhesion of parasitized RBCs to endothelium, and impact on the immune system. Indeed, *in vitro* studies of HbEE and HbAE RBCs have sometimes found reduced invasion (in HbEE (64, 65) and HbAE (65)) and growth (in HbEE (66) and HbAE (67)), sometimes not (in HbEE (68) and HbAE (64, 66, 68)), reviewed in (15). Clearly more work needs to be done to answer these questions.

The trade-off for harboring the HbE trait seems to be minimal, with mild anemia resulting from lower levels of expression of the β-globin gene due to the genetic mutation resulting in a splice site insertion that reduces mRNA stability and translation (reviewed in (15))

1.2.4 Thalassemias

Human hemoglobin is formed by a complex of two α-globin and two β-globin chains which are encoded by two alpha-globin genes (*HBA1* and *HBA2*) and one B globin gene (*HBB*). The term 'thalassemias' collectively refers to a number of different genetic mutations that result in reduced or absent expression of one or more of these globin alleles. Specifically, individuals described as having 'α-thalassemia' can have the loss of one or more α-globin allele(s): αα/α- (heterozygous), α-/α- (homozygous), or αα/-- (loss of both paired α-globin gene, also homozygous); there is also HbH disease (loss of 3 α-globin alleles, α-/--) and finally hydrops fetalis (loss of all 4 α-globin genes; incompatible with life). Individuals with mutations in the beta globin gene(s) can also have a range of genetic defects, including β-thalassemia minor (reduced expression of one β-globin allele), and β-thalassemia major (reduced expression of both β-globin alleles) (reviewed in (15)). Geographically, the thalassemias can be found worldwide, with mild α^{+}-thalassmias reaching prevalence of 10-20% throughout a belt spanning Sub-Saharan Africa, the Middle East, India, and Southeast Asia. β-thalassemia is predominantly prevalent in the Mediterranean and a few parts of Sub-Saharan Africa (see (15, 59)).

There is clear protection from malaria associated with each of the thalassemias. In fact, the original "malaria hypothesis" or "Haldane's hypothesis" connecting genetic hemoglobin alterations to

geographic malaria prevalence was formulated by Haldane in the 1950s as a result of his observations on the presence of β-thalassemia in West Africa (69).

To begin with α-thalassemia protection, meta-analysis of studies which examined prevalence of α-thalassemia in children with severe malaria and healthy children found an odds ratio for severe malaria of 0.63 for homozygote α-thalassemia and 0.83 for heterozygotes (14, 18, 70–72). Interestingly, such protection from severe malaria is eliminated by co-inheritance of the HbAS allele, perhaps providing an explanation for why α-thalassemia mutations are not as prevalent in many parts of Africa (26, 73). When it comes to protection from uncomplicated malaria, consensus is less clear – some studies found decreased incidence of malaria with any form of α-thalassemia (though consistently lower with homozygote versus heterozygote form) (73, 74), while others actually found no difference (75) or increased incidence (76) compared to normal genotype. Meta-analysis concluded there was no risk reduction of uncomplicated malaria for α-thalassemia carriers (14). Finally, there also seems to be inconclusive evidence of asymptomatic parasitemia protection with α-thalassemia, both in terms of presence and density of parasites – but most studies do not find an effect on parasitemia (75, 77, 78).

There are far fewer comprehensive studies relating to β-thalassemia malaria protection; in fact the majority of available evidence comes from 2 studies, one finding uncomplicated malaria cases were less likely to involve people with β-thalassemia (79) and the other finding upon population screening similar parasitemia prevalence amongst those with β-thalassemia as in those with normal genotype, albeit lower parasite densities were observed in those with β-thalassemia (80).

As with most of the aforementioned hemoglobinopathies, the molecular mechanisms by which the thalassemias protect against malaria disease are less clear than the clinical protection itself.

(1) Effects on growth and development. Minimal changes have been observed in invasion and growth with heterozygote α-thalassemia (64, 81–83). Similarly for RBCs from individuals with homozygote α-thalassemia, studies show conflicting and thus inconclusive evidence regarding growth and invasion differences (64, 82–85). RBCs from those with HbH disease more consistently exhibited reduced growth and invasion (65, 67, 82). The same phenomenon holds true for β-thalassemia – largely no invasion or growth differences have been observed (64, 84) except one study finding reduced growth (67). The exception is significantly reduced growth when examining RBCs missing either one copy of α-globin or β-

globin when cultivated at high oxygen tension (83). Interestingly, invasion is frequently observed to be reduced in RBCs harboring both a β-globin thalassemia mutation and HbE (64, 65, 67) and reduced growth has been observed here as well (81) (all reviewed in (15)). To explain these findings, it was predicted that the thalassemias resulted in loss of ability to counter oxidative stress (15, 83).

(2) Effects on endothelial cell adhesion. Others observed reduced rosetting with RBCs from individuals with α-thalassemia and β-thalassemia and also reduced binding of α-thalassemia and β-thalassemia RBCs to endothelial cells (81, 86–88), although the findings of reduced cytoadhesion and PfEMP1 expression with α-thalassemia is conflicted and inconsistent (43, 85, 89). Interestingly, as mentioned above, there is a reduction in protection against malaria in individuals who carry both HbAS and α-thalassemia (18, 23, 73). Investigating this phenomenon further with *in vitro* studies, Opi *et al.* found that co-inheritance abrogated the reduced PfEMP1 expression, cytoadhesion, and rosetting typically observed with HbAS. The mechanism for this remains unknown, though there was some speculation it might have to do with α-thalassemia reducing the levels of HbS production, perhaps especially if the hypothesis that HbS affects intracellular PfEMP1 and other membrane protein trafficking in infected RBCs is true (43).

(3) Effects on the immune system. Studies have found that anti-malarial immunoglobulins bind thalassemic infected RBCs more than normal infected RBCs (84, 89) and undergo increased phagocytosis (48), which may result in enhanced immune clearance of parasitized RBCs from individuals with thalassemias (discussed in (15)).

The drawbacks of maintaining the thalassemic genetic mutations within a population can be significant. For example, hydrops fetalis (missing all α-globin genes) is incompatible with life and HbH disease (missing 3 copies of α-globin) as well as β-thalassemia major result in severely reduced lifespan with chronic and severe anemia and other hematological disruptions. β-thalassemia minor and the α-thalassemia traits are much less consequential, and are often asymptomatic, only producing mild anemia (15).

1.3 ENZYMATIC DEFICIENCIES

1.3.1 Glucose-6-Phosphate Dehydrogenase Deficiency

Glucose-6-Phosphate Dehydrogenase (G6PD) catalyzes the first step in the pentose phosphate shunt, which ultimately produces NADPH, a reducing agent. NADPH is a critical co-factor for glutathione reductase which generates the reduced form of glutathione and is critical for lowering oxidative stress in RBCs.

X-linked genetic mutations causing G6PD deficiencies are among the most common genetic mutations globally and affect an estimated 400 million people (reviewed in (90)). Given the distribution of mutations throughout Africa, the Mediterranean, Middle East, and Asia, and their independent development in multiple distinct populations, G6PD deficiency has been hypothesized to have arisen due to malaria pressure (discussed in (91, 92)). Yet, despite this long standing hypothesis and a large number of studies, there is quite conflicting evidence about whether or not G6PD protects against falciparum malaria infection. There is a great deal of controversy surrounding the question of whether or not heterozygous and homozygous females are protected, and even the previously commonly held belief that hemizygote males are protected is no longer clear cut. Even very recent comprehensive studies report nearly the exact opposite results: that heterozygote females are the only ones protected against severe malaria (21, 93, 94) or that hemizygote males and homozygote females are the only ones protected against severe malaria (95).

A descriptive review of the dozens of studies that have been done to date to examine the question of whether or not G6PD deficiency protects against clinical malaria is beyond the scope of this chapter; instead, I will discuss some of the reasons why these studies have often conflicted. There are the typical issues: many of the studies have relatively small sample sizes, vary in the immune status of the subjects, and some are village surveys while others are hospital studies. This is all further complicated by the tremendous diversity in the G6PD gene, which results in a large diversity of enzyme levels – even amongst donors with the same genetic variant of G6PD, there is diversity in the levels of enzyme activity. In addition, since the gene is carried on the X chromosome, X inactivation of one allele in the RBCs results in a heterogeneity within the RBC population of heterozygous females. Finally, there are a wide variety of inconsistencies in the methodologies used to characterize G6PD deficiency (genetic

sequencing versus analysis of enzymatic activity) in the epidemiological studies linking malaria and G6PD deficiency. All of the aforementioned differences and complications are compounded by the fact that many of the different studies use different definitions of and categories of clinical malaria, which makes it difficult to draw definitive conclusions and compare studies, not to mention practically impossible to systematically review this research area.

The human gene for G6PD contains 13 exons and encodes 515 amino acids. G6PD is a monomer, but the active enzyme is a dimer which requires both NADPH and FAD (riboflavin) for its activity. Over 140 mutations have been identified (90, 96, 97) that each result in various degrees of change in the enzymatic activity and over 400 variants have been characterized that have normal levels of G6PD activity (98). To make things more confusing, all of the 400 variants with normal G6PD levels are considered wild type and are referred to as G6PD B. One common variant, which is found in 20-30% of Black Africans and is therefore often discussed in studies linking malaria and G6PD deficiency, is referred to as G6PD A+. G6PD A+ has a single amino acid substitution at position 376 of an asparagine for aspartate (98). Another common variant often referred to in the malaria literature is G6PD A- and it is found in 10-15% of African-Americans. All G6PD A- variants have a mutation at nucleotide 376 (A→G), which also is the nucleotide substitution characteristic of G6PD A+. However, the G6PD A- variants have a second mutation, which is usually at nucleotide 202 (G→A) or at nucleotide 680 (G→T) or at nucleotide 968 (T→C) (98, 99).

The World Health Organization has classified the different G6PD variants into clinically important categories according to the magnitude of the enzyme deficiency and the severity of hemolysis associated with each variant (100). Class I variants, which are rare, have severe enzyme deficiency (less than 10% of normal) and have chronic hemolytic anemia. Class II variants also have severe enzyme deficiency, but there is usually only intermittent hemolysis associated with infection, drugs, or chemicals. Class III variants have moderate enzyme deficiency (10-60% of normal) with intermittent hemolysis usually associated with infection, drugs, or chemicals. Note that most G6PD A- variants fall into Class III. Class IV variants have no enzyme deficiency or hemolysis. Class V variants have increased enzyme activity (98).

The proposed mechanisms for G6PD deficiency protection against malaria infection are unclear. Unfortunately, the literature on this topic, too, is plagued by the same issues discussed above for the

clinical studies and there are many conflicting results. As reviewed by Ruwende and Hill, there is evidence for two main ways which the increased oxidative stress in G6PD deficient RBCs may protect against malaria: (1) it causes increased Heinz body formation and methemoglobin formation which results either RBC lysis or enhanced phagocytosis of infected RBCs, (2) it decreases the rate of intra-erythrocytic parasite replication (91). Certainly it is logical that reduced parasitemia could be the protective mechanism against severe clinical malaria.

To go into more detail: some original studies do indicate decreased parasite growth in G6PD deficient RBCs (101–104) though not decreased invasion (103, 105), but others did not find reduced growth (105), or only observed decreased growth with oxidative stress present (83, 102). One study looking at growth in individual RBCs from heterozygote women in which X-inactivation occurs found decreased growth in the G6PD deficient RBCs specifically (106). Indeed, as G6PD deficiency reduces a RBC's ability to combat oxidative damage, and with evidence that oxidative stress reduces parasite growth (107) (especially in G6PD deficient RBCs (108)), this is a plausible link. Others found no difference in parasite invasion or maturation in erythrocytes harboring G6PD deficiency, but that infected RBCs were more susceptible to phagocytosis due to increased expression of phagocytic markers on the G6PD deficient infected RBCs (109).

But there remains a confusing point about reduced parasitemia in G6PD deficient RBCs being protective, as reviewed by others (92): studies finding G6PD deficient RBCs support less parasite growth imply both hemizygote males and heterozygote females should be protected, although this has not consistently been the case in the clinical literature, where some have found only heterozygote females are protected (21, 93, 94). Guindo *et al.* similarly point out that speculated mechanisms of protection of increased phagocytosis or reduced pathogenesis in G6PD deficient RBCs would be expected to have increased protection in those "uniformly" deficient (i.e. hemizygote males or homozygote females), not heterozygote females (95). As a potential explanation for this conundrum about why some clinical studies find heterozygote females are most protected, Ugayo *et al.* speculate that increased severe malaria infection rates in G6PD deficient hemizygote males observed in some studies may have to do with increased potential for oxidative damage (induced by infection) resulting in increased RBC lysis and anemia. They then speculate protection in heterozygote females might result in part from protection by

normal G6PD status cells resisting oxidative stress/lysis, and in part from the presence of X-inactivated G6PD deficient RBCs that reduce the portion of habitable RBCs (93). Others have conducted research finding replication rates in G6PD deficient RBCs only remain low for a few cycles, surmising that the parasite can adapt to G6PD deficient conditions, thus speculating heterozygote females would maintain the most protection because there would not be enough pressure to induce parasite adaptation (103, 110).

Overall, much like the clinical outcomes, there is substantial conflicting evidence and still a great deal left to be learned about the protective molecular mechanisms of G6PD deficiency. It is entirely likely that several factors combine to mediate protection. Very likely much of the confusion and conflicting evidence of malaria protection in the G6PD literature arises because of the heterogeneity of the G6PD mutations themselves, not to mention the mutations' inconsistent impact on overall enzymatic activity within an individual, and furthermore the complications of X-inactivation in females.

The clinical consequences of G6PD deficiency include increased risks of neonatal jaundice and of extensive hemolysis induced by excess oxidative stress. The most common triggers for hemolysis in this patient population are medications (including some gametocytic antimalarials), particular foods (most famously fava beans), and several types of infections (reviewed in (90)).

1.3.2 Pyruvate Kinase Deficiency

Pyruvate kinase (PK) deficiency, the other enzymatic deficiency that has been found to associate with malaria protection, deserves mention, although it is much less well studied. This enzyme is involved in the rate limiting step of glycolysis and is thus very important in energy production in RBCs, which lack mitochondria. Several mutations are known in humans (111), though the consequences of being homozygous are severe (hemolytic anemias requiring transfusions), making homozygotes rare (discussed in (111, 112)). Ayi and co-workers studied *P.falciparum* growth in PK-deficient RBCs and found reduced invasion and enhanced phagocytosis, speculating that heterozygote individuals might have an advantageous protection against malaria with relatively little negative health effects (112). Mouse studies have found protection with PK deficiency against malaria (113, 114), as have *in vitro* studies using RBCs from PK deficient donors, where a mechanism of decreased ATP levels was proposed to impact parasite growth (115). Clinical research has also found reduced numbers of PK deficient individuals when

examining *P.falciparum* cases in Thailand (116), again suggesting this is yet another human genetic variant that is likely related to malaria pressure, and will result in further studies.

1.4 RBC MORPHOLOGY AND THE CYTOSKELETON

Another important type of RBC change involves membrane defects which affect the RBC shape. There are several different kinds of these genetic mutations; most frequently mutations are seen in cytoskeletal components and associated membrane proteins such as spectrin or band 3 or 4.1 proteins, although they can also be seen occasionally in the glycophorins as well (discussed in (117–119)). The network of proteins making up the RBC cytoskeleton and junctional membrane tethering sites is complicated to visualize. Essentially, there is a lattice-like structure underlying the RBC membrane made up of primarily of α- and β-spectrin heterodimers which associate end to end as tetramers. There are also clusters of transmembrane proteins and other associated proteins that bridge the gap between binding the spectrin-based sub-membrane network and the membrane lipid bilayer. There are two main complexes of proteins: the ankyrin complex (consisting of predominantly band 3 tetramers, protein 4.2, GPA, CD47, and Rh linked via ankyrin to the spectrin heterodimer junction sites), and the 4.1R complex (consisting predominantly of 4.1R, F-actin, tropomysin, and band 3 dimers, as well as GPC, Rh, DARC, and Kell linked to the sites of association between the actin cytoskeleton and the subsurface spectrin mesh-like lattice). These protein interactions between membrane and cytoskeleton largely determine RBC shape and deformability and ability to circulate efficiently in the microvasculature (124, 125).

Multiple categories of these morphological RBC disorders exist. This includes:

1) Hereditary elliptocytosis (a heterogeneous disorder in terms of genetics and manifestation, ranging from severe hemolytic anemia to absence of symptoms; most mutations arise in spectrin and cause issues with spectrin dimer and tetramer associations or junctional associations (119–121))
2) Hereditary pyropoikilocytosis (a severe hemolytic anemia type of hereditary elliptocytosis, often from a spectrin deficiency (120, 121))
3) Hereditary spherocytosis (another heterogeneous cytoskeletal defect in spectrin, ankyrin, band 3, or protein 4.2, and the most common inheritable cause of hemolytic anemia in Caucasians (118, 119, 122))

4) Southeast Asian ovalocytosis (SAO, a disorder characterized by RBCs with oval shape and decreased deformability, possibly linked to a deletion in band 3 protein, and occurring at quite high rates in certain geographic areas (118, 119, 123)).

Most of these disorders are inherited in an autosomal dominant fashion (118–120) and are classified not based on genetics, but phenotypes. Generally speaking, mutations causing these RBC disorders result in reduced integrity of the RBC cytoskeleton, such that the RBC loses its biconcave shape and appears morphologically different by microscopy. Speculation that some of these conditions evolutionarily arose due to malaria pressure first arose due to the observed prevalence of hereditary elliptocytosis in people of African descent and areas where malaria was traditionally endemic. For example, hereditary elliptocytosis was determined by molecular epidemiology to be 30 times more prevalent in black people than Caucasians (117, 125, 126). Prevalence of hereditary elliptocytosis reached levels as high as 1.6% in Benin (126), and the prevalence of hereditary ovalocytosis can reach 35% in parts of Southeast Asia (117, 127). Overall there are several different cytoskeletal genetic mutations, especially in Africa, making it difficult to pinpoint exact correlations with predicted malaria disease protection. However, as discussed, it is hypothesized that these types of mutations are highly prevalent (particularly alpha spectrin haplotypes) and were maintained in human populations because they protect against malaria (117, 120).

Not all is understood, but there is clear evidence of malaria protection both in patients and *in vitro*. Clinically speaking, most of the documented association with protection comes from case studies with Southeast Asian Ovalocytosis, where reduced severity and prevalence of disease as well as reduced parasitemia was found (128–132). Protection against severe malaria was often profound (sometimes with odds ratio of 0 reported due to absence of severe malaria cases amongst those with the band 3 deletion) (128, 130, 131), and protection against malaria was not just afforded to falciparum but also vivax and malariae (129, 131, 132). Ovalocytosis was also found to reduce parasite growth *in vitro* (133–135). Despite little published evidence of clinical protection with the other types of morphological disorders, *in vitro* studies with *P.falciparum* have suggested reduced parasite growth in RBCs from people with mutations such as hereditary spherocytosis and elliptocytosis (134). Dhermy *et al.* studied falciparum growth in RBCs harboring different elliptocytic spectrin mutations and found overall significantly reduced

growth, altered development, and reduced invasion, and that the growth and invasion deficits were more apparent with each cumulative replication cycle (117). This is similar to what others observed, finding severely decreased growth and invasion in elliptocytes with spectrin mutations (121, 134), as well as in elliptocytes with 4.1 mutations (134, 136). As Facer speculated, the relationship between parasite growth deficiencies and hereditary elliptocytosis could very plausibly deal with RBC invasion inhibition, either for mechanical reasons (reduced deformability, stability, endocytosis), or due to cytoskeletal mutations having downstream effects such as altered sialic acid presentation on membrane glycophorins important for merozoite attachment and entry (121). Similarly, SAO RBCs are known to be less deformable (137), and Gallagher discusses the likelihood of reduced merozoite invasion playing a role in ovalocytosis protection, either from poor band 3 binding or inability of band 3 to properly cluster and allow for membrane and cytoskeletal dissociations necessary for the parasite to enter the RBC (120). Certainly there are many plausible links between cytoskeletal defects and reduced parasite growth or ability to cause disease, which will continue to be elucidated with further study.

For most people harboring cytoskeletal mutations, these morphological RBC changes cause few problems and remain asymptomatic throughout their lifetime, however there are some cytoskeletal mutations where hemolytic anemias and splenomegaly occur as the less deformable mutant RBCs get “caught” in the microvasculature of the spleen (118). Given the commonalities between so many other types of RBC mutations arising in malaria endemic areas and the realization that they afford malaria protection, the likelihood of these cytoskeletal defects having a similar evolutionary role in terms of malaria protection is clear. This is further backed up considering many plausible links between parasite pathophysiology and the impact of cytoskeletal defects, such as potential problems with RBC invasion, trafficking of parasite proteins to the RBC surface, or altered membrane antigens and deformability. Indeed the case of Southeast Asian Ovalocytosis is especially compelling. Autosomal dominance is so severe that only heterozygosity is compatible with life – and yet the prevalence of SAO remains high, indicating it must exist to confer evolutionary benefit (120).

1.5 BLOOD GROUPS

1.5.1 ABO Blood Groups

The ABO blood groups are most commonly discussed in the context of blood typing to determine compatible blood transfusion donors. Blood type A, B, and AB contain A and/or B antigens on the surface of RBCs which blood type O RBCs lack. Specifically, RBCs with A and B antigens express trisaccharides and RBCs with O antigens express disaccharides because individuals with Type O lack a specific glucotransferase enzyme that adds the third sugar on to the trisaccharide (138). All blood group types exist globally (albeit at different frequencies), so perhaps the connection to malaria protection is less intuitively obvious than many of the RBC mutations presented above. Early studies were conflicted: some researchers found significant associations with severe malaria in blood group A, B, and/or AB, and/or that type O was protective (139–141); others did not find an association between blood group type and malaria severity (142–144). A 2007 systematic review concluded there were poor controls and small sample sizes but likely a real association with ABO blood group type and malaria susceptibility (145). Since then, more comprehensive studies have taken place, namely (146, 147), more definitively indicating type O individuals are less susceptible to severe malaria and non-type O individuals are at increased risk in Sub-Saharan Africa and India (146–153). The correlations appear to be strongest between severe clinical disease outcomes and blood type, not simply parasitemia levels or uncomplicated infection. The protection is significant, with odds ratio for increased risk of severe malaria in non-O type blood groups ranging from 1.26 – 2.95 ((146, 147, 152)and reviewed in (146)).

In investigating the molecular pathophysiology behind differential susceptibility to severe malaria in the case of the ABO blood groups, the consistently arising factor is reduced rosetting. Rosetting refers to infected RBCs adhering to and clumping with uninfected RBCs in the bloodstream. This contributes to disease pathology through inflammation and obstruction of blood flow in small vessels, with rosetting overall shown to correlate with severe disease (reviewed in (154)). In the case of the ABO blood group, many *in vitro* studies examining rosetting potential of different blood types found larger and more stable rosettes, as well as increased percent of infected RBCs forming rosettes, in non-O blood types (81, 155–158). It was found that parasite protein PfEMP1 exported to the infected RBC surface binds to the A and B blood group antigens to increase rosetting susceptibility (158, 159). And in fact, this has been backed

up clinically by correlative case control studies examining rosetting frequency in parasites harvested directly from infected individuals with type O and non-type O blood (146). Others report increased hemolysis of infected RBCs with type O blood (153), so perhaps there are additional mechanisms that could contribute to malaria protection, but overall the evidence seems quite conclusive that blood group O benefits from relative protection against severe malaria due to reduction in rosetting potential of infected RBCs. The obvious question thus remains, why is blood group O not nearly universally present in areas of high malaria endemicity? As discussed in Rowe *et al.* (160), this condition, too, is likely under balancing selection, as there is evidence of increased association of other infectious agents (i.e. cholera, *E. coli*) with type O blood group (161, 162).

There is also some indication of altered rosetting susceptibility in certain Knops groups (which comprises various antigen presentations on the complement receptor (CR1) molecule), as well as altered immune complex formation and removal, though the data is quite preliminary (reviewed in (160)). Blood groups affecting the presence of glycophorin B and C have also been preliminarily speculated to relate to malaria susceptibility, but little has been as of yet investigated (reviewed in (160)).

1.5.2 Duffy Antigen Receptor for Chemokines (DARC)

Although *P.vivax* malaria is not the focus of this chapter, perhaps one of the most compelling pieces of evidence for RBC evolution against malaria is the Duffy antigen receptor for chemokines (DARC) found on the RBC surface. DARC is nearly totally absent from individuals in West and Central Africa. Molecularly speaking, the Duffy blood group is determined by two co-dominant alleles (Fy*A and Fy*B) coding for FyA and FyB proteins. The phenotype found across most of Sub-Saharan Africa is FyA-FyB-, and this results from a mutation in the promoter of the DARC FY gene which affects GATA-1 transcription factor binding – specifically, the GATA-1 transcription factor for erythroid lineage cells (thus expression of DARC in other tissues such as endothelium is unaffected) ((163) and reviewed in (160, 164)). There are also mutations found in Papua New Guinea resulting in essentially heterozygotic deficiency of DARC (FyA null) in a small population (165).

The association between vivax malaria protection and African populations originally arose from work in the United States where researchers were investigating infection of malaria species with RBCs of various blood groups, finding that FyA-B- RBCs from African Americans resisted infection with the non-

human primate parasite *P.knowlesi* (166). Subsequent research by this group looking at voluntary vivax exposure of inmates (through mosquito feeding) found African Americans with the FyA-B- blood group were resistant, while the Caucasians were not (167). This soon became hypothesized to be the reason *P.vivax* was largely absent from the African continent (reviewed in (168)). As further clinical evidence of the association between Duffy antigen presence and vivax susceptibility, it has been determined that those populations in Asia with essentially half the amount of Duffy antigen (FyA null) are also approximately half as at risk for vivax infection and less likely to have severe disease or high levels of parasitemia ((165, 169) and reviewed in (160)). Though *P.vivax* cell culture remains impossible to this day, it was eventually determined that closely related knowelsi parasites require the Duffy antigen for merozoite tight junction formation for invasion of RBCs (170). And although extensive *in vitro* vivax studies are restricted, inhibition of invasion has been shown with the presence of antibodies targeting the vivax parasite's Duffy antigen binding protein ((167, 171, 172) and reviewed in (160, 168)). This provides a very clear mechanism for the relationship between Duffy antigen absence and vivax absence in much of Sub-Saharan Africa.

Despite the seemingly obvious connection for the existence of Duffy negative blood groups (established at near 100% frequency in most of Africa) to have arisen as an evolutionary benefit to resist *P.vivax* infection, this association has still been called into question (reviewed in (168, 173)). For one thing, some researchers point out that vivax malaria (at least in its current form) is rarely as severe or lethal, questioning how such a strong phenotype regarding the existence and prevalence of the Duffy antigen negative blood group could have arisen from evolutionary drive for a comparatively weak disease. However, the notion of vivax being a rather "benign" form of malaria is also a point of recent debate, with many arguing vivax infection takes a significant clinical toll even if it is not as deadly, plausibly enough to drive evolutionary adaptation for resistance to infection (reviewed in (164, 168, 174–176)). Other evidence has arisen suggesting vivax originated in Asia where it is most prevalent today, which again calls into question how vivax could have possibly influenced absence of the Duffy antigen across most of Africa ((177, 178) and discussed in (173)). Neither is this point uniformly agreed upon, however. In fact, very credible and compelling recent research claims the exact opposite, that vivax malaria originated in

non-human primates in Africa (179). If true, this of course allows for simple explanation of why DARC is absent from most Africans.

Regardless of vivax origin and DARC prevalence, as Howes *et al*. extensively investigate and assess by meta-analysis, the belief that *P.vivax* is totally absent from Africa is far from accurate, with evidence for widespread (albeit quite low level) infection in many areas (180). Vivax existence in Africa is due to a combination of factors: a small population of Duffy positive individuals (particularly in parts of East Africa and Madagascar), possible zoonotic infection, and also parasite exploitation of alternative invasion routes in Duffy negative individuals (reviewed in (180)). In fact, definitive evidence has developed proving Duffy antigen negative individuals can support vivax infection – coming from such diverse regions as Mauritania, Equatorial Guinea, Cameroon, Ethiopia, Angola, Madagascar, Kenya, and even Brazil (reviewed and mapped in (168, 180)), which is cause for renewed scrutiny of vivax burden potential in Africa.

1.6 NEW AREAS OF RESEARCH REGARDING CHANGES IN RBC PHYSIOLOGY

1.6.1 Nutritional Immunity, Iron Deficiency, and Anemia

A newer area of research, at least in terms of framing it in the context of RBC alterations that impact malaria disease severity, has to do with nutritional immunity. Nutritional immunity refers to the host withholding essential nutrients from invading pathogens; often these nutrients are crucial growth or virulence factors for pathogens and the host gains an advantage by devising ways to make them less accessible. Though the term nutritional immunity can encompass several nutrients, particularly metal ions such as iron, zinc, copper, and manganese (reviewed in (181–183)), in the context of malaria, iron is central (183–185). In the bacterial field, the host-pathogen competition for iron is well-known to be a molecular arms race. To make it less freely available, the host binds iron in the blood and other secretions (either directly or as part of heme/hemoglobin, via proteins such as transferrin, lactoferrin, hemopexin, and haptoglobin), stores it in tissues (via the iron storage protein ferritin), and regulates dietary absorption and body storage (via inflammation-induced hepcidin signaling). Bacteria have devised several ways to sequester back iron from transport molecules or access intracellular iron stores. As examples, several bacteria express siderophore proteins or transferrin binding proteins that can chelate iron away from transferrin or access transferrin-bound iron in the blood. However, humans have further

evolved to possess the siderocalin/lipocalin2 protein which can sequester bacterial siderophores. And yet still, some bacteria have developed stealth siderophores to avoid siderocalin binding (reviewed in (186)). Such strong evidence of back and forth evolution over iron access is also seen with findings of specific, targeted, point mutations developing over time in host transferrin and bacterial transferrin binding protein (to either evade or improve binding each other, depending on the organism's goal) (187). Clearly, iron access can play an important role in human and pathogen evolution.

Beyond bacteria, iron is also a well-known growth factor for malaria (reviewed in (188)). Iron of course also directly effects erythropoiesis and is essential for hemoglobin formation. With growing epidemiological evidence over the past decades that iron deficient people are more resistant to malaria infection (189–192) and iron supplementation possibly puts them at increased risk for infection (193–197), the idea of nutritional immunity playing a role in malaria susceptibility is very plausible. Given the geographic overlap between prevalence of iron deficiency and malaria endemicity in the developing world, there exists a public health conundrum of how to best treat anemia (itself not an inconsequential health deficit that can significantly affect child development and pregnancy outcomes), if iron deficiency is protective and iron supplementation increases malaria risk. Yet, evidence from these epidemiological studies is inconclusive and questions remain about exactly how iron deficiency and iron supplementation may impact malaria pathogenesis. Iron is not just a growth factor for malaria; iron deficiency significantly impacts several physiological properties of the RBC and hence could alter malaria infection susceptibility in many ways.

To better address this question, we have worked to model *in vitro* the growth of malaria in RBCs from relevant donor populations. The crux of this dissertation thus involves investigating the degree of protection of iron deficiency anemia and the safety of iron supplementation via *in vitro* characterization of infection and growth parameters of the malaria parasite in RBCs from iron deficient individuals before and after iron supplementation. This research is directly applicable to the concept of RBC alterations conferring resistance to malaria and contributes to our broader understanding of malaria pathogenesis.

1.7 CONCLUSIONS

In focusing on human RBC factors contributing to malaria infection, admittedly missing from this discussion are potential linkages of malaria protection to human genetic alterations and physiology

beyond the RBC. This would include immunity and inflammatory related factors, such as HLA haplotypes and MHC complexes (198, 199), TNF-α (200), CR1 (201), NOS2 (202), CD40L (203), and IL22 (204), or factors that affect binding of infected RBCs to the endothelium like CD36 (205), ICAM-1 (116), and EPCR (206) variants – all of which have been published as potentially affecting malaria susceptibility. Nor have I discussed new areas of research involving GWAS looking for novel RBC or other factors associated with reduced malaria severity that are as of yet unidentified or underappreciated.

Still, as is clear from all of the above evidence, humans and *Plasmodium* species have been at war for centuries, and one of the most important battlegrounds is indisputably the human RBC. The work in this dissertation was designed to better understand the interaction of the falciparum malaria parasite with iron deficient and anemic RBCs, a relatively new line of interest in the long standing quest to characterize parasite-RBC interactions in the context of modified human physiology and genetic adaptations. Of further importance, the research described in this dissertation also addresses the important public health issue of how to safely correct anemia in malaria endemic areas. Better understanding of malaria pathogenesis in the face of altered RBC physiology will ultimately help improve our strategies to combat the disease.

1.8 DISSERTATION OUTLINE

To orient the reader, the work presented in the remainder of this dissertation includes:

Chapter 2: Initial *in vitro* modeling using UNC-based blood donors to examine how iron deficiency and iron supplementation conspire to mediate host susceptibility to erythrocytic stage malaria infection;

Chapter 3: Development of improved long-term RBC storage methods for *in vitro* falciparum culture in order to better utilize valuable RBC samples, in anticipation of initiating field work using blood donors enrolled in an iron supplementation clinical trial;

Chapter 4: Evaluation of the degree of protection afforded by iron deficiency anemia and the safety of iron supplementation through *in vitro* erythrocytic stage infection modeling, using RBCs from anemic Gambian children enrolled in an iron supplementation trial;

Chapter 5: Evaluation of the degree of protection afforded by iron deficiency anemia and the safety of iron supplementation through *in vitro* erythrocytic stage infection modeling, using RBCs from pregnant Gambian women enrolled in an iron supplementation trial; and

Chapter 6: Summary and discussion of ways to advance research regarding the relationship between iron deficiency, iron supplementation, and malaria infection.

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CHAPTER TWO: HOST IRON STATUS AND IRON SUPPLEMENTATION MEDIATE MALARIA SUSCEPTIBILITY TO ERYTHROCYTIC STAGE PLASMODIUM FALCIPARUM[1]

2.1 OVERVIEW

Iron deficiency and malaria have similar global distributions, and frequently co-exist in pregnant women and young children. Where both conditions are prevalent, iron supplementation is complicated by observations that iron deficiency anemia protects against falciparum malaria, and that iron supplements increase susceptibility to clinically significant malaria, but the mechanisms remain obscure. Here, using an *in vitro* parasite culture system with erythrocytes from iron deficient and replete human donors, we demonstrate that *Plasmodium falciparum* infects iron deficient erythrocytes less efficiently. In addition, owing to merozoite preference for young erythrocytes, iron supplementation of iron deficient individuals reverses the protective effects of iron deficiency. Our results provide experimental validation of field observations reporting protective effects of iron deficiency and harmful effects of iron administration on human malaria susceptibility. Because recovery from anemia requires transient reticulocytosis, our findings imply that in malarious regions iron supplementation should be accompanied by effective measures to prevent falciparum malaria.

2.2 INTRODUCTION

The interactions between falciparum malaria and iron deficiency anemia (IDA) are complex and bi-directional. Malaria causes acute anemia by destroying both infected and uninfected red blood cells (RBCs) (1), whereas persistent sub-clinical infection causes a milder anemia of infection by blocking iron recycling to the bone marrow (2). Conversely, once established, IDA protects both pregnant women (3–5) and children (6–8) from malaria. In addition, supplemental iron, given alone or in combination with other micronutrients, predisposes children to malaria (8, 9) and other serious adverse outcomes (10). Iron homeostasis has been implicated in regulating liver stage *P.falciparum* infection; in murine studies,

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erythrocytic stage malaria infection initiates hepcidin-mediated hepatic hypoferremia, which blocks superinfections by sporozoites from competing plasmodial strains (11). Mathematical modelling suggests that this can explain the low levels of superinfections in young children (11), but this mechanism cannot account for observed reductions in the risk of primary malaria infection in children with IDA. It has also been speculated that transient peaks in non-transferrin-bound iron caused by administration of highly absorbable iron supplements (12) could promote intra-erythrocytic parasite growth (13) or bacterial septicemia (a common cause of death in malaria patients (14–16) but definitive evidence is absent.

As iron deficiency and iron supplementation of iron deficient individuals profoundly alters erythropoiesis, RBC physiology, and RBC population structure, we hypothesized that iron deficiency and iron supplementation directly impact the disease causing erythrocytic stage of *P.falciparum* infection. In our investigations, we minimize the confounding factors that have complicated prior field studies of the relationship between host iron status, iron supplementation and falciparum malaria by utilizing an *in vitro* system with freshly isolated RBCs from donors with well-defined, physiologic iron states recruited through our US-based hospital clinic. This approach eliminated the influence of acquired and innate immunity to malaria, hemoglobinopathies and concurrent inflammation. Our study reveals that RBCs from donors with IDA confer malaria protection by impairing *P.falciparum* invasion and intra-erythrocyte propagation. This protective effect was reversed when donors with IDA received iron supplementation. We go on to show that when iron deficient RBCs are replaced with iron-replete (IR) RBCs *in vitro* (as occurs in individuals with IDA following iron supplementation) the susceptibility to *P.falciparum* infection is increased. These findings support well-described clinical patterns of differential susceptibility to malaria. Taken together, they indicate that therapeutic iron supplementation conspires with host iron status to mediate host RBC susceptibility to malaria infection by altering the dynamic structure of the host's RBC population.

2.3 MATERIALS AND METHODS

Clinical: Study participants included healthy, HIV-negative, non-pregnant donors over 18 years of age with and without IDA. Exclusion criteria included: on-going inflammation or infection, previous history of malaria, travel to malaria endemic areas, malignancy, sickle cell disease (or trait), thalassemia (or trait for either thalassemia-α or -β). This study was approved by the University of North Carolina Institutional Review Board, Protocol #09-0559, and informed consent was obtained from all subjects.

Study participants with Hgb >11g/dl and ferritin >12 ng/ml were classified as IR and participants with Hgb <11g/dl and ferritin <12ng/ml were classified as IDA. IDA+Fe donors were identified by their personal physicians for participation in our study. Subjects were included in the IDA+Fe group if they fit the criteria for IDA and had been prescribed high-dose oral ferrous sulfate, 60 mg (9–12.6 mg/kg) elemental iron orally three times per day or intravenous iron at a dosage determined by their personal physician using the following equation: Dose=0.0442 [desired Hgb – observed Hgb] x LBW + [0.26 x LBW]. Healthy donors took 325 mg of ferrous sulfate once daily for the 2-month duration of the study. These donors donated 40 ml of blood on three occasions— at enrollment and two subsequent monthly intervals. An additional group of healthy donors served as the IR control group. Non-anemic donors with low-iron stores (Hgb >11g/dl, ferritin <12ng/ml) were excluded. Full iron panels were obtained for each study participant and mean values for each group are reported in Table 2.1.

Parasite culture: *P.falciparum* parasite strains 3D7, Dd2 and FCR3-FMG were cultured in RBCs from O+, IR donors at 2–3% hematocrit and Albumax Complete Media (ACM)–RPMI 1640 (Sigma-Aldrich) with 10% AlbuMAX II (Gibco), 1mM hypoxanthine (Sigma-Aldrich), 20mM L-glutamine (Cellgro), 0.45% glucose (Cellgro) and 0.01 ng/ml gentamicin (Sigma-Aldrich). AlbuMAX II was used to supplement the media in place of human serum to isolate the effects of the RBCs from different experimental groups. All RBCs used for parasite culture were obtained from well-characterized IR O+ donors and used within 14 days of being drawn. Cultured parasites were maintained between 0.5 and 10% at 37ºC, in an atmosphere of 5% O_2, 5% CO_2 and 90% N_2 with continuous shaking. Early ring stage parasites were synchronized with 5% (w/v) D-sorbitol. Synchronization was repeated at 20 and 40 h to achieve a tightly synchronized parasite population.

Growth assay: *P.falciparum* parasites from routine cultures were seeded as rings at 0.5% initial parasitemia in 1% hematocrit in ACM in triplicate in 96-well plates. Parasites were maintained for 96 h under standard culture conditions and the media was changed daily. At 96 h, parasite cultures were split back to 0.5% parasitemia and maintained as described for an additional 96 h (Supplementary Fig. S2.1). Parasites were stained at all 0 and 96 h time points with 1X DNA dye SYBR Green I (Invitrogen) as described in ref. (17), and fixed in 1% paraformaldehyde and 0.0075% glutaraldehyde (Electron Microscopy Sciences) in Alsever's Solution (Sigma-Aldrich) for 30 min at 4ºC. Fixative was removed and

cells were stored in PBS at 4ºC until analysis by flow cytometry. Growth rate reflects the fold increase in RBC parasitization between 0 and 96 h. To identify parasitized reticulocytes, cultures were stained with 0.5 mM DNA dye SYTO 61 (Invitrogen) (13, 18) and PE-conjugated mouse anti-human CD71 antibody (Miltenyi Biotech) and analyzed by flow cytometry.

Barcoded RBC invasion assay: RBCs were labelled with 5 mM of either CellTrace Violet or CellTrace Far Red DDAO-SE (Invitrogen) as described (18). Violet- and DDAO-labelled RBCs were combined in ACM and delivered in triplicate into 96-well plates and subsequently seeded with schizonts to achieve 1.5–2% parasitized RBCs. Parasites were maintained for 12–18 h under standard culture conditions to allow for schizont rupture and subsequent invasion of CellTrace-labelled RBCs. The invasion of *P.falciparum* into Violet- and DDAO-labelled RBCs was directly compared by measuring the SI, defined as the ratio of the prevalences of infected Violet RBCs to infected DDAO RBCs (19). Note that the SI is different from the previously described selectivity index (20, 21). The selectivity index has been used to report the number of multiply infected cells, whereas the SI defines the relative susceptibility to invasion of two different types of RBCs.

Parasite erythrocyte multiplication rate assay: Trophozoite-infected cultures were magnetic activated cell sorting (MACS) purified and seeded into experimental RBCs to achieve 1.5–2% pRBCs and incubated for 48 h to allow for invasion of merozoites into experimental RBCs and their subsequent development into trophozoites inside the experimental RBCs. Experimental RBC parasite density was determined and the same number of infected experimental RBCs was seeded into separate wells containing target RBCs (from an IR donor) to achieve 1.5–2% pRBCs. Cultures were then incubated for 12–18 h to allow merozoites produced within experimental RBCs to invade target RBCs, allowing for assessment of the PEMR (20, 22). Following invasion of target RBCs, cells were stained with SYBR Green I and analyzed by flow cytometry to determine the total number of ring pRBCs. PEMR reflects the number of merozoite invasions of target RBCs per schizont infected experimental RBC.

Density separation: RBCs were separated into five fractions with a modified version of previously described density gradient centrifugation method (23). Briefly, blood was collected into acid citrate dextrose and plasma was subsequently removed by centrifugation for 15 min at 800g. Packed cells were resuspended at 50% hematocrit in RPMI, and passed over a 2:1 (w/w) α-

cellulose/microcrystalline cellulose column to remove lymphocytes (24). Following lymphocyte depletion, RBCs were washed twice with RBC buffer (10mM HEPES, 12mM NaCl, 115mM KCl, 5% BSA). RBCs were layered onto a 65, 60, 55 and 50% discontinuous Percoll gradient and then centrifuged for 25 min at 1,075g. Each of the five fractions was removed, washed twice with RBC buffer and stored at 4ºC for up to 5 days. Decreasing MCV, reticulocyte content and Calcein fluorescence (25) of the five fractions confirmed the age separation of RBCs (Supplementary Fig. 2.3c–g). For barcoded RBC invasion assays and growth assays, which utilized density separated RBCs, recombined RBCs were included as a 'total' RBC control.

Flow cytometry: Growth, invasion and infectious merozoite production assays were analyzed by flow cytometry using either a modified FACS-Calibur with two lasers 30mW 488 Diode Pumped Solid State laser and a 25-mW 637 red diode laser (FACS-Calibur; Becton Dickinson, modified by Cytek Development) or a Beckman-Coulter (Dako) CyAn ADP. Channels and probes used on the FACSCalibur included: SYTO 61 (637 nM, 666/27 bandpass), PE (488 nM, 585/42 bandpass) and SYBR Green I (488 nM, 530/30 bandpass). FACS-Calibur data were collected using FlowJo CE and analyzed with Summit v5.1. Channels and probes used on the Dako cyan included: CellTrace Violet (405 nM, 450/50 bandpass), SYBR Green I (488 nM, 530/40 bandpass) and CellTrace DDAO-SE (635 nM, 665/20 bandpass). Data from the Cyan cyotometer were collected and analyzed with Summit v4.3.01. Linear amplification of forward scatter was used to set event threshold in order to exclude cell debris, microparticles and doublets. Electronic volume of uninfected RBCs and pRBCs was assessed on a Beckman Coulter Cell lab Quanta (Beckman Coulter). Channels and probes used on the Quanta included: SYBR Green I (488 nM, 525/40 bandpass). National Institute of Standards and Technologies-certified beads standard L2 2 mm, L5 5 mm and L10 10 mm (Beckman Coulter) were used to calibrate electronic volume. Calibrated data were expressed as both electronic volume (mm3) and diameter (mm). Quanta data were collected with Cell Lab Quanta Collection Software for Instrument Control and analyzed with Kaluza (Beckman Coulter). For all experiments, samples were diluted to 0.001–0.002% hematocrit and 100,000–500,000 total events were collected.

Statistical methods: All experiments were performed in triplicate. Results are from either one representative experiment or the combined results of at least three independent experiments. Parasite

growth rate and infectious merozoite production experiments were analyzed with two-tailed Student's t-test and one-way analysis of variance (GraphPad Prism 5). Data from all *in vitro* growth studies were pooled and analyzed using random effects regression (Fig. 2.1d,e). The dependent variable in the analysis was the logarithm of the ratio of the percent pRBCs at 96 h (final) and 0 h (initial). In addition, to the usual variation independently affecting each observation, we fitted two higher levels of variance: variation between individuals and day-to-day variation in parasite 'preparations' taking account of the fact that in this data set these two variance components were cross (rather than the more usually encountered nested design). We fitted two exposure variables, iron status and iron supplementation, both as binary variables, and their interaction, focusing on three contrasts: supplemented IDA versus supplemented IR donors; supplemented versus non-supplemented IDA donors; supplemented versus non-supplemented IR donors. All experiments were performed in triplicate with three *P.falciparum* strains (3D7, Dd2 and FCR3-FMG) and consisted of three serial 96 h growth assays (Supplementary Fig. S2.1); *P.falciparum* strain, growth assay number and their (highly significant) interaction were fitted as binary covariates. We noted that when 0 h (initial) percent pRBCs was greater than 1, the second growth assay always gave anomalous low results (most likely due to a saturation effect). As these data were uninformative and yet added noise to the analysis, we omitted all such cases while noting that although their inclusion increased the standard errors, it did not change the same general conclusions of the analysis. This model was fitted using Stata's xtmixed procedure (v12, Stata Corp). To compare the susceptibility of different RBC types to invasion by *P.falciparum*, an unadjusted odds ratio was used to calculate the SI. All statistical analyzes for invasion experiments were performed with Stata/IC (v10, Stata Corp). Linear regression was employed to investigate the associations between host iron status and parasite invasion *in vitro* using percent of RBCs from IDA or IR donors as the independent variable and total invasions/1x10^5 RBCs as the dependent variable (Fig. 2.3e). Analysis of covariance was conducted to determine whether the invasion into the two groups were the same. An α of 0.05 was set a priori to determine statistically significant differences. Similar analysis was done to better understand associations between RBC age and parasite invasion (Fig. 2.4e).

2.4 RESULTS

2.4.1 Malaria growth is reduced in RBCs from individuals with IDA.

To determine the effect of IDA on the growth of erythrocytic stage *P.falciparum*, we enrolled donors with and without IDA from a non-malaria endemic area through our US-based hospital clinic. Donors were classified as IR (hemoglobin (Hgb) >11g/dl, mean corpuscular volume (MCV) >80 fL, ferritin >12ng/ml) or as IDA (Hgb <11g/dl, MCV <80 fL, ferritin <12ng/ml) (Table 2.1). Non-anemic donors with low iron stores (Hgb >11g/dl, ferritin <12ng/ml) were excluded. *P.falciparum* (strains 3D7, Dd2 and FCR3-FMG) were grown in either RBCs from the IR (n=10) or IDA (n=7) donors in up to three consecutive 96 h growth assays (Supplementary Fig. S2.1). We observed that parasite growth rates were reduced in RBCs from IDA donors as compared with growth in RBCs from IR donors by 48.8% (standard deviation (s.d.)±23.9), 34.3% (s.d.±22.2) and 50.0% (s.d.±20.4) for strains 3D7, Dd2 and FCR3-FMG, respectively (Fig. 2.1a). These findings clearly show that *P.falciparum* propagation is reduced within RBCs from IDA individuals, but that variability may exist in the degree to which different *P.falciparum* isolates are affected by IDA.

2.4.2 Malaria growth is increased in RBCs from iron-supplemented donors.

Given field evidence that supplementation of children with 12.5 mg of iron (1–1.5 mg/kg) and 50 ng of folic acid may potentiate the risk of malaria (9), we next investigated the effects of iron supplementation of IDA and IR individuals on *in vitro* growth of erythrocytic stage *P.falciparum*. We first collected RBCs from IDA patients who were receiving iron supplementation (IDA+Fe); these individuals met the above criteria for IDA and were receiving either high-dose oral ferrous sulfate (60 mg elemental iron orally three times per day (9–12.6 mg/kg)) or intravenous iron (at a dosage determined by their personal physician using the following equation: Dose=0.0442 [desired Hgb—observed Hgb] x LBW + [0.26 x LBW]). IDA+Fe group Hgb values ranged from 6.6 to 9.8 g/dL and MCV values ranged from 75 to 98 fL. Additionally reticulocyte counts and red cell distribution width (RDW) were elevated; and average mean corpuscular hemoglobin concentration (MCHC), total iron, and ferritin values were greater than that of the IDA group but still lower than that of the IR group (Table 2.1). Together, these values are indicative of an erythropoietic response to iron supplements, but not full recovery from IDA. Comparison of the growth rate of *P.falciparum* (strains 3D7, Dd2 and FCR3-FMG) within RBCs from the IDA+Fe donors to

the growth rate of parasites within RBCs from IR donors revealed increases in *P.falciparum* growth of 17.3% (s.d.±22.7), 17.6% (s.d.±14.0) and 26.3% (s.d.±16.1) for 3D7, Dd2 and FCR3-FMG in RBCs from IDA+Fe donors (Fig. 2.1b).

We additionally assessed the effect of iron supplementation of IR individuals on *P.falciparum* growth. For this study, IR individuals donated blood at enrollment (baseline) and were then prescribed daily oral iron supplementation (325 mg ferrous sulfate). Iron-supplemented IR study participants (IR+Fe) subsequently returned at one and two months following initiation of daily iron supplementation to donate blood. At each donation (enrollment, 1 month and 2 months), the growth rate of *P.falciparum* (strains 3D7, Dd2 and FCR3-FMG) within RBCs from IR+Fe donors was determined and then compared with the corresponding parasite growth rates within RBCs from a non-supplemented IR donor. Compared with RBCs from IR donors, we observed in RBCs from IR+Fe donors that 1 month of iron supplementation increases of 17.5% (s.d.±16.1), 11.3% (s.d.±15.7) and 6.6% (s.d.±8.1) in growth for 3D7, Dd2 and FCR3-FMG, respectively. There was no change in parasite growth rate in RBCs collected 1 and 2 months after administering iron supplements (Fig. 2.1c). Analyzes of Hgb, hematocrit, MCV, MCHC, transferrin saturation, ferritin and reticulocyte count of IR+Fe donors revealed no significant change in their iron status following iron supplementation (Table 2.1).

To comprehensively compare and ultimately quantify the impact of IDA and iron supplementation on the growth of *P.falciparum in vitro*, we integrated data from all growth experiments and fit a multilevel random effects model to the pooled data (Fig. 2.1d). The outputs of this analysis were growth rates of any parasite strain in experimental RBCs plotted against the growth rate in IR RBC controls. Values were adjusted for variation between study participants, day-to-day differences in parasite preparations and differences in *P.falciparum* strain growth rates. The Y=X line was fit to the growth rate of *P.falciparum* in RBCs from IR donors. Data above the Y=X line indicate growth rates greater than that of parasite growth in RBCs from IR donors and data below the Y=X line indicate growth rates lower than that of parasite growth RBCs from IR donors. Based on this analysis, we estimate that compared with IR RBCs, *P.falciparum* growth is reduced (59.8% (95% confidence interval (CI)=51.9–68.8)) in RBCs from IDA donors, and that there is a slight increase in the growth of *P.falciparum* in RBCs from IDA+Fe donors (22.8% (95% CI=2.7–46.7)) and RBCs from IR+Fe donors (18.9% (95% CI=5.0–33.9)); no difference was

observed between *P.falciparum* growth in RBCs from IRþFe donors and IDAþFe donors (Fig. 2.1e). These data clearly indicate that IDA substantially attenuates the growth of *P.falciparum* parasites and that iron supplementation of donors with IDA reverses the protection provided by IDA against falciparum infection. Furthermore, these data suggest that iron supplementation of IR individuals may slightly increase propagation of erythrocytic stage *P.falciparum*.

2.4.3 RBCs from donors with IDA are refractory to malaria infection.

Propagation of the erythrocytic stage of *P.falciparum* may be impeded at the point of (i) invasion, (ii) maturation or (iii) production of infectious daughter merozoites. To determine why *P.falciparum* infection of RBCs from IDA donors is reduced, we systematically assessed the capacity of *P.falciparum* to progress through each of these rate-limiting steps within RBCs from IDA donors compared with RBCs from IR donors. To assess invasion, we directly compare invasion of *P.falciparum* strains 3D7, Dd2 and FCR3-FMG into RBCs from IDA and IR donors with a barcoded RBC flow cytometry based invasion assay. To express the differential invasion of RBCs, we computed the susceptibility index (SI), which is the ratio of the relative risk of invasion of RBCs from IDA donors to that of RBCs from IR donors. An SI of 1.0 indicates no difference in parasite invasion between two RBC populations. In experiments with strains 3D7, Dd2 and FCR3-FMG, the mean SI of RBCs from IDA donors relative to RBCs from IR donors was 0.56 (95% CI=0.56–0.57), 0.52 (95% CI=0.52–0.53) and 0.70 (95% CI=0.69–0.71), respectively, indicating consistently reduced invasion of RBCs from IDA donors (Fig. 2.2a). We next assessed parasite maturation within RBCs from IDA donors by analyzing Giemsa-stained thing blood smears, which were made every 6 h during the course of a 48-h intra-erythrocytic lifecycle. We observed that parasites matured normally in iron deficient RBCs, indicating that the reduced overall parasite growth in iron deficient RBCs did not result from delayed maturation (Fig 2.2b,c). Finally, we measured the parasitized erythrocyte multiplication rate (PEMR) (20, 22) of *P.falciparum* within RBCs from IDA donors as compared with RBCs from IR donors. For parasite strains 3D7, Dd2 and FCR3-FMG, RBCs from IDA donors (relative to RBCs from IR donors) had a reduced PEMR of 48.0% (s.d.±12.2), 25.7% (s.d.±2.2) and 39.9% (s.d.±9.3), respectively (Fig. 2d). In accordance with the PEMR data, we additionally observed fewer merozoites within IDA as compared with IR RBCs by microscopy (data not shown). Taken together, these data indicate that *P.falciparum* matures normally within RBCs from IDA donors, but that invasion

into and production of infectious merozoites within RBCs from IDA donors are significantly reduced. These data additionally reveal that different *P.falciparum* isolates may exhibit different invasion and PEMR phenotypes in RBCs of IDA individuals.

2.4.4 Replacement of iron deficient RBCs increases malaria growth.

The erythropoietic rate of iron deficient individuals increases dramatically in response to iron supplementation, and elevated erythropoietic rate is hypothesized to increase an individual's susceptibility to malaria19. As the iron biomarkers of individuals in the IDA+Fe group were indicative of an erythropoietic response to iron (Table 2.1), we hypothesized that the replacement of iron deficient RBCs with IR RBCs would explain the recovered growth of *P.falciparum* in RBCs from IDAþFe donors (Fig. 2.1). To determine whether the replacement of iron deficient RBCs with young IR RBCs could explain the recovered growth of *P.falciparum* in RBCs donated by IDA+Fe individuals, we first compared *P.falciparum* infection of reticulocytes (CD71+) and mature RBCs (CD71-) from IDA+Fe donors. Consistent with *P.falciparum*'s reported preference for young RBCs, we observed 8.6% (s.d.±0.1) parasitization of reticulocytes (CD71+) and 4.5% (s.d.±0.4) parasitization of mature RBCs (CD71-) (Fig. 2.3a). However, (CD71+) reticulocytes accounted for only 1.6% (s.d.±0.9) of all RBCs and parasitized (CD71+) reticulocytes only contributed to 3.0% (s.d.±0.1) of the total number of parasitized RBCs (Fig. 2.3b).

Having clearly demonstrated that reticulocytes from IDA+Fe donors are more highly infected by *P.falciparum*, but that reticulocytes themselves only contribute marginally to the total infection, we next sought to definitively determine whether the replacement of iron deficient with IR RBCs could explain the recovered growth of *P.falciparum* in RBCs from IDA+Fe donors. However, low incidence of IDA in our study setting, the difficulty of following iron-supplemented IDA individuals longitudinally through full recovery from iron deficiency, as well as the inability to use the common surrogates of RBC age (volume and density) against the background of changing host iron status, prevented us from studying the impact of an elevated erythropoietic rate on erythrocytic stage *P.falciparum* infection. Therefore, we modelled the effect of iron supplementation- mediated changes in RBC population dynamics on erythrocytic stage *P.falciparum* infection by assessing the impact of replacing RBCs from IDA donors with RBCs from IR donors on *in vitro P.falciparum* growth. Replacing 10, 25, 50 and 75% of the RBCs from IDA donors with RBCs from IR donors resulted in a steady increase in parasite growth rate; 75% replacement recovered

3D7, Dd2 and FCR3-FMG *P.falciparum* growth rate to 88.2% (s.d.±2.0), 89.7% (s.d.±2.5) and 92.3% (s.d.±1.1) that of the growth rate of in RBCs from IR donors, respectively (Fig. 2.3c).

As the first step in the erythrocytic life cycle of the malaria parasite, erythrocyte invasion is a pivotal determinant of the magnitude of infection. Therefore, we utilized the barcoded RBC invasion assay to determine how iron deficient and IR RBCs interact to shape *P.falciparum* invasion. Like growth rate, *P.falciparum* invasion rate increased as IDA RBCs were replaced with IR RBCs. Rate of invasion was fully recovered to IR levels once 80% of IDA RBCs were replaced with IR RBCs for all three parasite strains (Fig. 2.3d and Supplementary Fig. S2.2a). Furthermore, employment of the barcoded RBC invasion assay allowed for the full characterization of the kinetics of *P.falciparum* invasion into IDA and IR RBCs, as the frequency of each changed relative to the total RBC population. We observed that the number of *P.falciparum* FCR3-FMG invasions into IDA and IR RBCs increased linearly, as each RBC population increased in frequency, R^2=0.984 and 0.931, respectively, and *P.falciparum* invasion as a function of IDA RBC abundance was significantly less than that of IR RBCs (Fig. 2.3e). Similar trends were observed for *P.falciparum* strains 3D7 and Dd2 (Supplementary Fig. S2.2b and c). Together, these results support the hypothesis that replacing an individual's iron deficient RBC population with IR RBCs would increase the host's susceptibility to erythrocytic stage *P.falciparum* infection and clearly illustrate the impact RBC population dynamics have on potential parasite biomass and pathogenesis. Moreover, our use of the barcoded RBC invasion assay allowed for the definitive determination and characterization of the distribution of parasites in IDA and IR RBC populations, as the frequency of each changed.

2.4.5 Effect of RBC population age structure on malaria infection.

Reticulocytes and young RBCs are preferentially invaded by *P.falciparum* (26, 27), and theoretical models predict that elevated reticulocytosis may increase the risk of high parasite density (28, 29). Because of the shortened lifespan of iron deficient RBCs, there is a period in the course of an individual's recovery from IDA at which point protective iron deficient RBCs have been cleared from circulation and the remaining circulating RBCs are on average younger than that of an IR individual. To determine the capacity of young RBCs to shape *P.falciparum* infection, we (i) directly compare *P.falciparum* infection of young RBCs and RBCs of increasing age and (ii) model the effect of replacing young IR RBCs with old IR RBCs on *P.falciparum* growth and invasion *in vitro*. For these studies, we

utilized two proxies for RBC age: RBC volume, which decreases with age (23) and is unaffected by ring stage parasitization (30), and RBC density, which increases with increasing RBC age (23). We observed that when RBCs from an IR donor were infected with *P.falciparum*, parasite infection increased with increasing RBC volume (Fig. 2.4a). To directly compare *P.falciparum* invasion of RBCs of different ages, we density separated RBCs from IR donors into four fractions of increasing RBC age: young, young adult, mature adult and old (Supplementary Fig. S2.3A). Decreasing MCV, reticulocyte content and Calcein fluorescence (25) confirmed the age separation of the RBCs (Supplementary Fig. S2.3b–d). We observed that the SI of young adult, mature adult and old RBCs to parasite invasion as compared with young RBCs was 0.85 (95% CI=0.82–0.90), 0.58 (95% CI=0.56–0.62) and 0.28 (95% CI=0.27–0.30), respectively (Fig. 2.4b). In accordance with previous reports (22, 26, 31), young RBCs sustained a significantly greater growth rate than young adult, mature adult and old RBCs, with young RBCs supporting a growth rate 50% greater than old RBCs (Supplementary Fig. S2.3e), and compared with young RBCs the PEMR was reduced by 10% (s.d.±4.78), 15% (s.d.±1.16) and 19% (s.d.±2.23) in young adult, mature adult and old RBCs, respectively (Supplementary Fig. S2.3f). These data clearly demonstrate the preferential invasion of *P.falciparum* into young RBCs and show that the risk of RBCs to *P.falciparum* invasion relative to young RBCs decreases with increasing RBC age. All together we have confirmed (i) *P.falciparum* infection is more prevalent in young RBCs (ii) the increased capacity of young RBCs to support *P.falciparum* invasion and growth.

To determine the capacity of the age distribution of a RBC population to shape *P.falciparum* infection, we examined the effect of replacing young IR RBCs with old IR RBCs on *P.falciparum* growth *in vitro*. We observed 15.7% (s.d.±3.1) greater growth of *P.falciparum* in young IR RBCs as compared with density separated and then recombined (total) IR RBCs, and growth remained significantly greater when 10% of young RBCs were replaced with old RBCs. Following replacement of 33, 50, 66, 80 and 90% of young RBCs with old RBCs, *P.falciparum* growth rate steadily decreased (Fig. 2.4c). Consistent with *P.falciparum* growth, we observe that the rate of *P.falciparum* invasion into young RBCs is significantly greater than that of total IR RBCs (29.0% (s.d.±2.1)). Furthermore, we observed that as the frequency of young RBCs decreased from 100 to 50% and the frequency of old RBCs increased from 0 to 50%, the total rate of invasion decreased by only 4.9% (s.d.±0.7), maintaining a *P.falciparum* invasion rate

significantly greater than that of the invasion rate of total RBCs. However, when the frequency of young RBCs fell from 50 to 0% and old RBCs increased from 50 to 100%, *P.falciparum* infection decreased steadily, ultimately falling by 45.7% (s.d.±1.9) (Fig. 2.4d).

As young IR RBCs are at the greatest risk of *P.falciparum* invasion, we speculated that an insufficient merozoite inoculum might be responsible for the observed plateau in *P.falciparum* invasion. However, invasion experiments with double the inoculum of merozoites also resulted in a plateau in the rate of *P.falciparum* invasion when young RBCs accounted for more than 50% of the total RBCs population (Supplementary Fig. S2.4a). Moreover, the rate of *P.falciparum* invasion achieved with the higher inoculum was less than that of the lower inoculum invasion experiments (Fig. 2.4d and Supplementary Fig. S2.4a). We subsequently characterized the kinetics of *P.falciparum* invasion into young and old RBCs as the frequency of each changed relative to the total RBC population. We observed that like *P.falciparum* invasion of IDA and IR RBCs, the number of *P.falciparum* invasions into old IR RBCs increased linearly as old IR RBCs increased in frequency, R^2=0.991. In contrast, the number of *P.falciparum* invasions into young IR RBCs as a function of young IR RBC abundance was best fit by a logarithmic function, R^2=0.976 (Fig. 2.4e). The same kinetics of *P.falciparum* invasion were observed when experiments were performed with double the merozoite inoculum (Supplementary Fig. S2.4b). These results demonstrate that replacement of young IR RBCs with old IR RBCs reverses the elevated growth and invasion rate sustained by young IR RBCs. In addition, we show that the rate of *P.falciparum* invasion only begins to dramatically drop off once 50% of young IR RBCs have been replaced with old IR RBCs, and that this can be attributed to the logarithmic nature of *P.falciparum* invasion of young IR RBCs. Together, these data support the hypothesis that the effects of iron deficiency and iron supplementation on RBC physiology and erythropoietic rate are at least partially responsible for determining an individual's risk of malaria infection (Fig. 2.5).

2.5 DISCUSSION

Iron supplementation has clear nutritional benefits for children and pregnant women (2), but iron is also an essential nutrient for most pathogens and as a result is a critical mediator of host–pathogen interactions (32). Activation of the host innate immune system by the malaria parasite or other infectious organisms triggers reduction in iron absorption, redistribution of existing iron stores and decreases

erythropoiesis, which effectively limits the availability of iron to invading pathogens. It is unknown what host iron (mosquito or human) *P.falciparum* is able to access and utilize nor how the parasite circumvents the host's attempt to restrict iron. It has been previously postulated that as occurs with other pathogens (33), iron deficiency inhibits *P.falciparum* infection via iron deprivation. Although the malaria parasite may find iron less readily available in an iron deficient host, our work reveals an alternate cellular mechanism by which iron deficiency may protect against malaria. Our study of the relationship between iron deficiency, iron supplementation and erythrocytic stage *P.falciparum* infection highlights how by altering the dynamics of the human hosts RBC population iron deficiency and iron supplementation shape erythrocytic stage *P.falciparum* infection.

Clinical studies in different field sites have reported that iron deficiency correlates with protection from malaria. In Malawian children, baseline iron deficiency was associated with significant reductions in the subsequent risks of both parasitemia (45%) and malaria (51%) (34). Similarly in Tanzanian children, baseline iron deficiency significantly decreased the odds of subsequent parasitemia (23%) and severe malaria (38%) (6). In addition, in two studies of pregnant women, iron deficiency was associated with a decreased prevalence of placental malaria, a major cause of neonatal and maternal morbidity (4, 5). Our results—that iron deficient RBCs impair parasite propagation *in vitro* (Fig. 2.1)—are consistent with these clinical findings, and provide valuable insight into a cellular mechanism for the observations made in the clinical setting. In our study of *P.falciparum* growth in RBCs from IDA donors, we reveal that RBCs from IDA donors are refractory to *P.falciparum* invasion and support a lower PEMR but that parasite maturation is normal (Fig. 2.2). There are multiple physiological differences between iron deficient and IR RBCs that may contribute to the impaired invasion into and replication within iron deficient RBCs. These include greater osmotic fragility and membrane rigidity, accelerated ageing in vivo (35–38), lower Hgb content and smaller size (microcytosis).

Iron supplementation has long been hypothesized to increase malaria risk, and this issue has garnered recent attention after a large-scale nutritional supplementation study in Tanzanian children was halted owing to significantly increased mortality among those receiving iron (8). Although it remains unclear whether the increased mortality rate was secondary to malaria, this potential for harm has complicated recommendations for widespread supplementation and has caused iron supplementation

programs in malaria endemic countries to be suspended. In a more recent randomized trial of Tanzanian children, iron supplementation increased the risk of malaria by 41% in iron deficient but not in IR children (8). Notably, our studies are consistent with the Tanzania study. Specifically, we observed increased parasite growth in RBCs donated by iron-supplemented IDA individuals and a modest effect in RBCs donated by iron-supplemented IR individuals (Fig. 2.1). As individuals in the iron-supplemented IDA group were observed to be undergoing an erythropoietic response to iron supplementation (Table 2.1), we proceeded to investigate the effect of replacing iron deficient RBCs with IR RBCs and young RBCs with old RBCs on *P.falciparum* erythrocyte infection. We demonstrate that *P.falciparum* growth and invasion rates increase when iron deficient RBCs are replaced with IR RBCs (Fig. 2.3). Furthermore, we show that young RBCs support greater *P.falciparum* growth and invasion rates than total IR RBCs and that the replacement of young RBCs with old RBCs reverses the effect of young RBCs on parasite growth and invasion (Fig. 2.4). Finally, in the course of these experiments, our use of barcoded RBC flow cytometry invasion assay has provided novel insight into the relationship between the frequency of a RBC subset in the total RBC population and *P.falciparum* infection (Figs 2.3e and 2.4e). In summary, our results demonstrate that the changes in the RBC population that occur during recovery from IDA enhance parasite propagation.

It should be noted that our work exclusively focuses on the influence of iron deficiency and iron supplementation on the susceptibility of the human host's RBC to malaria infection, and does not address the potential effect of serum iron (13) or additional factors, which may function *in vivo*, including growth of the hepatic stage of the parasite (39), rosetting and cytoadherence to the endothelium, accelerated clearance of parasitized RBCs (40, 41), effects of innate immune factors such as hepcidin (11) and lipocalin 2 (42), or adaptive immune function. We have previously reported that both transferrin and ferric citrate increase the bioavailable iron pool of trophozoite-infected RBCs but not that of uninfected RBCs (13). It is possible that parasite growth may be enhanced by the transient increase in serum iron that is observed in IR individuals who are given oral iron supplementation (12). Further investigations need to be conducted to explore the extent to which iron deficiency and iron supplementation shape other aspects of malaria pathogenesis.

Our findings, taken together with those from field studies, raise the important medical and public health question: How can iron supplementation be safely administered to IDA children in malarious areas? A critical implication of these observations is that reconstitution of red cell mass in anemic patients would be expected to transiently enhance susceptibility to malaria (Fig. 5), which may inform the on-going debate as to whether fortification with iron would be safer than supplemental iron. Our data implies that, where *P.falciparum* is endemic, treatment of anemia with iron supplementation should be accompanied by malaria preventive measures, such as malaria prophylaxis, bed nets and increased active surveillance and access to health care. Additional questions raised by this study are: Does iron deficiency in African children represent an evolutionarily advantageous phenotype that derives from polymorphisms in iron homeostasis? What molecular mechanisms confer protection from malaria in the setting of microcytosis, and can these protective mechanisms be exploited by medical interventions? Future clinical and translational studies will be needed in order to design safe and effective interventions to address the twin burdens of iron deficiency and falciparum malaria.

2.6 TABLES AND FIGURES

Table 2.1. Iron parameters and values of study participants. Tests were performed by McClendon Clinical Laboratory on samples taken from each donor at the same time points that blood samples were drawn for experiments. Values in the Normal Range column are the normal or healthy range for each parameter as defined by the McClendon Clinical Laboratory. Numerical values reflect the mean value of all individuals in each group and values in parentheses indicate standard deviation.

Variable	Normal Range	Iron Deficiency Anemia (IDA) (N = 7)	Iron Deficiency Anemia on Iron supplementation (IDA+Fe) (N = 6)	Iron Replete (IR) (N = 10)	Iron Replete after 1 month Iron supplementation (IR+Fe) (N = 4)	Iron Replete after 2 months Iron supplementation (IR+Fe) (N = 4)
White Blood Cell ($x10^9$/L)	4.5-11.0	4.84 (1.78)	4.45 (1.15)	6.40 (1.61)	6.3 (1.41)	6.2 (1.12)
Red Blood Cell ($x10^{12}$/L)	4.0-5.2	3.96 (0.60)	3.42 (0.35)	4.78 (0.52)	4.94 (0.52)	4.91 (0.39)
Hemoglobin (g/dL)	12.0-16.0	8.2 (1.56)	8.73 (1.23)	14.60 (1.40)	14.9 (0.57)	14.63 (0.53)
Hematocrit (%)	36.0-46.0	28.13 (4.32)	29.11 (4.25)	42.80 (4.31)	43.85 (2.62)	42.88 (2.81)
Mean Corpuscular Volume (fL)	80-100	71.20 (6.63)	84.78 (8.28)	89.80 (2.74)	89.67 (4.24)	87.75 (2.50)
Mean Corpuscular Hemoglobin (Pg)	26-34	20.79 (2.54)	25.50 (2.35)	30.70 (0.95)	30.5 (2.12)	30.0 (1.83)
Mean Corpuscular Hemoglobin Content (g/dL)	31-37	29.20 (1.50)	30.27 (0.86)	34.20 (0.63)	34.50 (0.71)	34.25 (0.96)
Red Cell Distribution Width (%)	12.0-15.0	17.41 (1.44)	18.13 (2.71)	13.22 (0.85)	13.90 (0.28)	13.2 (0.45)
Mean Platelet Volume (fL)	7.0-10.0	8.56 (1.05)	8.78 (0.65)	7.63 (0.54)	8.45 (0.92)	8.15 (0.52)
Platelet Count ($x10^9$/L)	150-440	294.57 (59.70)	344.0 (168.63)	251.80 (36.70)	212.5 (89.80)	256 (79.31)
Iron Total (mg/dL)	35-165	21.60 (10.55)	32.0 (16.79)	103.60 (43.34)	105.75 (32.71)	87.5 (17.99)
Transferrin (mg/dL)	200-380	343.60 (74.72)	305.20 (63.55)	264.30 (41.33)	275 (29.50)	276.75 (31.83)
TIBC (mg/dL)	252-479	438.67 (92.08)	384.60 (80.29)	333.0 (52.20)	346.0 (37.43)	348.75 (40.07)
Transferrin Saturation (%)	15-50	4.71 (3.15)	8.0 (7.45)	31.60 (13.04)	31.0 (9.49)	25.75 (7.63)
Ferritin (ng/mL)	30-151	5.71 (2.75)	17.17 (25.44)	42.01 (24.28)	46.75 (38.75)	33.33 (18.45)
Reticulocyte (%)	0.5-2.7	1.42 (0.55)	3.52 (1.62)	1.48 (0.48)	2.4 (0.71)	1.4 (0.38)

Figure 2.1. *P.falciparum* growth is reduced in iron deficient RBCs and iron supplementation eliminates growth attenuation. (a–c) Growth experiments with RBCs from IDA donors (n=7), IDA+Fe donors (n=5) and IR+Fe donors (n=4) were performed. Growth rate in RBCs from an IR donor served as the control. Bars represent growth of *P.falciparum* (strains 3D7, Dd2 and FCR3-FMG) in indicated RBCs, normalized to growth in RBCs from IR donors (% Pf growth in IR RBC). Error bars represent the s.d. (a) Bars represent growth in RBCs from IDA donors. Significance was determined by two-tailed paired Student's t-test. *P<$3E^{-10}$ as compared with *P.falciparum* growth in RBCs from IR donors. (b) Bars represent growth in RBCs from IDA+Fe donors. Significance was determined by two-tailed paired Student's t-test. *P<0.0003 as compared with *P.falciparum* growth in RBCs from IR donors. (c) Bars represent growth of *P.falciparum* in RBCs from IR+Fe donors at enrollment, 1 month and 2 months on iron, normalized to growth in RBCs from IR donors (% Pf growth in IR RBC). Significance was determined by one-way analysis of variance. $^{+}$P<0.02 for strain 3D7and nonsignificant (n.s.) for strains Dd2 and FCR3-FMG. (d) Mean growth rate of *P.falciparum* in RBCs from each individual IDA (white square), IR+Fe (x) and IDA+Fe (black square) donor plotted against the growth rate of *P.falciparum* in corresponding control RBCs from non-supplemented IR donors. Data were analyzed by mixed effects regression. The Y=X line was fit to the growth rates (in RBCs from IR donors). Points below the Y=X line indicate growth rates less than that within RBCs from IR donors. (e) Graphical summary of the mixed effects regression analysis shown in d. Donor and parasite preparation were fitted as crossed random effects. The bars show the estimated parasite growth of *P.falciparum* in RBCs from the IDA, IDA+Fe and IR+Fe donors as a percent of *P.falciparum* growth in RBCs from non-supplemented IR donors. Error bars represent the 95% confidence interval.

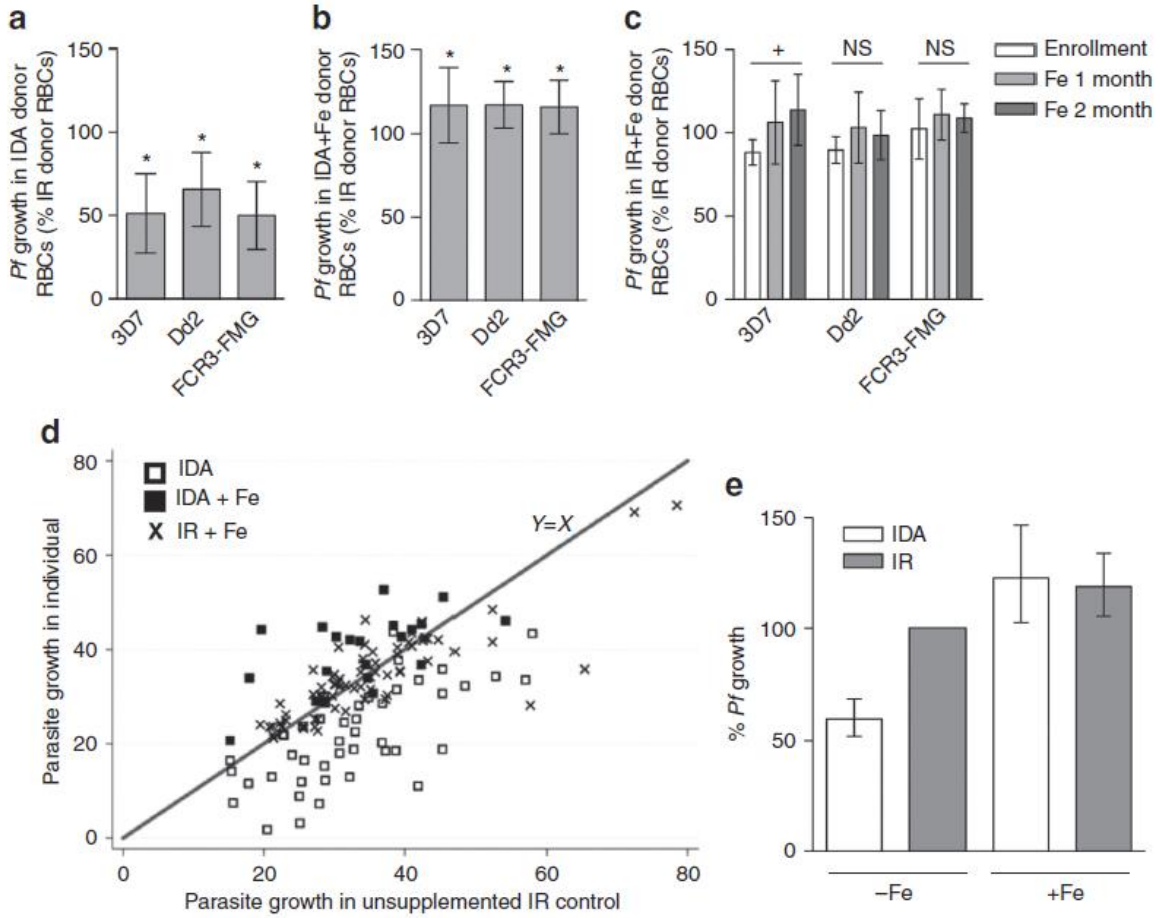


Figure 2.2. *P.falciparum* invasion and growth are reduced in RBCs from IDA donors. (a) Direct comparison of invasion into RBCs from either IDA or IR donors. Invasion experiments for RBCs from all IDA donors were performed independently and each experiment was performed in triplicate. Data show the mean SI of six independent experiments performed with RBCs from six IDA donors. The marker represents the SI point estimate and the bar represents the 95% CI. An SI of 1.0 indicates no difference in parasite invasion of the two RBC populations. (b,c) Comparison of the maturation of *P.falciparum* in RBCs donated by IDA and IR donors. Geimsa-stained thin blood smears were made every 6 h and 1,000 RBCs were counted by light microscopy to determine the percent of pRBCs as well as parasite intra-erythrocytic stage of maturation. Data are from a representative experiment (with strain FCR3-FMG) of three independent experiments performed with RBCs from three IDA donors infected with either *P.falciparum* strain 3D7, Dd2 or FCR3-FMG. (b) Giemsa-stained thin blood smears of *P.falciparum* ring, trophozoite and schizont stage parasites in RBCs from an IDA and an IR donor. (c) Bars indicate percent frequency of parasite ring, trophozoite and schizont stages in RBCs from an IDA and IR donor at each 6 h time point. Error bars represent the s.d. (d) Comparison of the parasite erythrocyte multiplication rate (PEMR) of *P.falciparum* within RBCs from IDA and IR donors. Bars represent PEMR of *P.falciparum* in RBCs from IDA donors, normalized to the PEMR of parasites in RBCs from IR donors (% IR PEMR). Data are the mean of three independent experiments performed in triplicate with RBCs from three IDA donors. Error bars represent the s.d. Significance determined by two-tailed paired Student's t-test.*$P<3E^{-6}$, compared with PEMR in RBCs from IR donors.

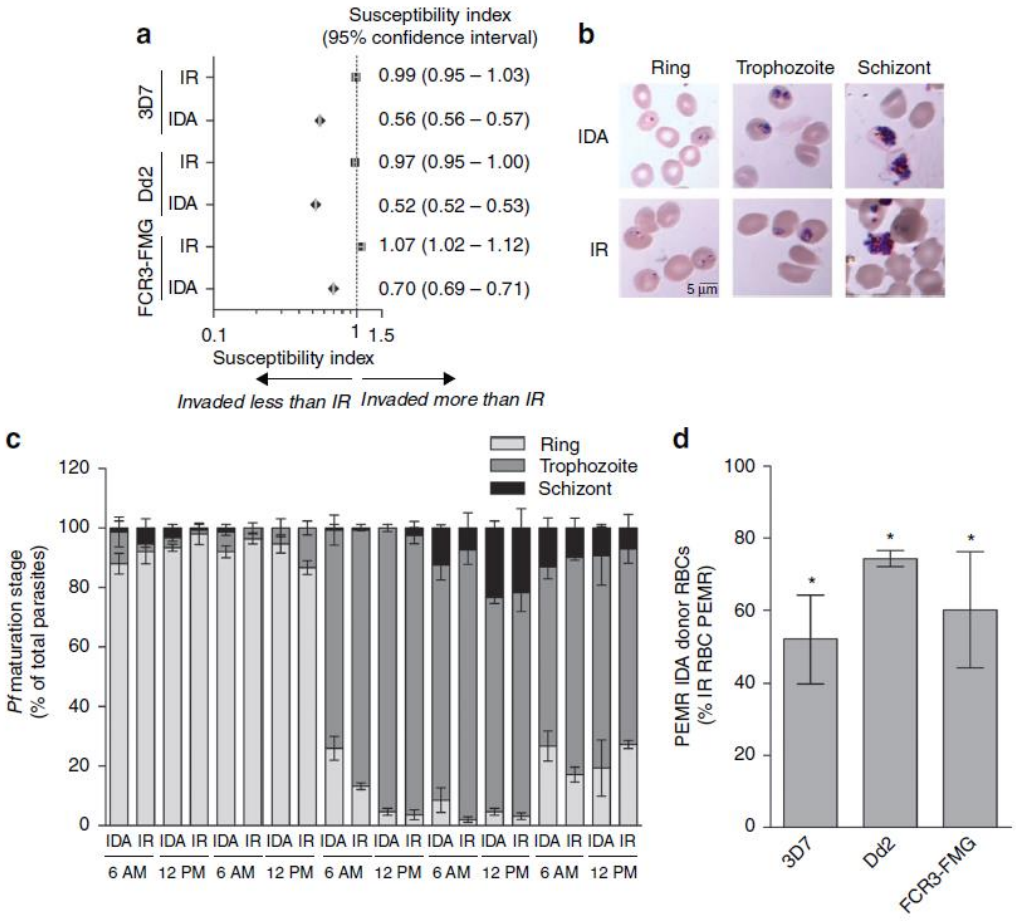


Figure 2.3. Replacement of iron deficient RBCs with iron-replete RBCs increases *P.falciparum* infection. (a,b) *P.falciparum* (strain 3D7) infection of reticulocytes (CD71+) and mature RBCs (CD71-) from an IDA+Fe donor. (a) Bars represent the percent of parasitized reticulocytes (CD71+) and mature RBCs (CD71-). Error bars represent the s.d. *P<0.0001. (b) Contribution of parasitized reticulocytes (CD71+) and parasitized mature RBCs (CD71-) to the total infection. Error bars represent the standard deviation. (c) Growth rate of *P.falciparum* in RBC populations in which IDA RBCs were replaced with IR RBCs. RBCs were inoculated individually or together in the same wells at different ratios (100% IDA; 90% IDA and 10% IR; 75% IDA and 25% IR; 50% IDA and 50% IR; 25% IDA and 75% IR; 100% IR) and subsequently infected. Elongated triangles below the x axis represent the percentage of IDA RBCs (white triangle) and IR RBCs (grey triangle) in the total RBC population. Bars represent parasite growth rates after one 96 h growth assay. Error bars represent the s.d. *P<0.01 and **P<0.0003. (d and e) Invasion rate of *P.falciparum* into RBC populations in which IDA RBCs were replaced with IR RBCs. Differentially labelled RBC donors were inoculated individually or together in the same wells at different ratios (100% IDA; 90% IDA and 10% IR; 80% IDA and 20% IR; 50% IDA and 50% IR; 20% IDA and 80% IR; 10% IDA and 90% IR; 100% IR). Each invasion condition contained $20x10^6$ total RBCs. (d) Bars represent parasite invasion rate. Elongated triangles below the x axis represent the percentage of IDA RBCs (white triangle) and IR RBCs (grey triangle) in the total RBC population. Error bars represent the s.d. *P<0.001, compared with *P.falciparum* invasion rate into 100% IR RBCs. (e) Number of invasions events into IDA (diamonds) and IR (circles) RBCs as the frequency of each increases. Linear regression was used to determine the best fit lines for the data (IR R^2=0.9842 and IDA R^2=0.9305). Analysis of covariance was performed to compare the slopes of the lines and calculated P<0.0001. The null hypothesis was no difference between the two RBC types (H_0: $\beta_{Iron\ replete} = \beta_{Iron\ deficient}$, α=0.05) n.s., nonsignificant.

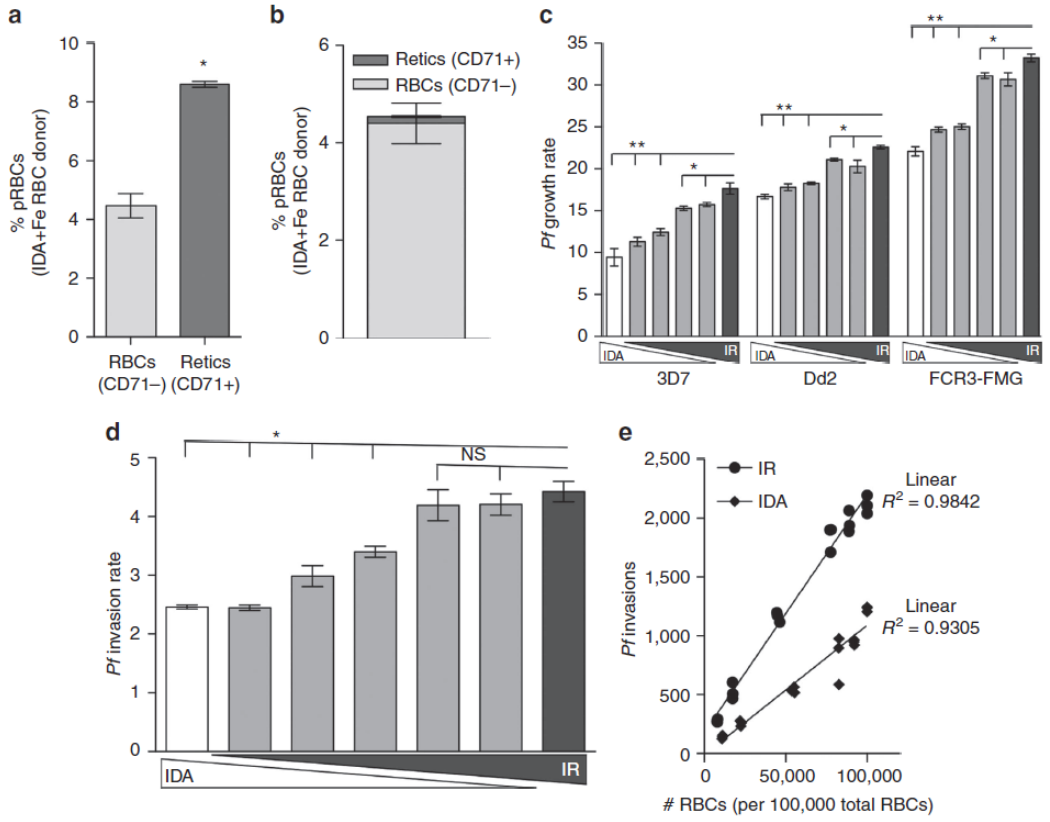


Figure 2.4. The elevated *P.falciparum* infection supported by young RBCs is reversed as young RBCs are replaced with old RBCs. (a) Percent *P.falciparum* (strain 3D7) infection of RBCs of increasing diameter. Data points represent the % pRBCs of five gated RBC populations of increasing volume. Error bars represent the s.d. (b) Direct comparison of *P.falciparum* (strain FCR3-FMG) invasion into RBCs of increasing age. IR RBCs were separated into five fractions of increasing density, a proxy for increasing RBC age (Supplementary Fig. S2.3a–d). The markers represent the SI point estimate and the bar represents the 95% CI. (c) Growth rate of *P.falciparum* (strain FCR3-FMG) in RBC populations in which young IR RBCs were replaced with old IR RBCs (0%, 10%, 20%, 33%, 50%, 66%, 80%, 90% and 100% replacement young RBCs with old RBCs). Elongated triangles represent the percentage of young IR RBCs (gray triangle) and old IR RBCs (white triangle) in the total RBC population. Bars represent parasite growth rates after 96 h. Error bars represent the s.d. *P<0.004 and **P<0.0003, compared to growth rate in 100% total IR and 100% young RBCs, respectively. (d and e) Invasion into RBC populations in which young IR RBCs were replaced with old IR RBCs. Differentially labelled young and old RBCs were inoculated individually or together in the same wells at different ratios (0%, 10%, 20%, 33%, 50%, 66%, 80%, 90% and 100% replacement young RBCs with old RBCs). (d) Bars represent invasion rates. Elongated triangles represent the percentage of young IR RBCs (gray triangle) and old IR RBCs (white triangle) in the total RBC population. Error bars represent the s.d. *P<0.05 and **P<0.003 (e) Number of invasions events into young (circles) and old (triangles) RBCs as the frequency of each increases. Linear regression was used to determine best fit lines. A linear function best fit old RBC data (R^2=0.9788) and a logarithmic function best fit young RBC data (R^2=0.9786). Analysis of covariance was performed to determine whether invasion data of old and young RBCs differed significantly, P<0.0001. The null hypothesis was no difference between the two RBC types (H_0: $\beta_{\text{Iron replete}} = \beta_{\text{Iron deficient}}$, α=0.05) n.s., nonsignificant.

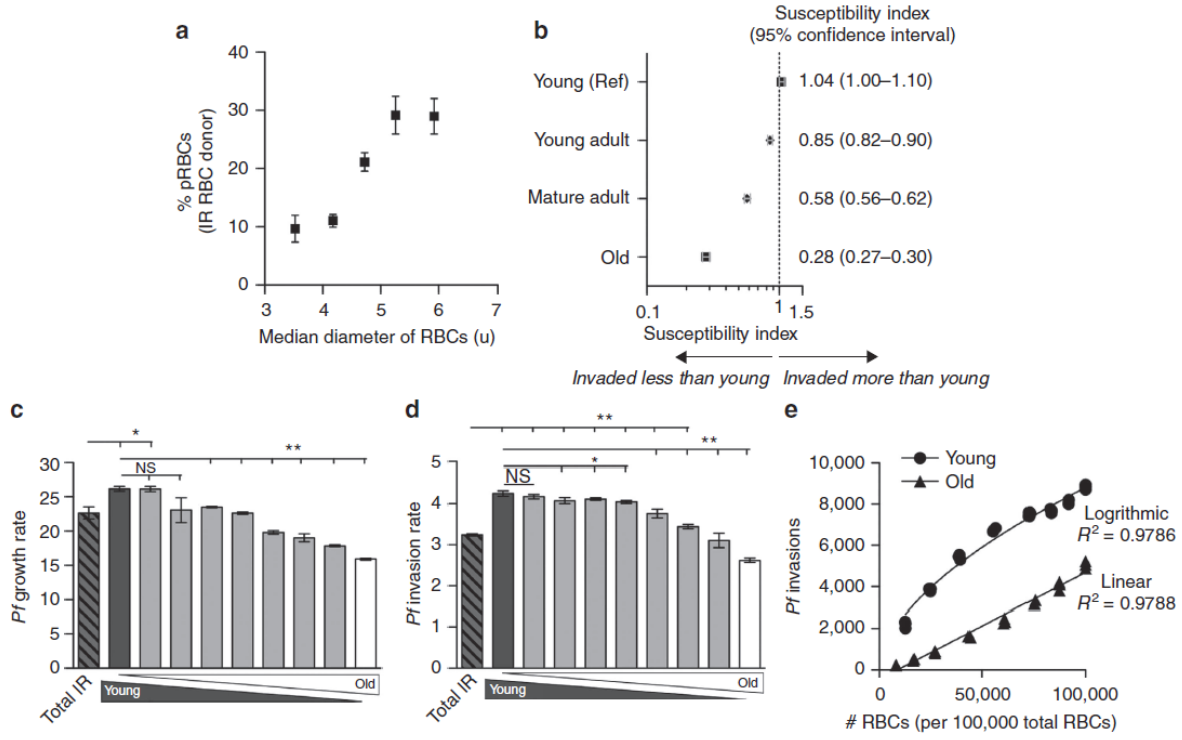
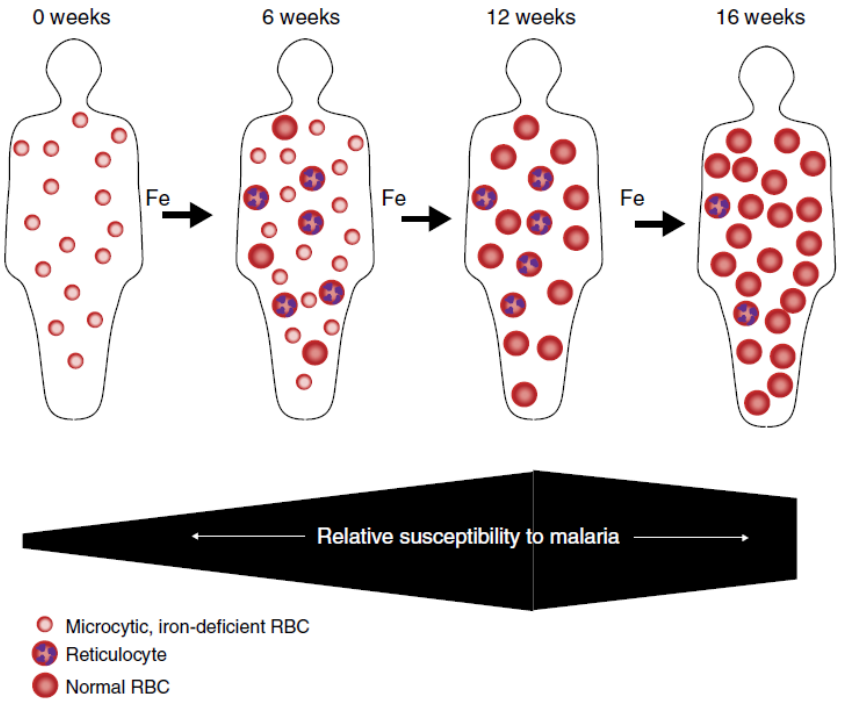


Figure 2.5. Hypothesized impact of iron deficiency and iron supplementation on host RBC population dynamics and susceptibility to erythrocytic stage malaria infection. Recovery from IDA is a complex process, which varies between individuals. Iron supplementation of an individual with IDA (0 weeks) will result in reticulocytosis and the production of young iron-replete RBCs (6 weeks). 12 weeks after the initiation of supplementation, the majority of the iron deficient RBCs, will have been cleared from circulation (iron deficient and iron-replete RBCs have 90 and 120 day lifespans respectively). After 16 weeks of iron supplementation, iron status has been corrected and the age structure of the RBC population will be restored. As shown above, we hypothesize that individuals with IDA will be less susceptible to erythrocytic stage malaria. The induction of erythropoiesis in these individual by iron supplementation and subsequent replacement of the iron deficient RBCs with young iron-replete RBCs will increase the susceptibility of the individual to erythrocytic stage malaria infection. The susceptibility to infection is predicted to peak at the point when all iron deficient RBCs have been replaced, but the age distribution of iron-replete RBCs is on average younger than a fully recovered iron-replete individual. Finally, restoration of the normal distribution of RBC age will return an individual's susceptibility to a normal level.



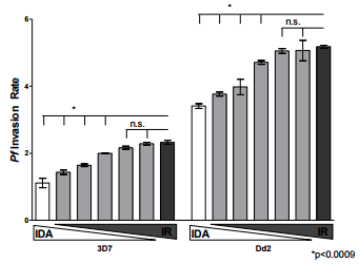
2.7 SUPPLEMENTARY FIGURES

Supplementary Figure S2.1. Serial *in vitro* growth assay design for *P.falciparum*. Synchronized *P.falciparum* cultured in RBCs from O+ iron replete donors were seeded as rings at 0.5% initial parasitemia at 1% hematocrit in triplicate into 96 well plates. Parasites were maintained for 96 h at 37ºC in 5% O_2, and media was changed daily. At 96 h, the parasite cultures were split back to 0.5% parasitemia and maintained for an additional 96 h in Growth Assay 2. At 192 h, the parasite cultures were split back again to 0.5% parasitemia and maintained for an additional 96 h in Growth Assay 3. Growth rate was determined for each growth assay independently. *seed experimental cultures with 0.5% ring stage pRBC. **parasites invade experimental cells of interest.

Growth assay	# replications in RBCs of interest	Time (h)												
		*0**	*24*	*48***	*72*	*96*	*120*	*144*	*168*	*192*	*216*	*240*	*264*	*288*
1	1	Ring	Troph	Ring	Troph	Ring								
2	3					Ring	Troph	Ring	Troph	Ring				
3	5									Ring	Troph	Ring	Troph	Ring

Supplementary Figure S2.2. Replacement of RBCs from IDA donors with RBCs from IR donors increases the invasion rate of erythrocytic stage *P.falciparum in vitro* (additional strains 3D7 and Dd2). (A – C) Invasion of *P.falciparum* (strains 3D7 and Dd2) into RBC populations in which DDAO labeled RBCs from an IDA donor were replaced with Violet labeled RBCs from an IR Donor. Differentially labelled RBCs from and IDA and an IR donors were inoculated individually or together in the same wells at different ratios (100% IDA; 90% IDA and 10% IR; 80% IDA and 20% IR; 50% IDA and 50% IR; 20% IDA and 80% IR; 10% IDA and 90% IR; 100% IR). Each invasion condition contained $20x10^6$ total RBCs and was subsequently inoculated with $0.3x10^6$ pRBCs. Data is from a representative experiment of three independent experiments performed in triplicate with RBCs from three different IDA and IR donors. (A) Bars represent parasite invasion rates into 100% IDA; 90% IDA and 10% IR; 75% IDA and 25% IR; 50% IDA and 50% IR; 25% IDA and 75% IR; and 100% IR RBCs. Elongated triangles below the X-axis represent the percentage of IDA RBCs (white triangle) and IR RBCs (gray triangle) in the total RBC population. Error bars represent the standard deviation. Significance determined by two-tailed paired Student's t test (GraphPad, Prism, v. 5.04, La Jolla, CA). *p<0.0009, compared to *P.falciparum* invasion rate into 100% IR RBCs. (B and C) Data shows the number of *P.falciparum* strains 3D7 (B) and Dd2 (C) invasions events into IDA and IR RBCs as the frequency of each increases in a RBC population containing 100,000 total RBCs. Circles and triangles represent the number of *P.falciparum* invasion events into IR and IDA RBCs respectively as each increases in frequency from 10% to 100% of the total RBC population. Linear regression was used to determine the best fit line for the data, IR R^2=0.9924 and IDA R^2=0.9789 for 3D7 and IR R^2=0.9884 and IDA R^2=0.9899 for Dd2. ANCOVA was performed to compare the slopes of the lines fit to the IDA and IR invasion data. The null hypothesis was no difference between the two RBC types (H_0: $\beta_{\text{Iron replete}} = \beta_{\text{Iron deficient}}$, α=0.05). ANCOVA performed with GraphPad, Prism, v.5.04, La Jolla, CA calculated a p value of <0.0001.

A.



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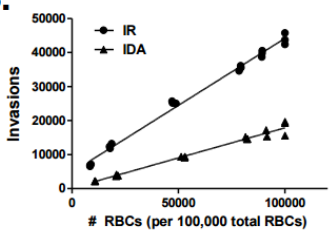
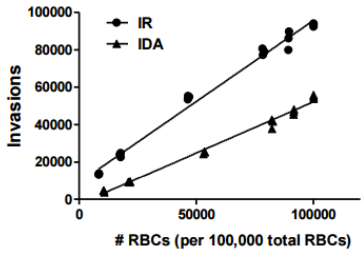


IR R^2=0.9924
IDA R^2=0.9789

p<0.0001

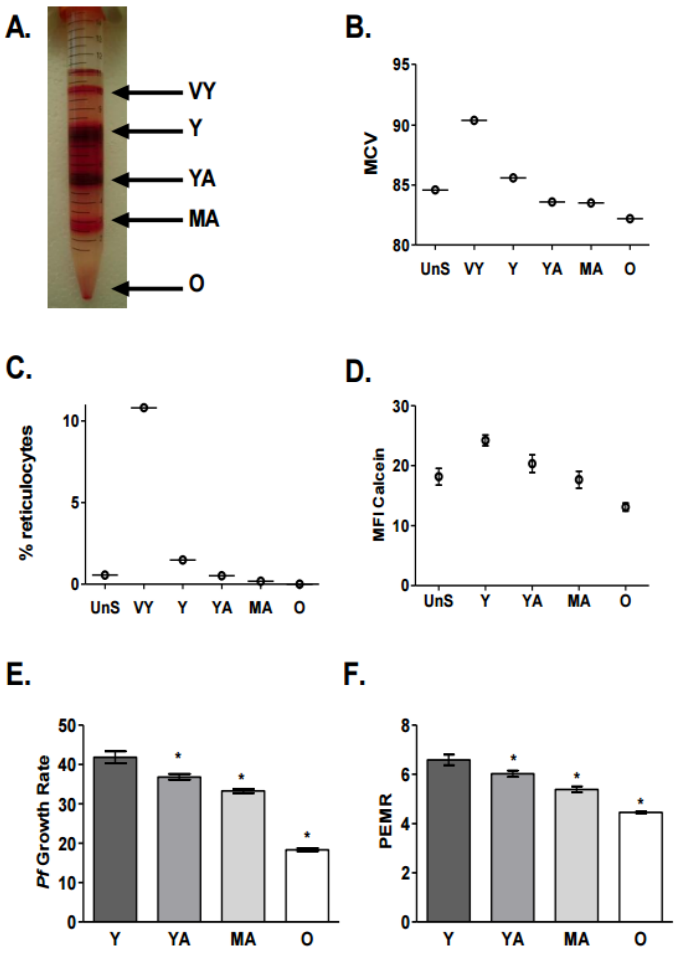
C.



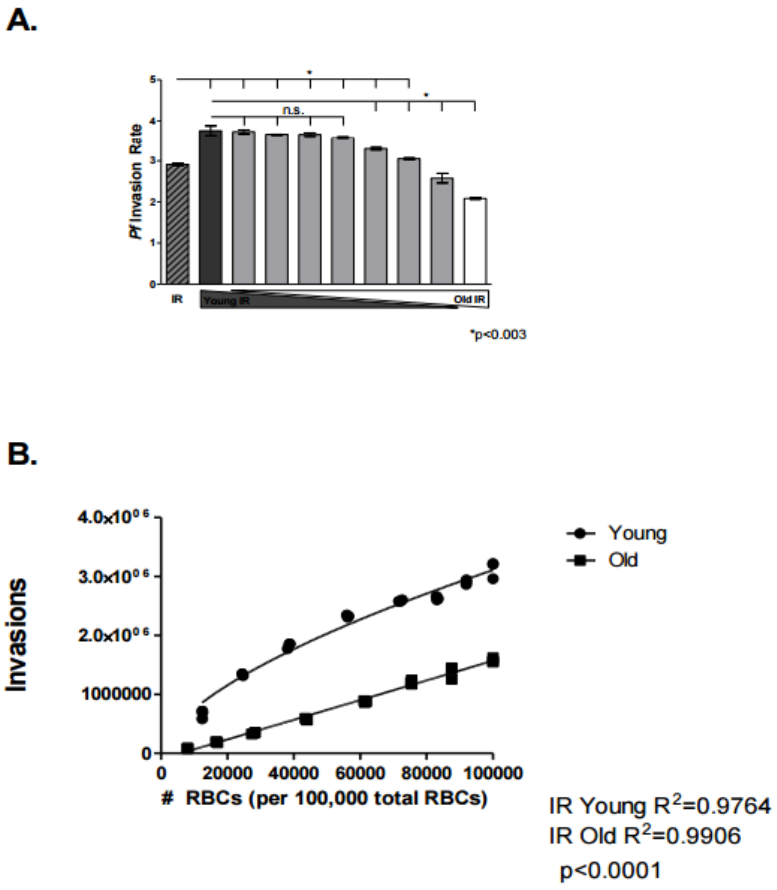
IR R^2=0.9884
IDA R^2=0.9899

p<0.0001

Supplementary Figure S2.3. Reticulocytes and young RBCs support greater *P.falciparum* growth rates and PEMRs *in vitro* than increasingly older RBCs. (A) Percoll density separation of RBCs from an IR donor into five fractions of increasing age: very young (VY), young(Y), young adult (YA), mature adult (MA) and old (O). Briefly, 1mL of RBCs at 50% hematocrit in RPMI were layered on top of a discontinuous Percoll gradient and spun at 1075g for 25 min. Layers were removed, washed, and stored at 4ºC for up to two days prior to use. Density age separation of RBC was confirmed by: (B) MCV, as measured by Beckman Coulter AcT diff2 (Brea, CA), (C) percent reticulocytes, as determined by new methylene blue staining and counting, and (D) fluorescence of density separated RBCs stained with 5 μM calceinAM for 30 min and assessed by flow cytometry. For *P.falciparum* experiments the very young RBC population was combined with the young RBC population and subsequently referred to as young. (E) Growth of *P.falciparum* (strain FCR3-FMG) within age separated RBCs. Data is from a representative experiment of three independent experiments performed in triplicate. Bars represent parasite growth rate after one 96 h growth assay in young, young adult, mature adult and old RBCs. Error bars represent the standard deviation. Significance determined by two-tailed paired Student's t-test (GraphPad, Prism, v.5.04, La Jolla, CA). *p<0.001, compared to *P.falciparum* growth rate in young RBCs. (F) Comparison of the Parasite Erythrocyte Multiplication Rate (PEMR) of *P.falciparum* (strain FCR3-FMG) within age separated RBCs. PEMR experiments were performed as described previously for RBCs from IDA and IR donors. Data is from a representative experiment of three independent experiments performed in triplicate. Bars represent PEMR of *P.falciparum* in young, young adult, mature adult and old RBCs. Error bars represent the standard deviation. Significance determined by two-tailed paired Student's t-test (GraphPad Prism 5, La Jolla, CA). *p<0.001, compared to infectious merozoites produced within young RBCs.


A.
VY
Y
YA
MA
O
B.
MCV
95
90
85
80
UnS VY Y YA MA O
C.
% reticulocytes
10
5
0
UnS VY Y YA MA O
D.
MFI Calcein
30
20
10
0
UnS Y YA MA O
E.
Pf Growth Rate
50
40
30
20
10
0
Y YA MA O
F.
PEMR
8
6
4
2
0
Y YA MA O

Supplementary Figure S2.4. *P.falciparum* plateau in invasion rate of RBC populations containing 50% to 100% young RBCs is independent of merozoites inoculum. (A and B) Invasion of *P.falciparum* (strain FCR3-FMG) into RBC populations in which Violet labeled young IR RBCs were replaced with DDAO labeled old IR RBCs. Differentially labeled young and old RBCs were inoculated individually or together in the same wells at different ratios (100% young; 90% young and 10% old; 80% young and 20% old; 67% young and 33% old; 50% young and 50% old; 33% young and 67% old; 20% young and 80% old; 10% young and 90% old; 100% old). Each invasion condition contained $20x10^6$ total RBCs and was subsequently inoculated with $0.6x10^6$ pRBCs. The invasion rate of *P.falciparum* into total IR RBCs was additionally assessed. Data is from a representative experiment of two independent experiments performed in triplicate with RBCs with *P.falciparum* strains 3D7 and FCR3-FMG. (B) Data shows the number of *P.falciparum* invasions events into young and old RBCs as the frequency of each increases in a RBC population containing 100,000 total RBCs. Circles and squares represent the number of *P.falciparum* invasion events into young and old RBCs respectively as each increases in frequency from 10% to 100% of the total RBC population. Linear regression was used to determine the best fit line for young and old RBC data. A linear function best fit old RBC data (R^2=0.9906) and a logarithmic function best fit young RBC data (R^2=0.9764). ANCOVA was performed to compare to determine whether invasion data of old and young RBCs differed significantly. The null hypothesis was no difference between the two RBC types (H_0: $\beta_{Iron\ replete} = \beta_{Iron\ deficient}$, α=0.05). ANCOVA performed with GraphPad, Prism, v.5.04, La Jolla, CA calculated a p value of <0.0001.



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CHAPTER THREE: BIOPRESERVATION OF RBCS FOR IN VITRO PLASMODIUM FALCIPARUM CULTURE[2]

3.1 OVERVIEW

The *in vitro* culture of *P.falciparum* in red blood cells (RBCs) is essential to studying the molecular and cell biology of the parasite. Current methodologies utilize RBCs within 4 weeks of collection. Here we show that storage of RBCs for greater than two weeks at 4ºC in each of four different media known to preserve RBC integrity, decreases the growth rate of the parasite. The decrease in growth is secondary to decreased invasion of the stored RBCs; there is no impact on the parasite erythrocyte multiplication rate. Finally, we show cryopreserved RBCs support growth of *P.falciparum* and are invaded equally well as freshly drawn RBCs. These data indicate that use of RBCs stored longer than 2 weeks may affect *in vitro* experimentation with *P.falciparum*, but that biopreservation of RBCs at -80ºC is an effective technique for preserving RBCs for *P.falciparum* culture. Our findings demonstrate the utility of biopreservation for RBCs obtained from individuals with rare blood types and for RBCs obtained during field studies.

3.2 INTRODUCTION

Falciparum malaria remains a devastating infectious disease, killing nearly 700,000 people annually. Further understanding of malaria pathogenesis will help identify molecular and cellular targets of next-generation therapeutics. The morbidity and mortality of malaria infection occur during the erythrocytic stage. Study of erythrocytic stage malaria is critical not only for new anti-malarial development, but also for increasing our understanding of the host pathogen relationship. For example, many host genetic polymorphisms known to impact red blood cell (RBC) physiology also alter malaria susceptibility. Methods for culturing erythrocytic stage Plasmodium falciparum were developed more than 30 years ago (1, 2). However, optimization of *in vitro P.falciparum* culture is still under investigation. The

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aspects of intra-erythrocytic development that are impacted by RBC storage, as well as the effects of RBC biopreservation on the intra-erythrocytic life cycle, remain unknown. Studies on RBC storage for human clinical use reveal a relationship between RBC storage and transfusion complications (3). Current blood banking standards involve RBC storage in SAGM (a solution containing saline, adenine, mannitol and glucose) or closely related solutions for up to 42 d at 4°C (4). Many RBC storage lesions have been documented in these acidic medias, including decreased intracellular ATP, 2,3-diphosphoglycerate (2,3-DPG) and potassium; increased intracellular NaCl; oxidative damage; lipid peroxidation; membrane phospholipid changes and vesiculation; decreased deformability; reduced glycolytic capacity; decreased vasodilatory capacity; and increased cytoadhesion (3, 5). Overall, these storage lesions are similar to physiological changes occurring with normal RBC ageing in the blood-stream (6) and could also impact parasite growth, as *P.falciparum* preferentially infects younger RBCs in circulation (7, 8). The use of biopreserved RBCs for human transfusion has been validated (9) and cryopreserved umbilical cord blood cells can propagate *Plasmodium vivax* (10). Here we examine the impact of RBC storage and biopreservation on *P.falciparum* growth and development *in vitro*. Prolonged RBC shelf-life and biopreservation could enhance malaria research by: (i) enabling standardization of the RBC source for multiple experiments, and (ii) increasing access to RBCs from individuals with unusual blood types, nutritional deficiencies, or from remote locations.

3.3 MATERIALS AND METHODS

***P.falciparum* culture.** *P.falciparum* parasite lines FCR3-FMG (MR4, MRA-736) and Dd2 (MR4, MRA-156) were routinely cultured in O-positive (O+) RBCs obtained from healthy individuals at the Clinical and Translational Research Center at the University of North Carolina, Chapel Hill, NC (IRB #09-0559, approved by the University of North Carolina Institutional Review Board). Cultures were maintained with 2% Hematocrit (hct) in complete media containing RPMI 1640 with 10% Albumax II, 1 mM hypoxanthine, 20 mM L-glutamine, 0.45% glucose, and 10 µg/l gentamicin (ACM). Cultures were incubated on a shaker at 37ºC in 5% O_2, 5% CO_2 and 90% Nitrogen. Parasite density was maintained between 0.5% and 10% *P.falciparum*-parasitized RBCs (pRBCs). pRBC cultures were synchronized to within 4-6 hours of each other by first treating cultures with 5% D-sorbitol to select for ring stage

parasites, followed by MACS (Miltenyi Biotec) isolation of hemozoin-containing trophozoite and schizont stage pRBCs.

Growth assay. Cultures were seeded as rings at 0.5% pRBCs and 1% hct in ACM in triplicate into 96 well plates and maintained for 96 hrs before staining. Duplicate plates were also stained at 0 hrs. Plates were stained with 1X DNA dye SYBR green I (Invitrogen) for 30 min at 37ºC and fixed in 1% paraformaldehyde and 0.0075% glutaraldehyde in Alsever's solution (Sigma) for 30 minutes at 4°C for flow cytometry analysis of pRBCs present. Parasite growth rates were determined by dividing final parasitemia at 96 hrs by initial parasitemia at 0 hrs (8).

Parasite erythrocyte multiplication rate (PEMR). Production of infectious daughter merozoites of FCR3 and Dd2 within fresh and stored RBCs was assayed beginning with trophozoite infected O+ cultures MACS purified and seeded into experimental RBCs to achieve 1.5-2% infected RBCs and maintained for 72 hrs to allow for a full growth cycle within each experimental RBC population. Trophozoites in experimental RBCs were MACS isolated and subcultured into a 96 well plate with 2% pRBC at 1% hct in fresh control RBCs in triplicate and maintained for 12-18 hrs to allow for invasion of control RBCs and comparison of infectious merozoites produced within different experimental RBCs. Wells were stained, fixed and analyzed by flow cytometry as above; calculating invasion events divided by initial schizonts seeded provides PEMR data, which represents replication efficiency within each blood condition (8).

RBC barcoding assay. As described in (11), RBCs were labeled at 2% hct in RPMI with 5 µM of either CellTrace Violet or DDAO-SE (Invitrogen) for 2 hrs with shaking at 37°C, washed twice with ACM, incubated 30 minutes in ACM with shaking at 37°C, and finally washed twice with RPMI and stored in RPMI at 4°C. All invasion assays were performed with within two days of labeling. For three color invasion assays, DDAO-SE and Violet labeled RBCs were combined in ACM and delivered in triplicate into a 96 well plate and subsequently seeded with MACS purified trophozoite pRBCs from standard, unlableled O+ cultures to achieve 1.5-2% pRBCs. Parasites were maintained for 12-18 hrs to allow for subsequent invasion of DDAO-SE and Violet RBC populations. Following invasion, cells were stained, fixed, and analyzed by flow cytometry as described above. *P.falciparum* invasion into Violet and DDAO-

SE labeled RBCs was directly compared by measuring the Susceptibility Index (SI), defined as the ratio of the prevalence of infected Violet RBCs to infected DDAO RBCs (8).

Flow cytometry analysis. Flow cytometry was performed at the UNC Flow Cytometry Core Facility on a Beckman-Coulter (Dako) CyAn ADP cytometer. Channels and probes used on the Dako cyan included: CellTrace Violet (405nM excitation, 450/50 bandpass), SYBR Green I (488nm excitation, 530/40 bandpass), and CellTrace DDAO-SE (635nm excitation, 665/20 bandpass). Detector gain settings varied between experiments to optimize signal but were kept constant within individual experiments and spectral overlap compensation was not necessary with this configuration. *P.falciparum* pRBCs were gated on SYBR Green I signal. Dako CyAn data was collected and analyzed with Summit v4.3.01. Linear amplification of forward scatter was used to set event threshold in order to exclude cell debris, microparticles and doublets. For all experiments, samples were diluted to 0.001-0.002% hct and 100,000 total events were collected.

RBC storage conditions. Blood for cultures was routinely drawn into ACD, packed by centrifugation with plasma removed, and stored at 4°C for 2 weeks; aliquots were washed with Alsever's Solution (4.2 g/l NaCl, 9.0 g/l Na-citrate, 20.5 g/l glucose) (Sigma) 3X prior to use, and used for experiments and culture within 4 days after washing. Variations in blood storage included storing packed RBCs at 4°C for up to 6 weeks, or after removal of plasma, overlaying packed RBCs with alternative storage buffers (CPDA, Alsever's Solution, a RBC Buffer (10 mM HEPES, 12 mM NaCl, 115 mM KCl, 5% BSA)) at a ratio of 2:1 packed RBCs:storage media.

RBC cryopreservation. To freeze RBCs, 200µl of fresh packed RBCs was mixed with 300µl plasma, then 500µl freezing media (28% glycerol, 3% sorbitol, 0.65% NaCl) was slowly added and cells were immediately frozen in liquid nitrogen. To use frozen RBCs, cells were quickly thawed, then 200µl of 12% NaCl was added dropwise, and cells were incubated at RT 5 min. Following addition of 10mL 1.6% NaCl, cells were centrifuged 10 min at 2500g. Pellets were mixed with 10ml 0.9% NaCl, and similarly centrifuged. Final pellets were washed 3X in Alsever's Solution prior to immediate culture use. Variations included washing in Alsever's Solution or ACM before freezing, using RBCs without washing post thaw, and storing RBCs at 4°C post-thaw before culture use.

Statistical Methods. All experiment conditions were tested in triplicate. Results depicted are from either one representative experiment of at least three independent experiments or the combined results of at least three independent experiments. Growth rates and PEMR were compared with two-tailed Students t-test and one way ANOVA using GraphPad Prism 5. Invasion assays utilize the SI (described above), an unadjusted odds ratio which allows the determination of the relative risk of the two RBC populations to *P.falciparum* invasion. All statistical analyses for invasion experiments were performed with Stata/IC (v10, Stata Corp, College Station, TX).

3.4 RESULTS

3.4.1 *P.falciparum* growth rates decline proportional to RBC storage length.

Given lingering discrepancies in standard *P.falciparum* culture protocols, we sought to definitively assess RBC shelf-life. To begin, fresh RBCs were collected into acid citrate dextrose (ACD) and stored in aliquots as packed RBCs at 4°C for up to 6 weeks. At 2-week intervals, fresh RBCs were obtained and identically stored to allow for simultaneous comparisons of parasite growth *in vitro* using blood stored for 0, 2, 4 and 6 weeks. Growth in RBCs stored for 2 weeks showed no decrease in standard 96 h growth assays (8). After 4 weeks of storage, growth rates diminished significantly (42% decline for Dd2, 65% for FCR3-FMG). In RBCs stored for 6 weeks, there was very little growth (over 90% decline for both strains Dd2 andFCR3-FMG) (Fig 3.1A).

3.4.2 Differential storage media does not prolong longevity of RBCs for use in *P.falciparum* growth assays.

We next compared parasite growth in RBCs stored for 0, 2 and 4 weeks at 4°C in four different storage buffers. Buffers tested were: (i) ACD; (ii) citrate-phosphate-dextrose-adenine (CPDA), commonly used for malaria culture; (iii) Alsever's Solution, a balanced salt solution routinely used for RBC washing prior to parasite culture; and (iv) "RBC buffer," an alternative balanced salt solution. Growth rates decreased proportionally to storage length in each of the buffers, with a significant decrease after 4 weeks of storage (62% for ACD,56% for CPDA, 54% for Alsever's and 51% for "RBC buffer") (Fig 3.1B). This confirms RBCs destined for parasite culture must be used within 2 weeks of collection and that differential storage media does not prolong their shelf-life fo r*P.falciparum* culture.

3.4.3 The decline in *P.falciparum* growth rates in stored RBCs is primarily due to decreased invasion.

We next sought to determine whether parasite replication and/or invasion were decreased in stored RBCs. To assess replication, we measured the parasite erythrocyte multiplication rate (PEMR), which reflects the number of infectious merozoites produced per schizont (8). We found no statistically significant differences in replication between RBCs stored for 0–4 weeks (Fig 3.1C). Invasion rates were assayed using a RBC barcoding assay (11) in which differentially labelled RBCs (with CellTrace membrane dyes; Life Technologies Corp., Grand Island, NY,USA) were combined in the same wells and seeded with unlabeled trophozoite stage parasitized RBCs. This assay allows direct comparison of parasite invasion into two different RBC populations. Invasion rates decreased as RBC storage time increased (Fig 3.1D). We hypothesize that this reduction in invasion is due to decreases in intracellular ATP content and RBC deformability, which are associated with RBC storage.

3.4.4 Biopreserved RBCs remain suitable for *P.falciparum* growth and invasion assays.

In an effort to increase the shelf-life of RBCs for malaria culture, we turned to biopreservation. RBC freezing/thawing resulted in minimal RBC lysis (Fig 3.2A). Biopreserved RBCs were able to support equally high rates of *P.falciparum* growth as fresh RBCs (Fig 3.2B). No variation of washing before or after freezing/thawing consistently increased biopreserved RBC shelf-life. Successful merozoite invasion of biopreserved RBCs was confirmed using the RBC barcoding assay. No differences were observed between invasion of fresh versus biopreserved RBCs (Fig 3.2C).

3.5 DISCUSSION

Regarding *P.falciparum* in vitro experimentation, the use of extensively stored RBCs could artificially indicate growth differences between donors with divergent RBC phenotypes, when, in reality, differential RBC storage length might primarily contribute to any observed difference. Routine use of outdated RBCs could also result in the artificial selection of strains that preferentially invade atypical RBCs. Our finding that biopreserved RBCs are suitable for P. falciparum assays has vast practical applications. Using biopreserved RBCs minimizes the need to obtain fresh blood, as fresh RBCs could be frozen in aliquots and individually thawed when needed. Our biopreservation method could also facilitate field studies in areas where immediate parasite growth is difficult. Additional studies are needed to

determine if this technique can be applied to ‘variant’ RBCs, such as RBCs from individuals with sickle cell trait or glucose-6-phosphate dehydrogenase deficiency. Freezing RBCs also allows for extensive evaluation of parasite growth in unique blood types and standardization in gene expression assays.

3.6 FIGURES

Figure 3.1. Effects of RBC storage conditions on *Plasmodium falciparum* growth *in vitro*. (A) Growth assays in RBCs stored 0, 2, 4, or 6 weeks in ACD a t4°C. Growth rates in stored RBCs are normalized to fresh RBCs. Error bars represent the standard deviation (SD). *P<0.01, compared to growth rates in fresh RBCs. (B) Growth rates in RBCs drawn into ACD and packed were either stored as is, or in alternative storage media (Alsever's, ACD,CPDA, RBC Buffer). *P<0.01, compared to growth rates in fresh RBCs (RBC Buffer = 10 mmol/l HEPES, 12 mmol/l NaCl, 115 mmol/l KCl, 5% BSA). (C) PEMR in RBCs stored 0, 2, and 4 weeks. PEMR is normalized to that in fresh RBCs. Error bars represent the SD. (D) Susceptibility Index (SI) for fresh RBCs compared to RBCs stored for 0, 2, 4 and 6 weeks. An SI of 1.0 indicates no invasion difference between two RBC populations. The marker represents the SI point estimate and the bars represent the 95% confidence intervals. RBCs, red blood cells; ACD, acid citrate dextrose; CPDA, citrate-phosphate-dextrose-adenine; PEMR, parasite erythrocyte multiplication rate; Dd2, *P.falciparum* Dd2 strain; FCR3-FMG, *P.falciparum* FCR3-FMG strain.

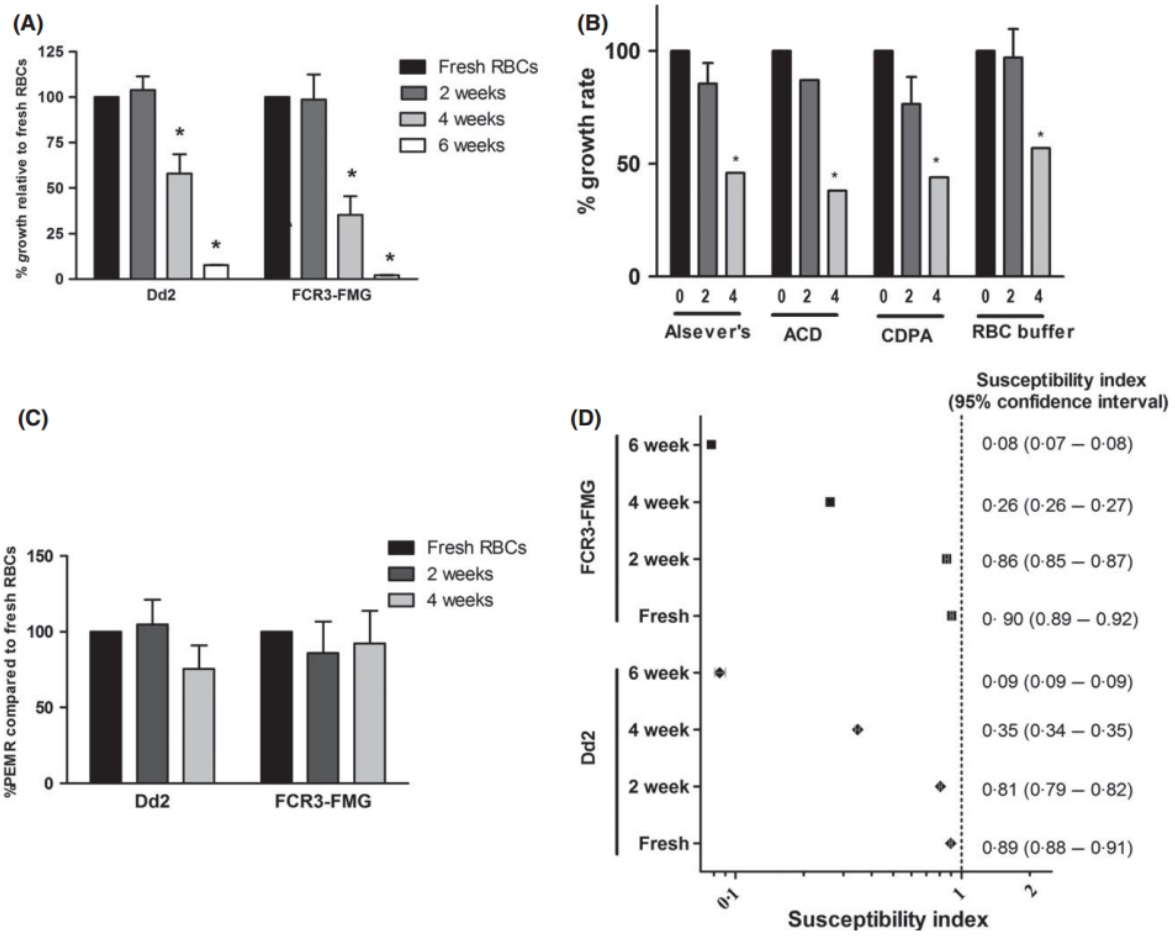
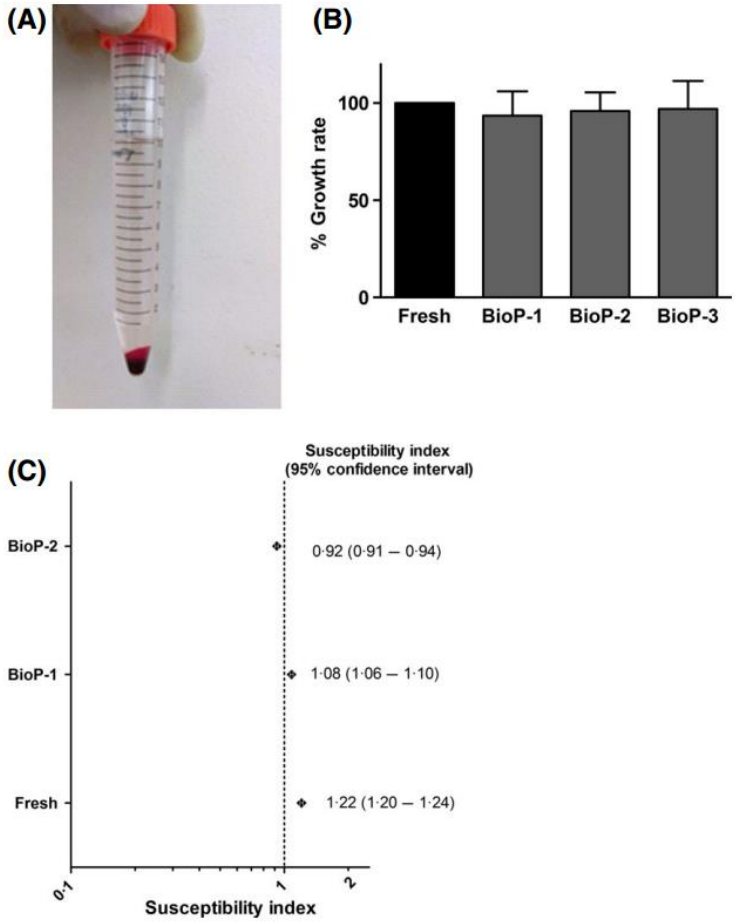


Figure 3.2. Biopreserved red blood cells (RBCs) are suitable for *Plasmodium falciparum* growth. (A) Biopreserved RBCs show minimal lysis after deglycerolization and centrifugation. (B) Comparison of growth rates between fresh RBCs and biopreserved RBCs differentially washed. To freeze RBCs, 200µl of fresh RBCs were mixed with 300µl human plasma, then 500µl freezing media (28% glycerol, 3% sorbitol, 0.65% NaCl) was added and cells were frozen in liquid nitrogen. To thaw, 200µl of 12% NaCl was added to the frozen RBCs, incubated for 5 minutes and pelleted by centrifugation. Subsequently, RBCs were washed with 1.6% NaCl, followed by 0.9% NaCl and used immediately for culture. Variations tested included: washing with Alsever's Solution (BioP-1) or parasite culture media (BioP-2) three times prior to freezing. Cells pre-washed in Alsever's solution were either used directly (BioP-1) or rewashed three times after thawing in Alsever's solution (BioP-3). Error bars represent the standard deviation. (C) Susceptibility Index (SI) for fresh versus biopreserved RBCs. The marker represents the SI point estimate and the bars represent the 95% confidence interval.



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CHAPTER FOUR: ANEMIA OFFERS STRONGER PROTECTION THAN SICKLE-CELL TRAIT AGAINST ERYTHROCYTIC STAGE MALARIA IN VITRO AND THIS PROTECTION IS REVERSED BY IRON SUPPLEMENTATION[3]

4.1 OVERVIEW

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 *P.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 <11g/dl) participating in an iron supplementation trial (ISRCTN07210906) in which they received iron (12mg/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.

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 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.

[3] This work was submitted to *EBioMedicine* on 30 Aug 2016 and was conditionally accepted on 13 Oct 2016 dependent upon formatting revisions (which have not yet been finalized and given back to the journal). The version that appears in this chapter is the version submitted to the journal on 30 Aug 2016, and also includes an extended discussion of data analysis and statistical methodology.

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.

4.2 INTRODUCTION

Malaria and iron deficiency anemia 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 (1). There is clear epidemiological evidence in both children (2–4) and pregnant women (5, 6) that, once established, iron deficiency anemia (IDA) is protective against malaria infection. In fact, in pregnant women, iron deficiency has been shown to reduce risk of placental malaria to a greater extent than multiparity (5).

Multiple studies have raised concern that iron supplementation in malaria-endemic areas may put people at increased risk of acquiring malaria (7–11). Most importantly, a large childhood nutritional supplementation study in Zanzibar was halted due to increased morbidity and mortality in the children receiving iron (12). Subsequently, the WHO abandoned its recommendation for universal iron supplementation and now recommends that, in malarious regions, iron supplements only be given where malaria management and prevention services are present (13, 14). 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 (15). Reducing the prevalence of IDA is one of the six priorities of the WHO's Comprehensive Implementation Plan on Maternal, Infant, and Young Child Nutrition (16). 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 insecticide-treated bed nets 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 (17, 18) – 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 RBCs from anemic African children before, during, and after 12 weeks of iron supplementation.

4.3 MATERIALS AND MEHTODS

Subject recruitment, study design, and blood samples for parasite assays. The children were recruited from 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) (19). (Note we also recruited children in the other two arms of this trial, but only for observation at baseline, pre-randomization/pre-intervention.) As per current WHO recommendations, children in the control arm received 12mg/d iron as ferrous fumarate, given 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 S4.1. Fresh RBCs were obtained from these anemic (Hgb<11g/dl) but otherwise healthy children (6-24m) living in rural Gambia (19). Blood was collected at Days 0 (baseline), 49, and 84 during 12wks of iron supplementation (Figure 4.1) 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 S4.2). For a full description of this embedded observational study, please reference the published protocol (19).

***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 (1). 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 outpatient clinic at MRC Fajara, both located within

the urban/periurban coastal area of The Gambia. Isolates were collected as part of a larger study during the annual malaria transmission seasons (September–January) from 2005–2011, as described in (20).

Growth assay. *In vitro* growth was assessed in fresh, washed RBCs as in (1) for 96h (performed in triplicate for RBCs from each study participant). RBCs from healthy, iron replete adult donors of normal hemoglobin genotype and G6PD status not undergoing iron supplementation served as controls for inter-assay variability. Growth rates represent final 96h parasitemia divided by initial 0h parasitemia (1), analyzed by flow cytometry (see Supplemental Methods). Growth rates in subjects' RBCs were normalized to that in control RBCs assayed simultaneously.

RBC barcoding invasion assay. The assay was performed and analyzed as in (21) using two different concentrations of CellTrace Far Red DDAO (Invitrogen Life Technologies/Molecular Probes): 1uM (high) or 0.1uM (low) (see Supplemental Methods and Supplemental Figure 2 for flow cytometry analysis).

Reticulocyte quantification. Reticulocyte (CD71+) levels in fresh subject RBCs were assessed using PE-conjugated anti-human CD71 antibody (Clone M-A712, BD) and isotype control (Clone G155-178, BD), and analyzed by flow cytometry (see Supplemental Methods) for reticulocyte percent relative to non-anemic control.

Statistics. All experiments were done in triplicate. Growth rates, invasion assays, and hematological data were compared by two-tailed Student's *t*-test, one-way ANOVA, and/or 95% CI values using GraphPad Prism 5.

Multivariate modelling. We employed linear regression to estimate the effect of hematological characteristics on *in vitro* parasite growth rates. First, bivariate associations and their respective 95% CI were calculated between growth rates and hematological and patient characteristics at Day 0. We then used multivariate linear regression – specifically, we estimated the effect of hemoglobin and hemoglobin genotype on growth rate along with their respective 95% CI. We used directed acyclic graphs to identify potential confounders and controlled for them in our modelling approach (22). An *a priori* alpha of 0.05 was used to determine statistical significance. All statistical analyses were performed using R software (RStudio Version 0.99.902).

Population level impact. We compared, at the population level, the relative protection offered by sickle-cell trait carriage and anemia using the following formula: pp(RG-1)/RG (modified from (22)), where pp is the proportion of the population exposed to the protective factor and RG is the relative growth rate associated with that factor. The RG values for sickle-cell trait and hemoglobin were based on the adjusted β coefficients from our multivariate modelling results. In this population the pp for anemia is 0.75 (derived from 688 children <3y in the Kiang West Longitudinal Population Study) (23) and the prevalence of AS is 0.159 (24), and there is no detectable confounding between AS and hemoglobin level.

Ethics approval. The trial from which children were recruited was approved by the MRCG Scientific Coordinating and The Gambia Government/MRC Joint Ethics Committees (SCC 1358) and the UNC IRB (#14-1551). Parents/guardians were given a full description of the study in their native language and provided written signed consent.

Role of the funding source. None of the funding sources had a role in study design, data collection or interpretation, writing of the manuscript, or the decision to submit for publication. The corresponding author had full access to all the data included in the study and assumed final responsibility for the decision to publish; all authors reviewed the report and agreed to submit for publication.

4.4. RESULTS

4.4.1 *P.falciparum* growth is reduced in RBCs from anemic children.

Evaluating *in vitro* parasite growth in RBCs from anemic children at baseline, we consistently found lower parasite growth rates than in RBCs from iron replete individuals. Furthermore, growth was lower in RBCs from those donors with the lowest hemoglobin concentrations (Hgb 7-9g/dl=mean relative growth rate (GR) 32.6%; Hgb 8.1-10g/dl=GR 45.9%; Hgb 10.1-11=GR 55.9%; $p<0.05$ by ANOVA) (Figure 4.2A). Iron panel data indicated some degree of iron deficiency in most participants (Table 4.1). However, as the diagnosis of iron deficiency in children with ongoing inflammation is controversial, we grouped subjects using several common definitions of IDA in an attempt to uncover any further differential impacts on malaria susceptibility. We observed decreased parasite growth in all anemic children independent of the type (e.g. with inflammation or without) and severity of iron deficiency, with no significant differences between groups (Supplemental Figure S4.1).

To further investigate potential confounding effects of inflammation and host genetics on parasite growth, we performed bivariate analysis using *P.falciparum in vitro* growth, hematological, iron, and inflammatory data obtained for subjects prior to iron supplementation to determine which variables influenced parasite growth in anemic children (Table 4.2). Several key variables commonly assumed to affect anemia and/or blood-stage malaria growth were tested. Hemoglobin genotype influence was evaluated solely based on β-globin sickle-cell trait (AS) mutation versus normal β-globin (AA), as other β-globin genotypes (homozygous sickle-cell anemia (SS), hemoglobin C (AC), and a heterozygous combination (SC)) were rare. Hemoglobin concentration, hemoglobin genotype, and mean corpuscular volume (MCV) all significantly influenced parasite growth. G6PD status (normal versus deficient) did not significantly affect parasite growth, nor did age, sex, ferritin, hepcidin, or CRP (Table 4.2). Parasite growth rate decreased 10.7% for every 1g/dl hemoglobin decrease. Additionally, we found parasite growth rate decreased 1.4% for every 1fL decrease in MCV and 18.3% in RBCs from children carrying sickle-cell trait. In order to compare the magnitude of these growth rate effects, we standardized the growth rate differences per standard deviation (SD) of each exposure variable, finding 8.6% and 10.8% decreased parasite growth per SD of hemoglobin and MCV, respectively (Table 4.2). Next, we performed multivariate analysis to determine if the effect of hemoglobin on malaria growth rate was confounded by hemoglobin genotype and vice versa. These variables retained significant effects on malaria growth independently of one other, highlighting the independent impact of both microcytic anemia and sickle-cell trait on malaria growth.

4.4.2 The population level impact on parasite growth is greater from anemia than from sickle-cell trait genotype.

Using our multivariate modelling results, we estimated the population level impact on parasite growth from both sickle-cell trait genotype and anemia in order to assess overall the risk of malaria infection in our study population. Given the prevalence of AS (15.9%) (24) and anemia (75%) (23), we thus calculated the population level impact of malaria growth reduction to be 3.5% from sickle-cell trait and 15.9% from anemia in these Gambian children. Note that this underestimates the protection by anemia because it simply compares anemic (defined as Hgb<11g/dl, 2 SD below the mean) versus non-anemic children. In fact, our population mean Hgb is 3.6 standard deviations below normative data (mean

12g/dl) from healthy African-American children (25); using this comparator the protection offered to the *average* Gambian child would be a 31% reduction in parasite growth rate (see Table 4.2).

4.4.3 *P.falciparum* clinical isolates exhibit decreased growth in RBCs from anemic children.

We additionally evaluated the growth of Gambian clinical *P.falciparum* isolates (952, 998, and 1029) to ensure the observed decreased parasite growth in anemic RBCs was not solely a phenomenon of laboratory adaptation. These field isolates assayed in parallel in RBCs from 5 randomly chosen anemic subjects at baseline (with normal hemoglobin genotype and CRP<5mg/ml) all exhibited decreased growth compared to RBCs from non-anemic individuals (Figure 4.2B). Mean growth rates for all strains were consistently below 100% (FCR3-FMG=51.88% CI= 29.33-74.43%; 952=74.43%, CI= 55.04-93.83%; 998=59.34%, CI= 42.51-76.16%; and 1029=74.94%, CI= 53.31-96.57%).

4.4.4 RBCs from anemic children are resistant to invasion by laboratory and clinical strains of *P.falciparum*.

Next, we used a RBC barcoding assay (21) adapted for field use (Supplemental Figure S4.2) to directly compare parasite invasion into RBCs from anemic children (n=15 for strain FCR3-FMG and n=10 for strain 3D7) versus non-anemic donors. Susceptibility Indices (SI) of RBCs from the anemic donors were significantly decreased using both strains (FCR3-FMG SI=0.77, CI=0.70-0.84; 3D7 SI=0.66, CI=0.54-0.78) (Figure 4.2C). *P.falciparum* clinical isolates from The Gambia (strains 952, 998, and 1029) also exhibited decreased invasion into RBCs from anemic donors (952 SI=0.65, CI=0.58-0.73; 998 SI=0.57, CI=0.42-0.77; and 1029 SI=0.62, CI=0.49-0.75) (Figure 4.2D). These assays confirm the clinical relevance of previous *in vitro* work examining laboratory parasite strains and iron deficient RBCs (1).

4.4.5 Malaria susceptibility increases transiently with iron supplementation.

In order to assess malaria susceptibility following iron supplementation, we investigated *in vitro* parasite growth 49d and 84d after daily iron supplementation compared to baseline. The children were monitored daily for changes in health status and underwent weekly malaria testing. Consistent with the fact that malaria incidence is now low in The Gambia (26), only two malaria cases occurred during our study. Hence, *in vitro* assays offered a way to examine the relationship between growth of malaria parasites in RBCs and changing hematological parameters and capture the window of increased susceptibility. Parasite growth rates in RBCs from study subjects were low on Day 0 (n=158, mean GR

48.51%, CI=42.88-54.14%), increased markedly by Day 49 (n=91, mean GR 120.3%, CI=106.6-133.9%), and then by Day 84 decreased back to levels closer to those seen in non-anemic individuals (n=87, mean GR 80.26%, CI=57.27-103.3%). One-way ANOVA confirmed significant differences in parasite growth rates across Days 0, 49 and 84 ($p<0.0001$) and post-hoc analysis using Tukey's test indicated significant differences between Days 0 and 49 ($p<0.001$), Days 0 and 84 ($p<0.01$), and Days 49 and 84 ($p<0.001$) (Figure 4.3A). Restricting the analysis to paired comparisons within the 35 children with growth measurements at all 3 timepoints, we confirmed the increased growth rate from Day 0 to Day 49 ($p<0.001$) (Supplemental Figure S4.3A**)**.

To further confirm changes in malaria pathogenesis in RBCs from anemic children taking iron, we performed invasion assays to assess subjects' RBC susceptibility before and after iron supplementation in a subset of randomly selected subjects (n=8). The mean SI values of these donors before iron supplementation (SI=0.72; CI=0.60-0.84) and post iron (SI=1.58, CI=1.17-1.99) were significantly different by student's t-test ($p<0.01$) (Supplemental Figure S4.3B).

4.4.6 The population of young RBCs increases in anemic children undergoing iron supplementation.

To assess RBC population age structure, we evaluated levels of CD71-positive early reticulocytes in circulation at Days 0, 49, and 84 for a subset of anemic children undergoing iron supplementation. Relative percent of CD71-positive cells at Day 0 (mean=129%, CI=82-175%) was comparable to non-anemic controls (standardized as 100%), and increased at Day 49 (mean=224%, CI=166-286%) and Day 84 (mean=180%, CI=148-211%). Means were significantly different by one-way repeated measures ANOVA ($p<0.01$), and Tukey's test showed significant difference between Days 0 and 49 only ($p<0.01$) (Figure 4.3B; Supplemental Figure S4.3C).

Further probing host factors which could increase parasite growth rates in RBCs from children undergoing iron supplementation, we assessed RBC surface markers from the same children over time (n=8). We examined changes in surface expression of: glycophorin A (GPA), a sialoglycoprotein affecting RBC charge; CD47, an anti-phagocytic RBC marker; C3b deposition on RBC surfaces; CD35, complement receptor 1; CD55, a decay accelerating factor regulating complement on the cell surface; CD147; and sialic acid, all of which can reflect RBC age and overall membrane integrity and/or have been

implicated in malaria merozoite invasion. We found significantly increased GPA, CD47, CD35 and CD147 levels and significantly decreased C3b deposition at Day 49 ($p<0.01$ for all analyzing means between Day 0 and Day 49 by ANOVA and Tukey's test) (Supplemental Figure S4.4). We were unable to detect differences in CD55 and sialic acid levels. Taken together, these surface marker findings support the idea that overall RBC population age and membrane physiology has shifted toward a younger, healthier RBC population following iron supplementation of anemic children.

4.5 DISCUSSION

Use of *in vitro* growth assays as our primary outcome provided a rare opportunity to systematically examine the cellular determinants of parasite growth in anemic and iron-supplemented children. We demonstrate here that blood stage *in vitro P.falciparum* growth is decreased in RBCs from anemic children and this effect is reversed by iron supplementation.

Defining iron deficiency in children with ongoing infections or inflammation is difficult, and has confounded previous clinical studies trying to determine the protective effect of iron deficiency on malaria susceptibility. Here we show protection offered by anemia is substantial (~10% per standard deviation shift in hemoglobin), and RBCs from children with iron deficiency – no matter the definition criteria nor the presence of potential confounders such as inflammation – consistently reduce parasite growth compared to RBCs from non-anemic individuals. The protection was mediated by anemia rather than iron deficiency. Additionally, the use of clinical parasite isolates from The Gambia confirm this is not merely an artefact of laboratory strains. Notably, at the population level, anemia was estimated to confer at least four times as much protection against blood stage parasite growth than sickle-cell trait. Taken together, this data is evidence that anemia exhibits a profound natural influence on parasite growth beyond even the mostly commonly studied and referenced RBC polymorphisms which evolved due to malaria pressure.

Furthermore, we demonstrate parasite growth increases dramatically relative to baseline in RBCs taken from children during iron supplementation, transiently rising at Day 49 to exceed growth rates in non-anemic controls and remaining elevated at Day 84 relative to baseline. Iron deficient RBCs have a shorter circulation lifetime (90 vs 120 days, on average) and exhibit physiological differences such as microcytosis, decreased deformability, and increased oxidative membrane stress, among other effects –

similar to changes in aged RBCs (27). As parasites preferentially infect young RBCs and reticulocytes (1, 28), we assessed surface markers reflecting RBC age and integrity to provide a picture of the overall health of RBCs in anemic children undergoing iron treatment. Our data suggests that erythropoiesis increased in response to iron, creating a younger population of circulating RBCs. These younger RBCs are most prevalent at Day 49, which matches the largest shifts in malaria growth rates and supports our hypothesis that parasite growth transiently increases following iron supplementation due to *P.falciparum's* preference for young RBCs (1). The study was constrained by the wide intervals between venous bleeds selected for the intervention. At Day 49, it is possible the main iron-induced erythropoietic surge already passed, in which case our data would underestimate the true extent of increased malaria risk.

We also examined merozoite invasion into RBCs from anemic and non-anemic individuals, as our previous work found invasion differences contributed significantly to reduced malaria pathogenesis in iron deficient RBCs (1). We expanded our previous findings to show that RBCs from anemic African children were resistant to invasion with both laboratory and clinical *P.falciparum* strains and that iron supplementation increased invasion susceptibility. Our RBC surface marker data corroborating a shift towards younger, healthier RBCs corresponds with our hypothesis that changes in RBC population structure influence overall malaria risk.

The public health implications of our study are significant, shedding light on the overarching question of whether iron supplements cause harm. We acknowledge that *in vitro* parasite growth might not translate directly to malaria susceptibility. Yet there are no other viable alternatives for addressing this safety aspect regarding iron supplementation in malarious regions. While our system only examined the RBC impact of anemia on malaria growth, eliminating the impact of serum iron or immune cells, the fact that we still observe such profound growth effects highlights the protection afforded by anemia and the need for caution regarding iron supplementation. Furthermore, our results provide insight into why other clinical studies on this topic produce such variable results – given we find increased malaria susceptibility is transient, other studies may miss the window of enhanced susceptibility. We detect significant changes in parasite growth rates despite relatively small changes in hemoglobin levels, emphasizing the impact of iron and RBC population dynamics on *P.falciparum* pathogenesis. Our data clearly show that the safety of iron supplementation must be addressed, even if additional unknown mechanisms contribute to increased

malaria susceptibility. We thus advocate temporary malaria prophylaxis should always accompany iron supplementation for anemic children in malaria endemic areas, though the period of enhanced susceptibility has not been accurately identified by this study. Finally, quantifying the sizeable contribution of anemia to population level protection against malaria, our research raises the question of whether consistently reduced hemoglobin and MCV values in people of African descent are genetic signatures of evolution under significant malaria pressure, much like the hemoglobinopathies.

4.6 TABLES AND FIGURES

Table 4.1: **Blood, inflammatory, and iron parameters of anemic donors whose RBCs were used for parasite growth assays before (Day 0), during (Day 49), and after (Day 84) iron supplementation.** Tests were performed in MRC Keneba laboratories using a Medonic M20M GP and Cobas Integra 400 plus, or in the field using a HemoCue 301. Values in the Normal Range column are the normal or healthy range for each parameter for 6-24 month-olds as defined by standard guidelines (29). Numerical values reflect the mean value of all individuals and values in parentheses indicate standard deviation. Note that control non-anemic donors had an average hemoglobin of 14.13 g/dl (standard deviation 0.85).

Variable	**Normal Range**	**Day 0 n=158 Mean (SD)**	**Day 49 n=91 Mean (SD)**	**Day 84 n=87 Mean (SD)**
White Blood Cell (x10^9 per l)	6-17.0	12.11 (4.34)	12.35 (4.80)	12.22 (3.86)
Hemoglobin (g per dl)	11.0-13.5	9.88 (0.81)	10.68 (0.94)	10.78 (1.04)
Hematocrit (%)	33-39	28.88 (6.34)	28.57 (3.68)	29.67 (5.97)
Mean Corpuscular Volume (fl)	70-86	62.90 (7.66)	64.39 (6.40)	64.80 (6.15)
Mean Corpuscular Hemoglobin Concentration (g per dl)	30-36	34.98 (1.47)	35.16 (1.32)	35.44 (1.18)
Red Cell Distribution Width (%)	12-14	18.06 (2.51)	18.24 (2.38)	17.52 (2.17)
Platelet Count (x10^9 per l)	150-300	430.01 (200.10)	417.44 (172.28)	372.45 (155.27)
Iron Total (µ mol per l)	9-21	4.99 (5.10)	9.24 (5.25)	14.97 (7.21)
Transferrin (g per l)	2-36	3.08 (0.62)	2.91 (0.52)	2.88 (0.56)
Transferrin Saturation (%)	15-39	8.10 (8.76)	13.22 (6.73)	21.75 (11.04)
Ferritin (ng per ml)	12-140	16.55 (17.30)	28.81 (46.50)	22.78 (23.74)
Alpha 1 Anti-glycoprotein (g per l)	<1	1.29 (0.52)	1.27 (0.46)	1.29 (0.46)
C Reactive Protein (mg per dl)	0.8-3.1	6.30 (13.70)	5.19 (7.90)	4.56 (7.61)
Soluble Transferrin Receptor (nmol per l) (30)	1.26-1.23	8.83 (3.84)	8.21 (2.67)	7.36 (3.17)
Soluble Transferrin Receptor: log Ferritin Index	N/A	8.57 (18.24)	7.95 (9.10)	5.62 (7.39)
Hepcidin (ng per ml)	N/A	12.07 (13.73)	13.23 (12.76)	14.42 (12.37)

Table 4.2. Effect of host hemoglobin, iron status, and other hematological characteristics on *in vitro P. falciparum* growth in RBCs from anemic children (Hgb<11g/dl) at baseline. Growth rates (GR) were calculated relative to growth in healthy, non-anemic donors. Growth assays were performed in triplicate for each donor and the average value was used for linear regression modelling; multivariate analyses represent the estimated association for a given variable while controlling for potential confounders. Hgb genotype was evaluated solely based on AA vs. AS classification (too few individuals for statistical evaluation of SS genotypes) and G6PD status was evaluated solely based on normal vs. deficient classification. For continuous variables, the β_1 value represents the %GR change (x100) for every 1 unit increase in the primary variable. For categorical variables, the β_1 value represents the %GR change (x100) based on yes-no genotype. For example, for Hgb AS, the %GR change is -18.3% relative to Hgb AA. Significant p values (<0.05) are bolded. The adjusted %GR change for Hgb and MCV is calculated based on the SD for the exposure variable of interest (see Table 1) multiplied by β_1 (x100%), to give the %GR change for every 1 SD change in the exposure variable; for Hgb genotype the adjusted %GR change is simply β_1 (x100%).

Condition	**β_1 Value**	**Lower CI**	**Upper CI**	**p Value**	**Adjusted % GR Change**
Bivariate analysis of measures affecting parasite growth					
Hgb (g/dl)	0.107	0.039	0.174	**0.002**	8.6%
Hgb genotype (AA vs AS)	-0.183	-0.318	-0.047	**0.009**	-18.3%
MCV (fL)	0.014	0.007	0.021	**<0.001**	10.8%
G6PD status (normal vs deficient)	0.051	-0.206	0.309	0.696	
Ferritin (ng/ml)	0.002	-0.002	0.005	0.290	
Hepcidin (ng/ml)	0.004	0.000	0.008	0.074	
CRP (mg/dl)	-0.002	-0.006	0.002	0.360	
sTfR:log ferritin ratio	-0.001	-0.004	0.003	0.702	
Transferrin saturation (%)	0.431	-0.307	1.169	0.255	
Multivariate analysis of significant measures affecting parasite growth controlling for possible confounders					
Hgb affects parasite growth controlling for Hgb genotype	0.103	0.036	0.170	**0.003**	8.3%
Hgb genotype affects parasite growth controlling for Hgb	-0.179	-0.312	-0.047	**0.009**	-17.9%

Figure 4.1: 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, 2.5mL of venous blood was drawn directly into microvette tubes containing CPDA-1 (Sarstedt, Germany). Unavailable donors include safety exclusion (Hgb<7g/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 9.3% at Day 0, 17.0% at Day 49, and 20.7% 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).

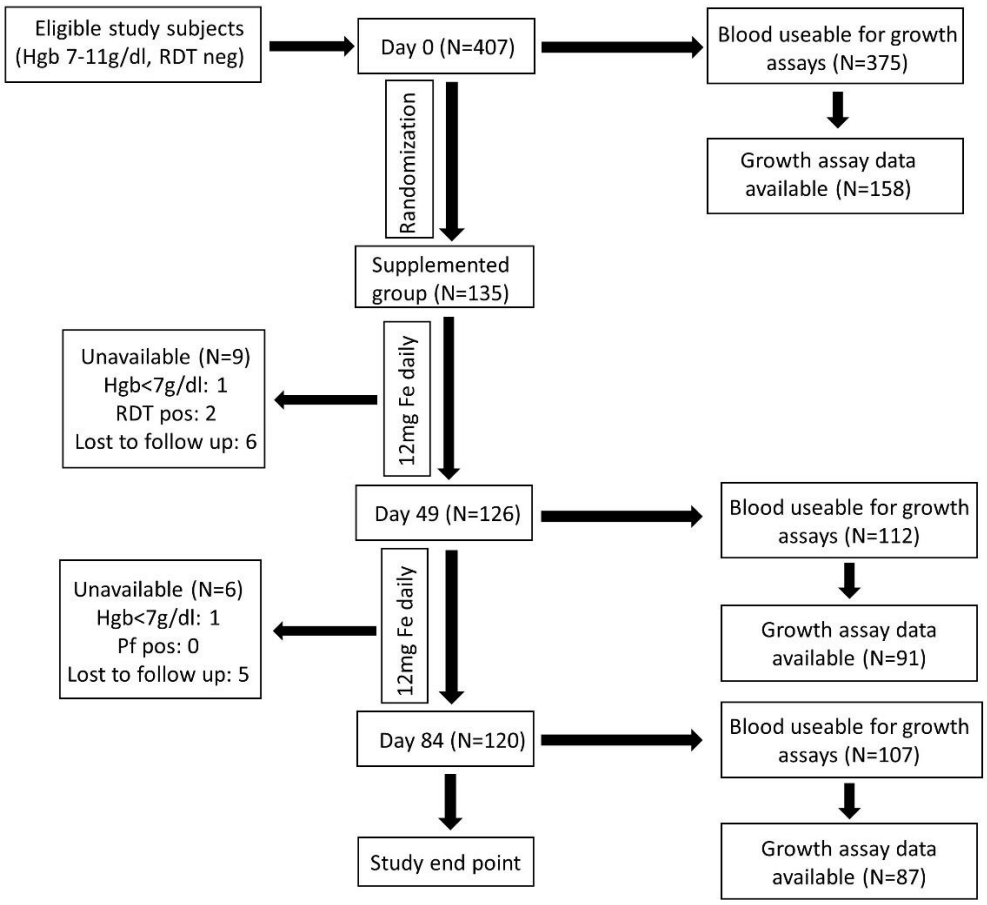


Figure 4.2: Parasite growth and invasion in RBCs from anemic children (Hgb<11g/dl) at baseline. A) *P.falciparum* (strain FCR3-FMG) growth rates are proportional to hemoglobin concentration. Growth assays were performed in RBCs drawn from anemic children at baseline (Day 0) and values are presented relative to growth in RBCs from non-anemic donors. Each dot represents the mean result of triplicate growth assays from each donor and the error bars represent 95% CI. One-way ANOVA indicates the means are significantly different between Days ($p<0.05$); specifically, post-hoc analysis with Tukey's test indicates significant differences between Hgb levels 7-9g/dl and 10.1-11g/dl (*$p<0.05$). B) *P.falciparum* clinical isolates from The Gambia exhibit decreased growth in RBCs from anemic children at Day 0. Growth of 3 different clinical strains (952, 998, 1029) was compared to growth of a laboratory strain (FCR3-FMG) in RBCs from five anemic children. Each dot represents the mean result of triplicate growth assays from each donor relative to growth in non-anemic RBCs and error bars represent the 95% CI. The mean relative growth rate in anemic RBCs for each strain is decreased compared to 100% growth in non-anemic RBCs. C) Direct comparison of invasion into RBCs from anemic and non-anemic donors using *P.falciparum* laboratory strains. Invasion experiments for RBCs from all anemic donors (drawn at Day 0) were performed independently and each experiment was performed in triplicate. Data show the mean SI using RBCs from 10 anemic donors for strain 3D7 and 15 for FCR3-FMG. The SI defines the relative susceptibility to invasion of two different types of RBCs. The marker represents the SI point estimate and the bar represents the 95% CI. An SI of 1.0 indicates no difference in parasite invasion of two RBC populations. Both strains 3D7 and FCR3-FMG give SI values significantly decreased from the control value of 1.0. D) Direct comparison of invasion into RBCs from either anemic or non-anemic donors using clinical strains of *P.falciparum*. Invasion experiments for RBCs from all anemic donors (drawn at Day 0) were performed independently and each experiment was performed in triplicate. Data show the mean SI using RBCs from 5 anemic donors for all strains (FCR3-FMG, 952, 998, 1029). The marker represents the SI point estimate and the bar represents the 95% CI. An SI of 1.0 indicates no difference in parasite invasion of two RBC populations.

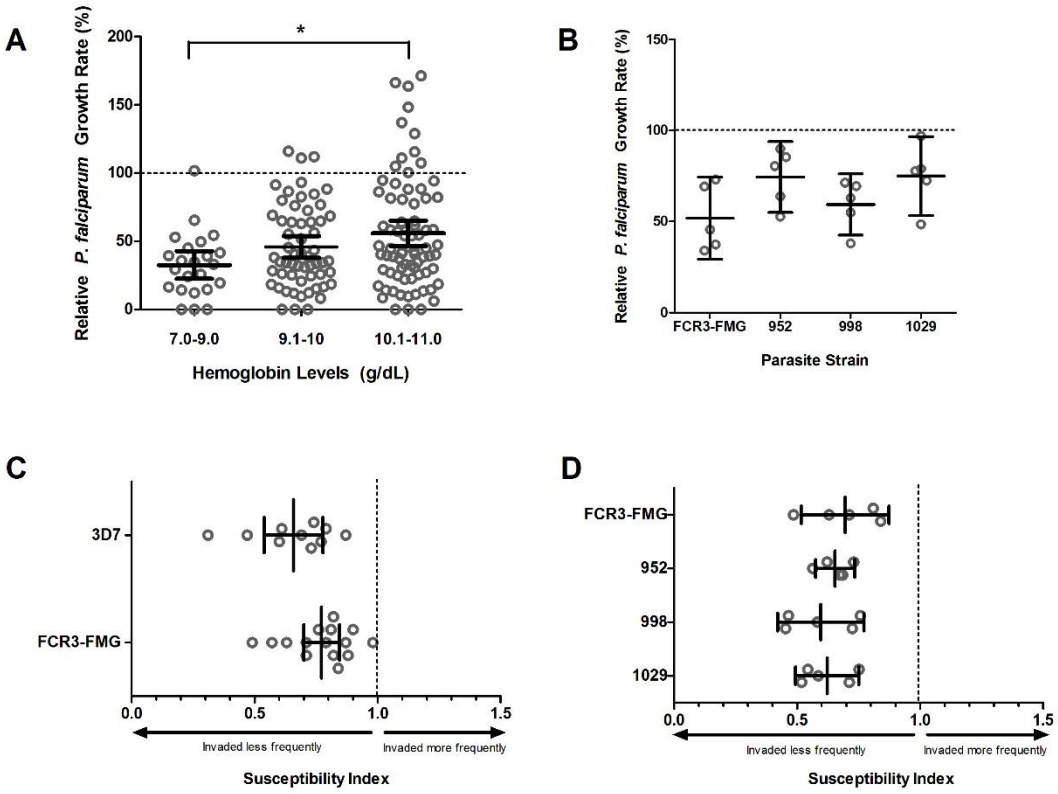
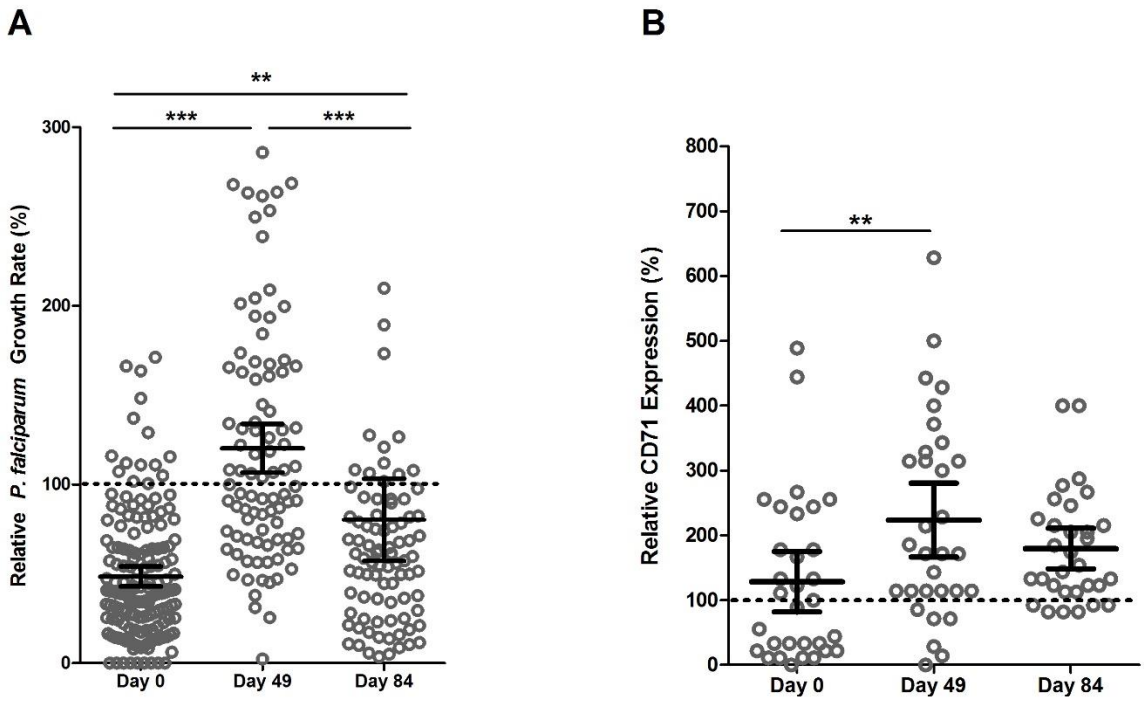


Figure 4.3: Malaria susceptibility increases transiently during iron supplementation and anemic children receiving iron supplements have increased numbers of young RBCs. A) *P. falciparum in vitro* growth rates in RBCs from anemic children increase over time with iron supplementation (12mg iron daily for 84d). Parasite growth assays were conducted in RBCs from children at Day 0, Day 49, and Day 84 using strain FCR3-FMG. Growth rates are reported relative to growth in RBCs from non-anemic donors. Each dot represents the mean of triplicate assays and error bars represent the 95% CI. Differences between growth rates at the different timepoints were significant ($p<0.0001$ by one-way ANOVA); specifically, post-hoc analysis with Tukey's test indicates significant differences between Day 0 and Day 49 (***$p<0.001$) and Day 49 and Day 84 (***$p<0.001$), as well as Day 0 and Day 84 (**$p<0.01$). n=158 children at Day 0, n=91 children at Day 49, and n=87 children at Day 84. B) Levels of CD71 positive RBCs increase over time in anemic children undergoing iron supplementation. Percent CD71-positive RBCs was measured by flow cytometry analysis of CD71 surface expression. Error bars represent the 95% CI; one-way repeated measures ANOVA indicates the means are significantly different between Days ($p<0.01$, n=31); post-hoc analysis with Tukey's test indicates significant differences between Day 0 and Day 49 (**$p<0.001$) but not between Day 49 and Day 84, nor Day 0 and Day 84.



4.7 SUPPLEMENTAL INFORMATION

4.7.1 Supplemental Methods

Micronutrient power. The iron supplementation product used in this trial is a micronutrient powder (MNP, MixMe WHO) produced by the DSM Company and distributed routinely by the United Nations Children's Fund and the World Food Program (contains 10mg of iron). For our study purposes the iron concentration was altered to contain 12mg iron/sachet (daily dose). The MNP contained Vitamin A (400µg RE), Vitamin D (5µg), Vitamin E (5mg), Vitamin C (30mg), Thiamine B1 (0.5mg), Riboflavin B2 (0.5mg), Niacin B3 (6g), Pyridoxine B6 (0.5mg), Cobalamine B12 (0.9µg), Folate (150g), Zinc (4.1mg), Copper (0.56mg), Selenium (17µg), and Iodine (90g).

Flow cytometry analysis. Flow cytometry was performed onsite at MRCG using a BD Accuri C6 flow cytometer. Channels and probes used included: SYBR Green I, FITC, and Alexa Fluor® 488 (488nm excitation with a 530/30nm bandpass emission filter, detector FL1); PE (488nm excitation with a 585/40nm bandpass emission filter, detector FL2); and CellTrace Far Red DDAO and Alexa Fluor® 647 (640nm excitation with a 675/25nm bandpass emission filter, detector FL4). Detector gain setting changes and compensation were not necessary with this configuration. Accuri C6 data was collected and analyzed with Accuri software (BD Accuri CSampler Analysis Software). Linear amplification of forward scatter was used to set event threshold in order to exclude cell debris, microparticles and doublets. For all experiments, samples were diluted to 0.001-0.002% hematocrit and ≥ 100,000 total events were collected.

RBC surface marker assessment. RBC surface protein levels were determined by staining with fluorescently tagged antibodies followed by flow cytometry analysis. The relative expression fluorescence of each surface marker for each study sample was calculated using the MFI values (corrected for background), relative to MFI in the non-anemic donor. The exception was C3b for which the relative expression was reported as a percent of positively stained RBCs relative to the non-anemic donor.

Antibodies. The following antibodies were used: for CD35 (Mouse Anti-Human CD35 Clone E11 (BD) primary 1:2000; Alexa Fluor® 647 Rabbit Anti-Mouse IgG secondary 1:2000); for CD47 (Mouse Anti-Human CD47 Clone B6H12 primary 1:2000; Alexa Fluor® 647 Rabbit Anti-Mouse IgG secondary 1:500); for CD55 (Mouse Anti-Human CD55-PE conjugate Antibody NaM16-4D3 (Santa Cruz Biotechnologies)

1:10); for CD147 (Mouse Anti-Human CD147 Clone HIM6 (BD) primary 1:500; Alexa Fluor® 647 Rabbit Anti-Mouse IgG secondary 1:500); for Glycophorin A (GPA) (primary Rabbit Anti-Human CD235a/Glycophorin A (ThermoFisher Scientific/Pierce) 1:500 and secondary Alexa Fluor® 647 Goat Anti-Rabbit 1:2000); for sialic acid residues (Wheat Germ Agglutinin Alexa Fluor® 488 Conjugate (ThermoFisher Scientific/Molecular Probes) 1:2000); and for C3b deposition (primary Mouse Anti-Human Complement C3b Antibody 10C7 (ThermoFisher Scientific) 1:200; Alexa Fluor® 647 Rabbit Anti-Mouse IgG secondary 1:500).

4.7.2 Supplemental Tables and Figures

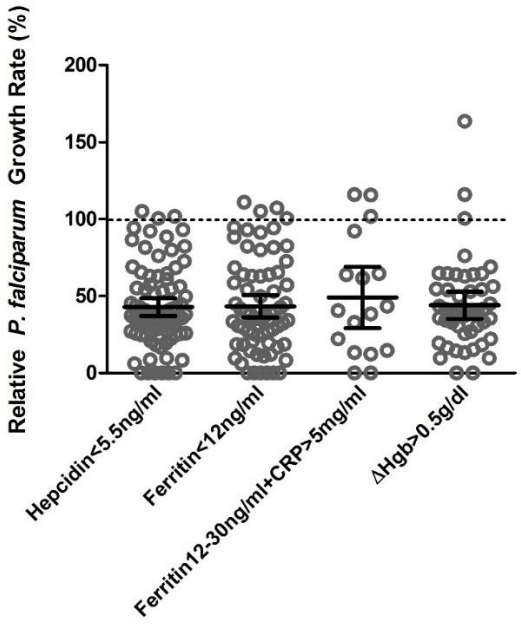
Supplemental Table S4.1: Description of subjects. Hemoglobin genotyping was performed by electrophoresis. G6PD enzyme activity was performed by commercial kit. *A portion of subjects (% of total) were unable to be tested for G6PD status or hemoglobin genotype so are not included in the denominator for other % of total calculations.

Description of study population at baseline			
		All participants (n=407)	Those to receive 12 mg iron daily (n=135)
Age, months, mean (SD)		15.4 (4.4)	15.5 (4.4)
Sex (%)			
	Female	209 (51.2)	71 (52.6)
	Male	198 (48.5)	64 (47.4)
Z score, Wt for ht, mean (SD)		-0.91 (0.89)	-0.76 (0.84)
G6PD Status, n (% of those tested)			
	Normal	312 (93.7)	99 (93.3)
	Deficient	21 (6.3)	7 (6.6)
	Untested*	74 (18.2)	29 (21.3)
Hemoglobin genotype, n (%)			
	AA	326 (81.5)	110 (83.3)
	AS	66 (16.5)	19 (14.4)
	SS	6 (1.5)	2 (1.5)
	AC	1 (0.3)	0 (0.0)
	SC	1 (0.3)	1 (0.8)
	Untested*	7 (1.7)	3 (2.2)

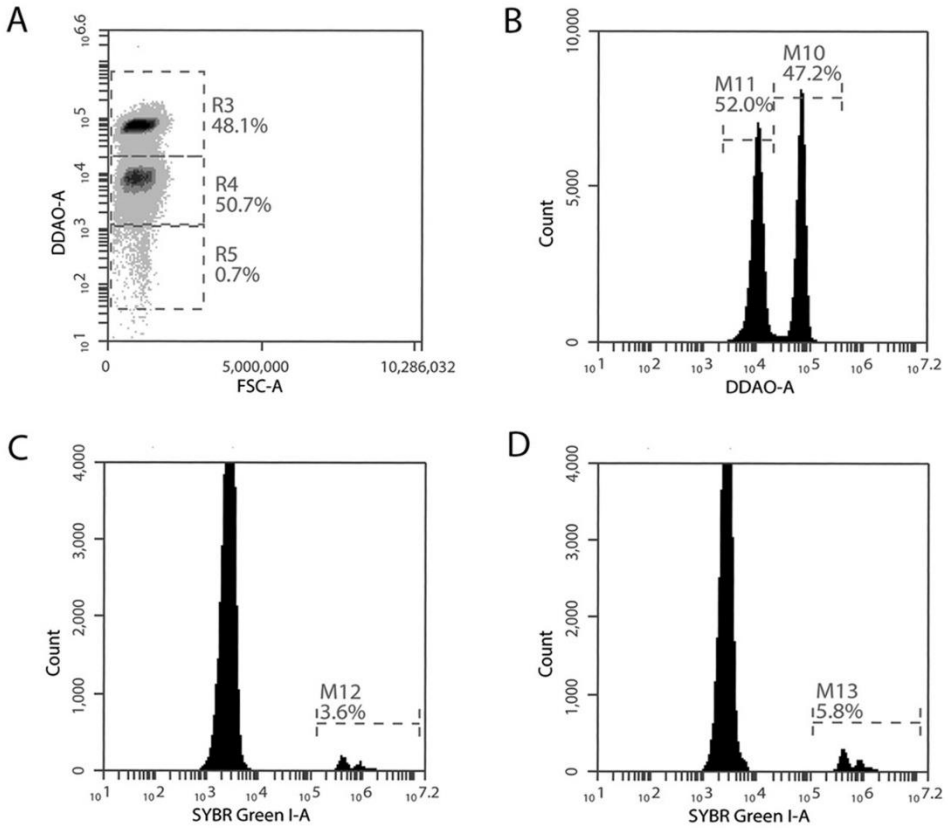
Supplemental Table S4.2: **Blood, inflammatory, and iron parameters of study participants with reportable parasite growth rate data versus those without.** Numerical values reflect the mean value of all individuals of a particular category and time point, and values in parentheses are the SD.

Variable	D0 w/ GR n=158 Mean (SD)	D0 no GR n=249 Mean (SD)	D49 w/ GR n=91 Mean (SD)	D49 no GR n=35 Mean (SD)	D84 w/ GR n=87 Mean (SD)	D84 no GR n=33 Mean (SD)
White Blood Cell (x10^9 per l)	12.11 (4.34)	11.83 (3.82)	12.35 (4.80)	11.21 (2.69)	12.22 (3.86)	10.86 (2.54)
Red Blood Cell (x10^12 per l)	4.62 (1.00)	4.38 (0.63)	4.64 (0.62)	4.58 (0.42)	4.59 (0.85)	4.34 (0.33)
Hemoglobin (g per dl)	9.88 (0.81)	9.85 (0.82)	10.68 (0.94)	11.05 (1.21)	10.78 (1.04)	11.32 (1.04)
Hematocrit (%)	28.88 (6.34)	27.30 (3.90)	28.57 (3.68)	30.35 (3.93)	29.67 (5.97)	29.44 (3.00)
Mean Corpuscular Volume (fl)	62.90 (7.66)	62.72 (6.84)	64.39 (6.40)	66.27 (6.21)	64.80 (6.15)	67.84 (5.72)
Mean Corpuscular Hemoglobin (pg)	22.02 (2.92)	22.17 (2.84)	22.66 (2.56)	23.11 (2.43)	22.97 (2.33)	23.63 (2.20)
Mean Corpuscular Hemoglobin Concentration (g per dl)	34.98 (1.47)	35.29 (1.39)	35.16 (1.32)	34.88 (1.11)	35.44 (1.18)	34.82 (1.30)
Red Cell Distribution Width (%)	18.06 (2.51)	17.58 (2.68)	18.23 (2.38)	18.13 (2.46)	17.52 (2.17)	18.69 (2.21)
Mean Platelet Volume (fl)	7.62 (0.62)	7.70 (0.48)	7.76 (0.60)	7.85 (0.55)	7.90 (1.19)	7.62 (0.44)
Platelet Count (x10^9 per l)	430.00 (200.10)	459.21 (196.49)	417.44 (172.28)	386.64 (187.22)	372.45 (155.27)	370.35 (152.33)
Iron Total (µ mol per l)	4.99 (5.10)	6.18 (4.88)	9.24 (5.25)	9.53 (4.89)	14.97 (7.21)	17.23 (12.77)
Transferrin (g per l)	3.07 (0.62)	3.22 (0.66)	2.91 (1.52)	2.90 (0.48)	2.88 (0.56)	2.94 (0.54)
Transferrin Iron Binding Capacity (µ mol per l)	66.31 (11.50)	72.06 (16.95)	69.21 (12.40)	76.19 (11.88)	70.27 (13.62)	74.82 (11.45)
Transferrin Saturation (%)	8.10 (8.76)	9.04 (7.01)	13.22 (6.72)	12.60 (6.43)	21.75 (11.04)	23.12 (15.31)
Ferritin (ng per ml)	16.55 (17.30)	20.89 (28.33)	28.80 (4.50)	20.88 (13.58)	22.78 (23.74)	30.95 (27.07)
Alpha 1 Anti-glycoprotein (g per l)	1.29 (0.52)	1.24 (0.52)	1.27 (0.46)	1.02 (0.40)	1.29 (0.46)	1.04 (0.30)
C Reactive Protein (mg per dl)	6.30 (13.70)	6.27 (14.99)	5.19 (7.90)	4.87 (11.55)	4.56 (7.61)	3.15 (5.76)
Soluble Transferrin Receptor (nmol per l)	8.83 (3.84)	8.99 (3.84)	8.21 (2.67)	7.84 (2.47)	7.36 (3.17)	6.91 (2.13)
Soluble Transferrin Receptor: log Ferritin Index	8.57 (18.24)	7.09 (28.79)	7.95 (9.10)	4.43 (13.08)	5.62 (7.39)	4.51 (4.80)
Unsaturated Iron Binding Capacity (µ mol per l)	61.33 (13.17)	66.54 (17.17)	60.97 (11.91)	66.80 (11.75)	55.53 (15.23)	57.59 (15.47)
Hepcidin (ng per ml)	12.07 (13.73)	11.79 (11.61)	13.23 (12.78)	11.49 (12.89)	14.42 (12.37)	12.12 (10.95)

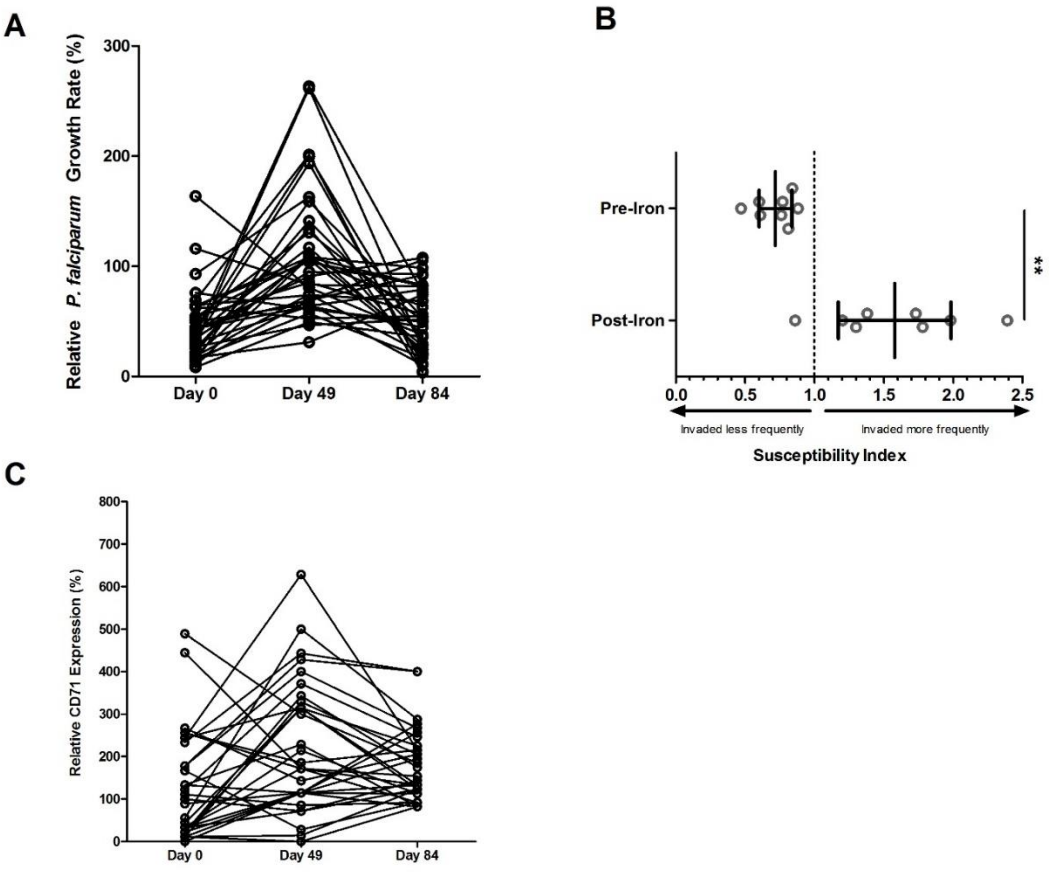
Supplemental Figure S4.1: Parasite growth rates in RBCs from children categorized by different definitions of anemia at baseline. In analysis of parasite growth rates in RBCs from children at Day 0, we stratified participants (all anemic) using four different definitions to categorize the severity and type of iron deficiency in the presence or absence of inflammation: those with 1) hepcidin<5.5ng/ml (n=82); 2) ferritin<12ng/ml (n=69); 3) ferritin12-30ng/ml with CRP>5mg/ml (n=17); 4) hemoglobin increase of >0.5g/dl from baseline after 49d or 84d of daily iron supplementation (n=46); definitions 1-4 are not necessarily mutually exclusive. Of note, everyone in our population had a raised serum transferrin receptor (sTfR):log ferritin index >2 which is highly suggestive of iron deficiency. Growth rate values are presented relative to growth in RBCs from non-anemic donors. Each dot represents the mean result of triplicate growth assays from each donor and the error bars represent 95% CI. Mean growth rate results (with 95%CI) are: hepcidin<5.5ng/ml=42.89% (37.11-48.67%); ferritin<12ng/ml=43.34% (36.01-50.68%); ferritin 12-30ng/ml with CRP>5mg/ml=49.08% (29.16-69.00%), ΔHgb>0.5g/dl=44.04% (35.14-52.93%). There are no significant differences between the means.



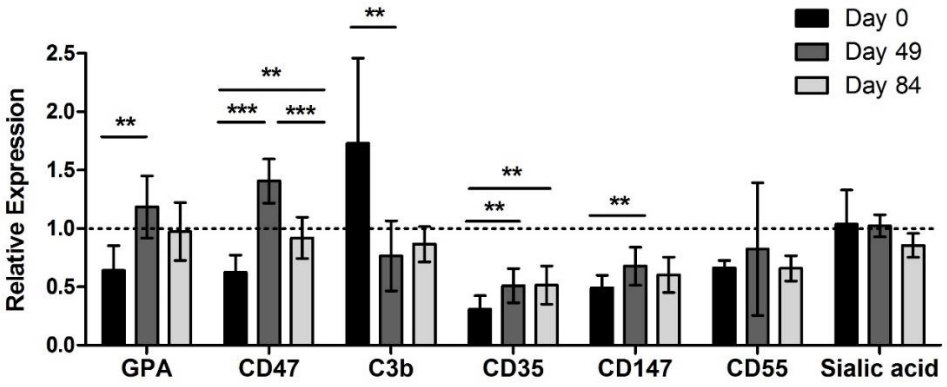
Supplemental Figure S4.2: **Gating strategy to highlight adaptation of RBC barcoding assay to the field setting using basic two-color flow cytometry.** A) RBCs from different blood donors are differentially labelled with CellTrace Far Red DDAO (1 µM (R3) or 0.1 µM (R4)) to distinguish between donor populations. Late stage purified parasites grown in unlabeled RBCs (R5) are seeded into the differentially labelled RBCs which have been combined in equal proportion. B) M10 represents the 1 µM Far Red DDAO labelled RBCs from a non-anemic donor and M11 represents the 0.1 µM Far Red DDAO labelled RBCs from an anemic donor. Gating cells on M11 (C) or M10 (D) allows for Sybr Green I DNA dye detection of parasite infected RBCs in the RBCs from anemic donors (M12, C) or from non-anemic donors (M13, D). Parasitemia in each cell population is compared to calculate the invasion SI.



Supplemental Figure S4.3. Changes in parasite growth, invasion, and reticulocytosis in RBCs from anemic children before and after daily iron supplementation. A) Levels of parasite growth rates increase over time in anemic children undergoing iron supplementation, as depicted by line graph in order to highlight changes for each individual that had data available at all timepoints (n=35 children with complete repeat growth measures at Day 0, 49, and 84, with 86% having increased growth rate at Day 49) One-way repeated measures ANOVA of growth rate values indicates the means are significantly different between Days ($p<0.0001$); post-hoc analysis with Tukey's test indicates significant differences between Day 0 and Day 49 means ($p<0.001$) and Day 49 and Day 84 means ($p<0.001$), but no significance between Day 0 and Day 84 for those children with repeat measures. B) Direct comparison of invasion into RBCs from non-anemic donors to RBCs from 8 anemic children either before or during 12mg daily iron supplementation. Each experiment was performed in triplicate for each blood donor. The marker represents the SI point estimate and the bar represents the 95% CI. An SI of 1.0 indicates no difference in parasite invasion of the two RBC populations. Student's t-test indicates significant differences between pre- and post-iron SI values (**$p<0.01$). C) Line graph of CD71 repeated measures (n=31 children with complete repeat CD71 measures at Day 0, 49, and 84). In 21 of these children, the relative percent CD71 positive cells increased from Day 0 to Day 49. See Figure 3B for repeated measures ANOVA statistics.



Supplemental Figure S.4.4: Surface markers of RBC age and integrity change in a pattern consistent with an increase in erythropoiesis in anemic children undergoing iron supplementation (12mg daily). We measured GPA (an abundant sialoglycoprotein which contributes to RBC surface charge and is found at higher levels on younger RBCs (31)), CD47 (an anti-phagocytic marker which influences RBC senescence and is found in lower levels in RBCs that have been in circulation longer or are less healthy (32)), surface deposition of complement factor C3b (higher levels of which would correlate with increased RBC time in circulation, or less healthy RBC membranes (33)), and levels of *P.falciparum* merozoite receptors (CD35, CD147, CD55, and sialic acid residues). Note that GPA is also a merozoite receptor, and CD35 and CD55 involved in the complement system have also been described as reflecting RBC age (more abundant on younger/healthier RBCs (33)), as has sialic acid abundance (reduced on older RBCs) (32). CD147, known as basigin, is the only known essential *P.falciparum* invasion receptor (34). Data represent relative expression based on anemic donor RBC MFI values (GPA, CD47, CD35, CD147, CD55, and sialic acid residues) or percent positive population values (C3b), compared to RBCs from a non-anemic donor not receiving iron supplementation (relative expression=1.0). RBCs from the same 8 donors were examined over time. Error bars represent the 95% CIs. If indicated, one-way repeated measures ANOVA with post-hoc Tukey's test analysis indicates significant difference between expression levels (*p<0.05, **p<0.01, ***p<0.001).



4.8 DISCUSSION ADDENDUM

4.8.1 Linear regression and population level impact: results, interpretations, and alternative analyses.

Dr. Meshnick and his Ph.D. student Jordan Cates have kindly offered some very specific points about the analytical approach used in the previous chapter. Below, we address these issues point by point and present the same data as analyzed by suggested alternative methods.

You are extrapolating from an in vitro finding (parasite growth) to a disease (malaria).

We understand this comment, and will modify the final published manuscript to make it clearer that this is a translational research study that was a sub-study within a clinical trial. It is not an epidemiological study; we did *in vitro* assays with clinical samples. We are not trying to make policy statements or clinical recommendations. We are contributing to the evidence base on malaria and iron. These results will help people design future clinical trials on the impact of iron supplementation and risk of clinical malaria and/or on the interaction of sickle-cell trait and anemia.

Instead of adjusting the β effect to be based on standard deviation, can you use something like "every 10 units" of a given variable? This might make your results more applicable to a broader population because currently your presentation of growth rate changes for every standard deviation of a given variable amongst your subjects is only relevant to your specific study population.

We see the point that it would be nice to have our growth rate changes be interpretable to any population of anemic children, not just the kids in our study. However we disagree with this method because attempting to compare 10 units of MCV (femtoliters) to 10 units of Hgb (g/dl), for example, the degree of growth rate changes become very difficult to interpret in relation to one another as the units of measurement can be so vastly different and have such different normal spread. Thus, results presented in this manner would not be clinically interpretable. We have renamed our "adjusted" growth rate which represents the change for every one degree of standard deviation change of that variable in our population to be the "standardized" growth rate to better fit with epidemiological terminology.

Multivariate linear regression analysis of growth rate versus hemoglobin or hemoglobin genotype, controlling for one another, doesn't completely make sense because hemoglobin cannot influence hemoglobin genotype.

As was correctly pointed out, hemoglobin cannot influence sickle-cell trait genotype because it is not possible for an individual's genotype to change based on hematological variables. However, in reverse, we do need to control for sickle-cell trait when examining the effect of hemoglobin on parasite growth, because sickle-cell trait carrier status can in fact influence hemoglobin levels. Fortunately, as our multivariate results controlling for hemoglobin and hemoglobin genotype do not affect the relationship of parasite growth to hemoglobin or sickle-cell trait genotype, in our case we can effectively ignore this issue.

It is impossible for you to say anything about the influence of "anemia" on parasite growth since all of your subjects are anemic, so you do not have growth rate data from non-anemic people within your dataset.

We understand this comment and it is certainly a limitation for our *in vitro* parasite study that the enrollment criteria for the clinical trial from which we obtained RBC samples was for children to be anemic (Hgb 7-11g/dl). We can make this distinction clearer in the final manuscript text, including statements like "within our study population of people with anemia, we find the degree of anemia affects parasite growth rate." The same statement would apply for the sickle-cell genotype: "within our study population of people with anemia, those with sickle-cell trait have lower growth rates than those without."

Non-anemic subjects were not enrolled in the iron supplementation study, but RBCs from non-anemic control donors were independently included as a reference in all of our experiments. The dependent variable of parasite growth in our linear regression model is a calculated number. Growth assays were incubated for 96 hours and parasite growth is calculated by dividing the final 96h parasitemia by the initial 0h parasitemia. The relative growth rate for each individual experimental RBC sample is then calculated by dividing the parasite growth rate in the experimental RBCs by the parasite growth rate in RBCs from control non-anemic RBCs assayed in parallel. Every growth assay we set up

included parasites grown in control non-anemic RBCs to be able to make these comparisons. We ultimately agree we must make it clear we were restricted to looking at people with anemia, even though all our data is normalized to non-anemic controls. Normalizing the data to non-anemic controls does not fully take care of the problem. Thus to make a specific inference about someone with Hgb>11 based on our linear regression results is wrong because we do not have data collected on subjects with Hgb>11; we only generally know that parasite growth at Day 0 prior to iron supplementation is lower in anemic versus non-anemic individuals. However this general knowledge, which relates back to our date being reported as "relative" growth rates, does allow us to still make statements saying that parasite growth is lower in RBCs from anemic study subjects than from non-anemic controls. There is further discussion of this point below in relation to our "population level impact" calculations.

Comparing the continuous variable "Hgb" and the dichotomized variable "Hemoglobin genotype" in your linear regression model is like comparing apples and oranges.

We chose to compare Hgb (continuous) vs. genotype (dichotomized) because we were interested in reporting our output as the effect on parasite growth per unit= 1g/dl of change in Hgb, similar to how we were using the unit=genotype for the genotype. We only analyzed AA and AS genotypes in our study population. For both Hgb and genotype, the dependent variable was parasite growth. We also wanted to begin by analyzing our results with hemoglobin as a continuous variable because sometimes categorizing a variable can mask its significance. Finally, we are aware of many other studies and statisticians who use both continuous and categorical variables in the same linear regression modeling (35).

In an attempt to make it easier for the reader to compare the impact of the various factors on the parasite growth, we presented information on the standardized %GR change for each of the variables. For Hgb and MCV this was calculated based on the SD for the exposure variable of interest (using the data from Table 1 in this chapter) multiplied by the β_1 effect (x100%), to give the %GR change for every 1 SD change in the exposure variable; for Hgb genotype the adjusted %GR change is simply β_1 (x100%). Please refer to Table 2 in this chapter for a refresher of the results.

Please reanalyze results with hemoglobin as a categorized variable, for the sake of comparison.

We have now redone the multivariate analysis using Hgb as a categorical variable and present the results here below. For comparison, we tried several different ways of breaking down the hemoglobin into categories. Admittedly, it makes the most sense to have the categories be clinically relevant or otherwise justifiable, in order to best demonstrate the clinical relevance of the results and interpretations. The WHO categories for anemia are severe (Hgb<7g/dl), moderate (Hgb 7-10g/dl), and mild (Hgb 10-10.9g/dl). The population of children enrolled in our study had hemoglobin values between 7-11g/dl. Thus, our data exclusively represents parasite susceptibility in children with mild and moderate anemia, and if we categorized hemoglobin based on these WHO-defined distinctions, we dichotomize the Hgb variable.

Below are the linear regression results assessing impact of moderate versus mild anemia on parasite growth rates (dichotomizing Hgb into two categories). Note that now the growth rate change for hemoglobin is negative, reflecting the difference between those with moderate anemia compared to those with mild anemia (the reference category). Also, standardizations for growth rate here are simple conversions of the β_1 effect to percent growth rate changes, and do not take into account SD of Hgb.

Addendum Table A4.1: Multivariate modeling with Hgb dichotomized

Condition	B_1 value	Lower CI	Upper CI	p Value	Standardized % GR change
Hgb dichotomized					
Hgb affects growth at Day 0	-0.130	-0.241	-0.020	0.0223	-13.0%
Hgb genotype affects growth at Day 0	-0.183	-0.318	-0.047	0.0091	-18.3%
Hgb affects growth at Day 0 independently of Hgb genotype	-0.126	-0.236	-0.016	0.0260	-12.6%

The effect of hemoglobin is thus still significant when dichotomized, although the overall spread between categories in terms of both Hgb means (10.45g/dl average amongst mildly anemic versus 9.19g/dl average amongst moderately anemic) and also potential growth rate differences (now only 13.0%) within our population is now much smaller. We know the potential spread of hemoglobin within our population is much greater, as is the growth rate spread, so this analysis minimizes the size of our results. Not only that, but the p value greatly increases compared to Hgb as a continuous variable.

If we further categorize Hgb values into tertiles, quartiles, or quintiles (or even slightly different divisions of tertiles and quintiles), we still see an effect of Hgb on parasite growth that is independent of genotype. Finally, we did not include Hgb genotype linear regression results in these tables because this remains the same no matter the Hgb classification (see main chapter text and Table 2 for results). Complete results for different Hgb categorizations are as follows:

Addendum Table A4.2: Combinations of hemoglobin categories tested

Hemoglobin categories						
Several different category groupings of hemoglobin were tried, to see if different divisions affected the data. Average hemoglobin values for each division are in parentheses.						
Category label**	Quintiles 1:	Quintiles 2:	Quartiles:	Tertiles 1:	Tertiles 2:	Dichotomized:
0	10.6-11 (10.74)	10.5-11 (10.69)	10.5-11 (10.69)	10.1-11 (10.50)	10.5-11 (10.69)	10-11 (10.45)
1	10.1-10.5 (10.33)	10-10.4 (10.22)	9.9-10.4 (10.17)	9.1-10 (9.64)	9.6-10.4 (10.05)	<10 (9.19)
2	9.6-10 (9.84)	9.5-9.9 (9.75)	9.3-9.8 (9.61)	<9.1 (8.38)	<9.6 (8.83)	
3	9.1-9.5 (9.30)	9.0-9.4 (9.22)	<9.3 (8.60)			
4	<9.1 (8.38)	<9.0 (8.29)				
**Note here "0" is always the highest Hgb value – so for every unit change in Hgb category in linear regression analyses, growth rate should decrease (as Hgb also decreases)						

Addendum Table A4.3: Number of people in each group for different Hgb categories

Quintiles 1:	n	Quintiles 2:	n	Quartiles:	n	Tertiles 1:	n	Tertiles 2:	n	Dichotomized:	n
10.6-11	24	10.5-11	34	10.5-11	34	10.1-11	68	10.5-11	34	10-11	77
10.1-10.5	44	10-10.4	43	9.9-10.4	50	9.1-10	58	9.6-10.4	70	<10	72
9.6-10	36	9.5-9.9	31	9.3-9.8	33	<9.1	23	<9.6	45		
9.1-9.5	22	9.0-9.4	21	<9.3	32						
<9.1	23	<9.0	20								

Addendum Table A4.4: Linear regression results for different Hgb categories

Linear Regression Results						
Condition	B1 value	St Err	Lower CI	Upper CI	p Value	%GR change*
Hgb by quintiles 1						
Hgb affects growth at Day 0	-0.072	0.021	-0.112	-0.031	0.001	-7.16
Hgb affects growth at Day 0 independently of Hgb genotype	-0.068	0.021	-0.108	-0.027	0.001	-6.75
Hgb by quintiles 2						
Hgb affects growth at Day 0	-0.065	0.021	-0.106	-0.025	0.002	-6.52
Hgb affects growth at Day 0 independently of Hgb genotype	-0.064	0.020	-0.103	-0.024	0.002	-6.36
Hgb by quartiles						
Hgb affects growth at Day 0	-0.084	0.026	-0.134	-0.033	0.001	-8.35
Hgb affects growth at Day 0 independently of Hgb genotype	-0.080	0.025	-0.129	-0.030	0.002	-7.97
Hgb by tertiles 1						
Hgb affects growth at Day 0	-0.108	0.039	-0.184	-0.032	0.006	-10.83
Hgb affects growth at Day 0 independently of Hgb genotype	-0.102	0.039	-0.178	-0.025	0.010	-10.16
Hgb by tertiles 2						
Hgb affects growth at Day 0	-0.124	0.037	-0.197	-0.052	0.001	-12.42
Hgb affects growth at Day 0 independently of Hgb genotype	-0.117	0.037	-0.189	-0.044	0.002	-11.65
Hgb dichotomized						
Hgb affects growth at Day 0	-0.130	0.056	-0.241	-0.020	0.022	-13.03
Hgb affects growth at Day 0 independently of Hgb genotype	-0.126	0.056	-0.236	-0.016	0.026	-12.58

While many of these categorizations are not as easily justifiable in practical terms, it is true that the more groups of hemoglobin we have, the more significant our results are in terms of spread of parasite growth in people within our population. We retain statistical significance for Hgb influencing parasite growth for every categorization we tried, but we lose the ability to report the impact of anemia in

terms of g/dl, which we feel is more relevant clinically. It is true the dichotomization of Hgb into mild and moderate is a well-known definition, but this distinction is still not clinically relevant as, at least in The Gambia, treatment regimens for children with mild and moderate anemia are the exact same.

Overall, we have proven with these additional analyses that categorizing hemoglobin maintains the significance of association versus parasite growth (which remained true for every permutation of hemoglobin categories we assessed). This speaks to the significance of our results in terms of the effect of hemoglobin, as sometimes categorizing a variable can even mask its effect.

Given the results from the different categorizations together show a linear response in comparison to one another, this further validates the original use of Hgb as a continuous variable. To simply dichotomize Hgb into mild and moderate anemia categories is statistically wasteful and furthermore it only compares the effect of a difference in Hgb between 10.45-9.19g/dl which vastly underestimates the predicted effect of anemia versus no anemia. These alternative analyses were very useful in helping validate our results, but overall we clearly show our initial results finding significant association between continuous Hgb and the degree of the effect were real.

***It is not possible to extrapolate linear regression results from this dataset to a "population level impact" because you didn't measure parasite growth in non-anemic people*.**

This relates to the discussion above about how we need to be careful making statements about effects of certain variables on parasite growth within the entire population versus within our population of anemic subjects. We understand the point that our linear regression model does not give any specific output/results for the effect of anemia on parasite growth. Presumably if we had included data from non-anemic people, we would certainly get a different β estimate. And we understand the point that therefore, we cannot calculate the "population attributable fraction". However, we are not in fact calculating the "population attributable fraction" – to be discussed further in the next item.

Furthermore, as argued above, we do still know everyone in our dataset has lower parasite growth than those who are non-anemic – because the definition of the "relative" growth rate we reported is that it was normalized relative to non-anemic controls. Also, given our extensive *in vitro* characterizations of parasite growth in RBCs from iron deficient versus iron replete donors previously (1),

it is not like we question that the parasite growth might potentially be greater in anemic than non-anemic Gambian children. Thus we feel it is still justifiable to argue for our attempt to use the population prevalence of anemia to make some conclusions about our growth rate changes seen with different Hgb levels in anemic children and extrapolate to what that could mean across the population. We are not calculating the "population attributable fraction."

In order to calculate a population attributable fraction for a subpopulation (i.e., anemic individuals), you need an odds ratio (or risk ratio) explicitly for that subpopulation, which involves information and measurements we do not have regarding this population. To be explicitly clear, here we use the β coefficient from our linear regression modeling as a proxy measurement for an odds ratio to generate our "population level impact" results, understanding that is not directly comparable to methods required for calculating population attributable fraction.

***If you are not calculating the "population attributable fraction," the term "population level impact" is too easily confused with this well-known epidemiological calculation*.**

We acknowledge this criticism and agree that the terms are easily confusable and that our citation of "population attributable fraction" as inspiration for our own "population level impact" equations was less than ideal. Our equation for population level impact is: prevalence of given trait x (relative growth rate for that trait – 100%/relative growth rate for that trait). We are trying to look at something similar to the relative risk for a trait, multiplied by the prevalence for that trait. As was pointed out, we should not reference Rothman for the epidemiological equation of "population attributable fraction" as inspiration, because for one thing he uses the prevalence of exposure *among cases* multiplied by the relative risk, and thus when our prevalence refers to the whole population (not just cases) it is not equitable. We fully recognize we are not calculating a "population attributable fraction" and that we should further distance ourselves from that term as we continue to include the "population level impact" calculations in our manuscript.

With our creation and use of the "population level impact" equation, we were simply trying to find a logical way to quantitatively compare the impact of anemia versus sickle-cell trait on malaria growth as measured in our *in vitro* system and to expand the significance of these effects in order to infer

information about malaria protection in Gambian children. Knowing that the growth rates are reduced comparably in those with anemia versus those with sickle-trait, and also knowing prevalence of anemia is much greater in Gambian children than sickle-cell trait, then it makes logical sense to conclude the overall protection from anemia is greater in the population. We do not think that this interpretation is wrong, and we will make an effort to further clarify the "population level impact" calculation in our manuscript.

Please reanalyze "population level impact" with categorized hemoglobin linear regression results. Taking the suggestion of using hemoglobin as a categorical variable will allow you to make more official "population attributable fraction"-like calculations, for the sake of comparison.

As just stated, we accept that we may have inadvertently led the readers to mistakenly think that our "population level impact" calculations were comparable to the "population attributable fraction" term. If we take the suggestion to categorize hemoglobin in our linear regression model, then we do have the ability to calculate "population level impact" of protection for each of the categories of hemoglobin, which does much more closely relate to the concept of "population attributable fraction." It is worthwhile to see these results in order to evaluate the relative effect of genotype versus level of anemia on growth rate in another light.

In order to do these calculations, we have to take into account the prevalence of each Hgb "category" amongst the population as a whole, not just general anemia prevalence, nor the prevalence of mild versus moderate anemia within our study population. We do, in fact, have population data that allows us to calculate this. This population data comes from the study enrollment screening done for children in the capture area for our study – all children were screened by Hgb test, and only those with Hgb 7-11g/dl were included in the study. 651 children in the study area were screened for possible enrollment. Of those, 30.3% were moderately anemic (hgb 7-9.9g/dl), 41.3% were mildly anemic (hgb 10-11g/dl), 0.5% were severely anemic (hgb <7g/dl), and 27.9% were non-anemic (hgb>11g/dl). This is an accurate representation of the children in this area, because another larger study a few years ago determined anemia prevalence (all levels) to be 75%, which is very close to our screening data (72% anemia prevalence) (23).

Addendum Table A4.5: Frequency of anemia type in Gambian children

Population data – categories of anemia	
Screened population - 651 total	Prevalence
3 with Hgb<7g/dl	0.46%
197 moderate anemia Hgb 7-9.9g/dl	30.26%
269 mild anemia Hgb 10-10.9g/dl	41.32%
182 with no anemia Hgb>10.9g/dl	27.96%
With mild or moderate anemia (study population)	71.58%
With any anemia (mild, moderate, or severe)	72.04%

To calculate the population level protection for Hgb categories, we have to use the new β_1 effects and standardized %GR changes presented above. To begin with dichotomized Hgb categories, there is a prevalence of 30.3% for moderate anemia at the population level, and we determined a 13.0% decrease in parasite growth comparing moderately anemic to mildly anemic children in our study population (p=0.023). Thus, the new "population level impact" calculation becomes (30.3*(13-100)/87) which is a 4.53% population level protection in moderately anemic kids.

Addendum Table A4.6: Population level impact using continuous versus dichotomized Hgb

Population Level Impact			
This is how we reported population level impact in our paper.			
Factor	Prevalence	Growth reduction	Population Impact
Sickle trait	15.90%	18.30%	3.50%
Anemia (Hgb<11 g/dL)*	75%	17.20%	15.90%
*This assumes anemic individuals are by definition 2 SD below the mean in healthy children, hence the growth reduction represents the effect across 2 SD in our population			
This is based on dichotomized Hgb, producing results only for those who aremoderately anemic.			
Factor	Prevalence	Growth reduction	Population Impact
Moderate anemia (Hgb 7-9.9g/dL)	30.30%	13.00%	4.50%

Therefore, the "population level impact" from sickle-cell trait and moderate anemia is roughly comparable, 3.5% vs. 4.5%, respectively. However, by doing the calculation this way, we ignore the effect on kids with mild anemia because they become our comparison group. In essence, we are using the "mild" anemia Hgb category as the denominator for calculating the risk ratios for moderate anemia, but this prevents us from doing any population level impact calculations for the mildly anemic group.. Not only

that, but by comparing those with moderate anemia to those with mild anemia, the growth rate difference becomes much smaller than we know it is in reality in our population. If we do use these "population attributable fraction"-like results based on dichotomized Hgb categories, we can still make a statement about how those with moderate anemia have comparable levels of protection in terms of malaria growth as those with sickle-cell trait. But, we would have to revert to descriptions to emphasize the importance of our findings, reminding readers that there are other levels of anemia which also provide some degree of protection against malaria not measured in this calculation (thus looking only at those with moderate anemia underestimates the effect), and that anemic people encompass a much greater portion of the population than do people with sickle-cell trait. Using the dichotomized Hgb categories for the population level equation and finding a condition (moderate anemia) that provides the same amount of protection against malaria as sickle-cell trait is still a very interesting statement, since sickle trait is the known gold standard for malaria protection. However, ultimately we dislike this method of categorizing Hgb to calculate something closer to a "population attributable fraction" because we know it vastly underestimates the true effect of anemia as a whole on the growth rate reductions in our study population.

It is possible we could calculate the "population level impact" for all other permutations of Hgb categorization we tried and add up the effects from each category, to try and reduce the loss of data from the highest level comparison Hgb category by expanding the number of categories used. However, we ultimately still believe that our original analysis (in which we keep Hgb as a continuous variable) is better, as it captures much more data describing the effect of anemia on parasite growth and is therefore a much more realistic and accurate representation. (Of course it goes without saying we all have to remember that these results are based on specific *in vitro* erythrocytic stage malaria cultures.)

The meaning of the "population level impact" results needs to be clarified – what does 3.5% or 15.9% protection mean?

We recognize that our *in vitro* parasite growth data are difficult to translate to clinical protection levels in terms of malaria, and this is not what we were trying to accomplish. Again, this speaks to the reason why we need to focus on distancing ourselves from the clinical epidemiology "population

attributable fraction" term. Reduction in relative growth rate *in vitro* might very plausibly be reflective of reduction in parasite burden in a population, and thus perhaps relate to clinical malaria cases – but this is not at all an official statement we can attempt to make, as there are obviously so many other factors involved in malaria infection in the real world that we have not even begun to account for in our *in vitro* system. All we can describe is sickle-cell trait affords a 3.5% reduction in parasite growth and anemia affords a 15.9% reduction in parasite growth relative to one another. In essence this "population level impact" is a completely artificial concept and we are ultimately just comparing relative units, simply as a means of evaluating the impact of anemia versus genotype on parasite growth.

***Please summarize appropriate alternative options for data presentation and analysis*.**

We wish to thank Jordan Cates for her assistance in discussing epidemiological methods and concerns regarding our data presentation. Together, she agreed these were plausible solutions for us going forward:

1) Change our "population level impact" results to be purely descriptive, arguing the impact of anemia on parasite growth must surely be greater than sickle-cell trait given what we know about *in vitro* growth rates and prevalence of these traits, but avoiding any attempts at quantitative calculations
2) Break our Hgb data into mild/moderate categories for linear regression analyses and use "population attributable fraction"-like calculations to make quantifiable statements about differences in parasite growth between mild and moderate anemics (but not anemia as a whole).
3) Continue using our current "population level impact" calculations, but make sure it is explicitly clear we are not attempting to make official epidemiology calculations (rather we have devised our own way to take into account the prevalence of a given condition and the relative growth rate for a that condition in our *in vitro* system using the β coefficient from the linear regression modeling), and better describe the interpretation of the outcome of X% population level protection as a relative unit merely providing a means of comparing our results from different traits.

4.8.2 Conclusions

Working with epidemiologists to better comprehend clinical epidemiological methods of data analysis and interpretation has been very useful in helping us interpret and clarify our own results and objectives, use alternative epidemiological analyses to confirm our data has significance, and gain more perspective on clinical epidemiological methods. Having extensively re-examined our results and interpretations, we feel confident the results we are presenting have real meaning based on the *in vitro* data we obtained. Naturally, any translation to population level clinical malaria only represents logic-based descriptions and hypotheses, which are not based on actual results, as we did not use clinical malaria as an outcome. It is useful to note that our linear regression data from a parallel study conducted in pregnant women (which included both anemic and non-anemic women and is presented in the following chapter) found correlations with growth rate and Hgb level at baseline exactly on par with the level of growth rate changes seen at baseline in this children's population. This gives us additional confidence that we are not inaccurately making assumptions about parasite growth in a non-anemic children's population being greater than that in our anemic population. However, this extensive conversation has certainly improved our presentation of results, knowledge of the topics at issue, and understanding of the importance of being able to back up your claims, and we are grateful for the critique and thoughtfulness dedicated to our work.

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CHAPTER FIVE: ERYTHROCYTES FROM GAMBIAN PREGNANT WOMEN ARE PROTECED AGAINST MALARIA IN VITRO AND THIS PROTECTION IS REVERSED BY IRON SUPPLEMENTATION[4]

5.1 OVERVIEW

Anemia, particularly iron deficiency anemia, is highly prevalent amongst pregnant women in the developing world and exhibits a profound health burden on both the mother and the developing fetus. The World Health Organization thus commonly recommends iron supplementation for pregnant women where anemia is common. Pregnant women are also particularly susceptible to placental malarial infection, which again affects both mother and fetus. Paradoxically, epidemiological studies suggest iron deficiency protects against malaria and administering iron to iron-deficient individuals may increase malaria risk. This has generated much debate in the public health field about how to best distribute iron supplements to pregnant women in malaria endemic areas. Our previous laboratory work demonstrated decreased P. falciparum growth in iron deficient red blood cells (RBCs) and increased infection susceptibility in young RBCs and reticulocytes in vitro. Here, our objective was to comprehensively evaluate P. falciparum pathogenesis in RBCs from pregnant women before, during, and after iron supplementation. We also sought to evaluate the hypothesis that malaria risk increases as erythropoiesis increases in response to iron supplementation. To do so, we investigated P. falciparum in vitro growth characteristics in RBCs from pregnant Gambian women participating in an iron supplementation trial. RBCs were collected from 165 pregnant women (2nd and 3rd trimester) before, during, and upon completion of 12 weeks of iron supplementation (60 mg daily, respectively). We used in vitro flow cytometry based assays to examine effects of iron deficiency and iron supplementation on overall parasite growth and merozoite RBC invasion. Our results demonstrate P. falciparum erythrocytic stage growth in vitro is low in pregnant

[4] We would like to emphasize to the reader that all of the research presented in this chapter remains a work in progress in terms of data analysis and presentation. We are awaiting our collaborators to officially lock the “Hepcidin and Pregnancy” iron supplementation study database before we can draft a manuscript for this work.

Gambian women at baseline but increases during supplementation. The elevated growth rates parallel increases in circulating reticulocytes and other markers of young RBCs which are suggestive of increased erythropoiesis. We conclude malaria growth in vitro corresponds with elevated erythropoiesis, an inevitable consequence of iron supplementation during pregnancy. Our findings imply iron supplementation of pregnant women in malarious regions should be routinely accompanied by effective preventative measures against falciparum malaria.

5.2 INTRODUCTION

Pregnant women represent one of the most susceptible populations to *P. falciparum* malaria infection, particularly primagravida women. This is because the parasite protein exported to the surface of trophozoite- and schizont-infected RBCs that allows for cytoadherence to the peripheral endothelium, *Pf* Erythrocyte Membrane Protein 1 (PfEMP1), has a variant (var2csa) that specifically binds to chondroitin sulfate A (CSA) on syncytiotrophoblasts present in the placental intervillous spaces. Thus parasites can accumulate in the placenta and cause severe consequences for both the mother and fetus, including a risk of severe maternal malaria and death, as well as stillbirth, preterm delivery, intrauterine growth restriction and low birth weight, and perinatal morbidity and mortality (1). Risk of placental malaria infection diminishes with subsequent pregnancies, as women develop immunity against the PfEMP1 variant that allows the parasite to cytoadhere in the placenta (2, 3). Because placental malaria can often go undetected for long periods, negative health consequences can become compounded and malaria in pregnancy is estimated to account for over 100,000 annual infant deaths in Africa as recently as 10 years ago (1), with tens of millions of pregnancies at high risk for malaria infection (4).

Another important risk factor for pregnant women, particularly in the developing world, is anemia: over 50% of pregnant women globally are estimated to have anemia, with the rates even higher for Africa and other developing regions(5). Iron deficiency anemia accounts for 50-75% of those anemia cases, largely due to inadequate diet and increased nutritional requirements during pregnancy (6) (and reviewed in (7)). Adequate maternal iron stores are essential as the mother's blood volume must expand accordingly to provide for a growing fetus, and erythropoiesis is heavily dependent on iron. In total, the maternal blood volume expands by 1-1.5 L, though plasma volume expands more so than red cell mass, such that there is an effective hemodilution peaking in the 2nd trimester which drops blood hemoglobin

concentrations by 1-2 g/dl (reviewed in (7, 8)). There are significant dangers associated with anemia for the mother, as the anemic woman is at much greater risk for life-threatening complications if excessive blood loss occurs during birth – as can be especially common in low resource settings (reviewed in (7)). Furthermore, anemia in the pregnant women is also very detrimental to the fetus, contributing to low birth weight and premature births (7, 9, 10). Not only that, but iron plays an essential role in developmental health, so babies born to anemic mothers begin life at increased risk for cognitive impairment and inability to reach their full potential – consequences of which can last far beyond time of birth (reviewed in (7)).

Given that pregnant women are vulnerable to malaria and also to iron deficiency, understanding the relationship between iron and malaria in pregnancy is important. Providing evidence of the profound impact that iron deficiency anemia has on reducing malaria infection, the effect of anemia on reducing placental malaria risk has been documented in observational studies (11–13). Despite the malaria protection afforded by anemia, anemia and iron deficiency during pregnancy must be treated – especially considering the lasting detrimental health defects on infants born to anemic and iron deficient mothers. Further complicating matters is the difficulty in accurately diagnosing iron deficiency anemia in pregnancy in the context of changing physiology, especially in developing world settings where many tests are not routinely available and high rates of inflammation due to infection and environmental exposures exist. Many routinely used markers of iron status are impacted by inflammation (e.g. ferritin, zinc protoporphyrin, transferrin saturation) (14).

Of course, ideally women would have adequate iron stores before becoming pregnant. But how to address iron deficiency complications in already pregnant woman? The obvious solution would appear to be iron supplementation. The WHO recommends 60mg iron daily as a supplement taken throughout pregnancy in areas where iron deficiency anemia is greater than 40% (i.e. much of the developing world) (15). A 2015 Cochrane review on iron supplementation in pregnancy concludes iron supplementation decreases rates of low birth weight and pre-term deliveries, though not at a statistically significant level, and that there was no evidence of differences in neonatal death (16). Women taking iron supplements during pregnancy had a 70% reduction in risk of anemia and 57% reduction in iron deficiency at birth, as well as higher Hgb levels at birth. Although there were no detectable differences regarding maternal infection rates, maternal mortality, or side effects, meta-analysis of these parameters was based on

studies determined to provide “low quality evidence” according to the Cochrane review (16). It should be noted that a 2009 Cochrane review on the same topic determined there was no evidence to conclude iron supplementation improved maternal and neonatal health outcomes despite improved anemia and iron status (17), and a 2012 Cochrane review did find evidence for reduction in low birth weight (18). Clearly the topic is open for research and discussion.

Despite the facts that iron supplementation during pregnancy does improve maternal anemia status and iron stores and likely also improves infant birth outcomes, there are still some areas of concern regarding negative health outcomes. Those who take iron during pregnancy are also at greater risk for hemoconcentration (Hgb >13g/dL) during late pregnancy (slight physiologically normal hemodilution during pregnancy serving to increase blood flow in the placenta) (16), potentially impacting maternal hypertension and birth weight for those non-anemic women receiving excess iron. Elevated iron stores in pregnancy may also be associated with preeclampsia and gestational diabetes (discussed in (19, 20)). Secondly, of particular concern in developing world settings, there is also reason to believe that iron supplementation during pregnancy may impact the risk of infection, particularly malaria.

The potential dangers of iron supplementation and the hypothesis that increased morbidity and mortality with iron was associated with malaria infection was most famously brought to light by a large nutritional intervention study in Tanzanian children (21). Other studies have also come to similar conclusions. The question about the impact of iron supplementation on malaria pathogenesis has not been definitively answered, but a 2016 Cochrane review concludes that iron supplementation in children in malaria endemic areas is safe if appropriate antimalarial public health measures are available (22). For pregnant women, less research has been done, but a full review of studies can be found in a 2014 meta-analysis on iron and malaria and pregnancy (23). This meta-analysis found association between iron deficiency and reduced malaria infection risk if iron deficiency was measured by ferritin; transient increased incidence of vivax malaria with iron supplementation; and no increased risk of falciparum malaria infection with iron supplementation (though ultimately the study concluded there was insufficient evidence to fully answer this question) (23). A few recent studies have also attempted to address this. Mwangi *et al*. concluded in pregnant Kenyan women there was a beneficial effect on birth weight and no differences in malaria infection rates for those taking 60mg daily iron versus those taking placebo (24).

Etheredge *et al*. found in Tanzanian pregnant women taking 60mg daily iron that there were increases in iron stores and hemoglobin versus those taking placebo, and no differences between placebo and iron groups in terms of placental malaria infection rates (25). However, as the authors of both papers discussed, it is nearly impossible to conduct a purely observational clinical trial regarding iron supplementation and malaria, ethically speaking, because of past evidence suggesting iron supplementation might put people at increased risk for malaria – hence they must work hard to provide extensive antimalarial prevention measures and may in fact bias their own results (24, 25).

As the level of risk of malaria infection with iron supplementation remains an important public health question, we thus attempted to better understand the precise relationship between iron deficiency, iron supplementation, and malaria in pregnancy. We have systematically assessed malaria susceptibility *in vitro* in RBCs drawn from pregnant women before, during, and after 12 weeks of iron supplementation initiated during the 2nd trimester of pregnancy. Having complete iron panels, full blood counts, and inflammatory marker data to match the malaria growth parameters at Day 0, 14, 49, and 84 of iron supplementation has allowed us to comprehensively evaluate the effect of iron supplementation on malaria risk in pregnancy. Our previous *in vitro* work led us to hypothesize that RBC population structures drive malaria susceptibility, given that iron deficient RBCs and older RBCs are more resistant to malaria infection and that younger RBCs are more susceptible. Hence, with iron supplementation in pregnant women we also hypothesized increased numbers of young RBCs following iron-induced elevated erythropoiesis rates would increase malaria growth rate *in vitro*, our proxy measure for malaria susceptibility (26). This is exactly what we observe in the data presented in this Chapter. Our work with pregnant women has taken place in The Gambia and was a sub-study within a larger iron supplementation trial (19).

5.3 MATERIALS AND METHODS

Subject recruitment. The pregnant women were recruited from the control arm of a randomized trial testing the efficacy and safety of a hepcidin-guided screen-and-treat strategy for combatting anaemia (see published protocol for full details (19)) and are 18-45 years old with estimated 14-22 weeks gestation at recruitment. (Note we also recruited pregnant women in the other two arms of this trial, but only for observation at baseline, pre-randomization/pre-intervention.) As per current WHO recommendations,

pregnant women in the control arm received 60mg/d iron as ferrous fumarate, given within a micronutrient capsule (UNIMMAP). Field workers visited the women frequently (biweekly) in order to check their health status and deliver the supplements. For baseline population characteristics, see Supplemental Table S5.1.

UNIMMAP Capsules. The iron supplementation product used in this trial is a micronutrient mixture (UNICEF/WHO/UNU international multiple micronutrient preparation (UNIMMAP)) produced by the DSM Company (South Africa) and previously determined to be safe in pregnancy. It contains: 60 mg iron, 400 µg folic acid, Vitamin A (800µg RE), Vitamin D (200 IU), Vitamin E (10mg), Thiamine B1 (1.4 mg), Riboflavin B2 (1.4mg), Niacin B3 (18g), Vitamin B6 (1.9mg), Vitamin B12 (2.6µg), Vitamin C (70mg), Zinc (15mg), Iodine (150µg), Selenium (65µg), and Copper (2mg).

Blood samples for parasite assays. Fresh RBCs were obtained from otherwise healthy pregnant women (age 18-45 years, gestational age 14-22 weeks at enrolment) enrolled in an iron supplementation study in rural Gambia (19). Venous blood was collected at Days 0 (baseline), 14, 49 and 84 during 12wks of iron supplementation (Figure 5.1). 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 5.2).

***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 (26). Parasite strains ΔPfRh1 B9, ΔPfRh2a, and ΔPfRh2b were kindly provided by the laboratory of Alan Cowman (WEHI, Australia).

Growth assay. *In vitro* growth was assessed in fresh, washed RBCs as in (26) for 96 h (performed in triplicate for RBCs from each study participant). RBCs from healthy, non-pregnant, adult iron replete donors of normal hemoglobin genotype and G6PD status not undergoing iron supplementation served as controls. Growth rates represent final 96 h parasitemia divided by initial 0 h parasitemia (26), analyzed by flow cytometry. Growth rates in subjects' RBCs were normalized to that in control non-anemic RBCs assayed simultaneously.

RBC barcoding invasion assay. The assay was performed and analyzed as in (27) using two different concentrations of CellTrace Far Red DDAO (Invitrogen Life Technologies/Molecular Probes):

1uM (high) or 0.1uM (low). Labeling took place at 1% hematocrit in serum-free RPMI for 90 minutes with thorough rocking at 25°C, followed by washing twice with ACM, incubating an additional 30 minutes in ACM with rocking at 25°C, and finally washing twice with RPMI before storage in RPMI at 2% hematocrit at 4°C. All invasion assays were performed with fluorescently labeled RBCs within four days of labeling. Differentially labeled RBCs were combined in equal density in triplicate into a 96 well plate and seeded with MACS purified (Miltenyi Biotec) late stage parasites to achieve 1.5-2% pRBCs in 1% hematocrit. Plates were maintained for 12-18 hours under standard culture conditions to allow for schizont rupture and subsequent invasion of labeled RBCs. Invasion into high and low DDAO RBCs was detected by DNA dye Sybr Green I, as analyzed by flow cytometry (see Chapter 4 for pictorial flow cytometry analysis strategy), and the ratio of the prevalences of infected high to low DDAO RBCs was calculated to generate the “Susceptibility Index” (described in (28)). Note that we also report data from some invasion assays which were performed without barcoding, which instead assesses invasion rates into anemic and non-anemic RBCs in parallel (not in the same well).

Reticulocyte quantification. Reticulocyte (CD71+) levels in fresh subject RBCs were assessed using PE-conjugated anti-human CD71 antibody (Clone M-A712, BD) and isotype control (Clone G155-178, BD), and analyzed by flow cytometry for reticulocyte percent relative to non-anemic control.

RBC surface marker assessment. RBC surface protein levels were determined by staining with fluorescently tagged antibodies and analyzed by flow cytometry. The following antibodies were used: For CD35 (Mouse Anti-Human CD35 Clone E11 (BD) primary 1:2000; Alexa Fluor® 647 Rabbit Anti-Mouse IgG secondary 1:2000); For CD47 (Mouse Anti-Human CD47 Clone B6H12 primary 1:2000; Alexa Fluor® 647 Rabbit Anti-Mouse IgG secondary 1:500); For CD55 (Mouse Anti-Human CD55-PE conjugate Antibody NaM16-4D3 (Santa Cruz Biotechnologies) 1:10); For CD147 (Mouse Anti-Human CD147 Clone HIM6 (BD) primary 1:500; Alexa Fluor® 647 Rabbit Anti-Mouse IgG secondary 1:500); For Glycophorin A (GPA) (primary Rabbit Anti-Human CD235a/Glycophorin A (ThermoFisher Scientific/Pierce) 1:500 and secondary Alexa Fluor® 647 Goat Anti-Rabbit 1:2000); For sialic acid residues (Wheat Germ Agglutinin Alexa Fluor® 488 Conjugate (ThermoFisher Scientific/Molecular Probes) 1:2000); and for C3b deposition (primary Mouse Anti-Human Complement C3b Antibody 10C7 (ThermoFisher Scientific) 1:200; Alexa Fluor® 647 Rabbit Anti-Mouse IgG secondary 1:500). The Relative Fluorescence Intensity (RFI) of each

surface marker for each study sample was calculated using the MFI values (corrected for background), relative to MFI in the non-anemic donor as described in (29) . The exception was C3b which was reported as a percent of positively stained RBCs instead of MFI.

Attachment assay. Assays and analysis of merozoite attachment to IDA vs. IR RBCs was performed based on (29, 30). In detail, tightly synchronized parasite cultures (15 ml of 4% hematocrit cultures containing 5% infected RBCs) were treated with 50U/ml of heparin as trophozoites. Approximately 12 hours later, upon visualization of schizonts by Geimsa stain, schizonts were purified by MACS. Resulting schizont concentration was quantified by Sybr Green I stain and flow cytometry, and schizonts were incubated in ACM containing 50U/ml heparin under standard culture conditions to allow for 3-4hrs of recovery. RBC concentrations of the donor samples were also quantified by flow cytometry. To set up the assay, schizonts were washed twice with 5ml heparin-free ACM and distributed equally amongst triplicate wells (of a 96-well plate) for each assay condition. RBCs from IR or IDA donors were then added to appropriate wells to bring the final concentration of schizonts to 10%. ACM with either no drug (to assay merozoite invasion), 1µM cytochalesin D (to assay merozoite attachment), or 200U/mL heparin (to block both invasion and attachment), was added to appropriate wells and plates were incubated under standard culture conditions. At each time point (0 h, 3 h, and 7 h), 50µl of each drug treatment condition from each blood sample were aliquoted in triplicate into 200µl of fixative (PBS containing 0.116M sucrose and 2% glutaraldehyde), with overnight fixation at 4ºC before staining with DNA dye Sybr Green I and analysis by flow cytometry. Giemsa stains were also made from each time point and condition to confirm attachment and invasion events were prevented by the various treatments and to verify that new events in the ring region of the flow cytometry plot for DNA quantification could correctly be counted as either invasion or attachment events depending on the treatment condition (as attachment and invasion are indistinguishable by flow otherwise). Flow cytometry analysis allows the use of DNA levels in infected parasite cultures to distinguish seeded schizonts present apart from new invasion or attachment events which occur at 3 and 7 hours. To calculate attachment or invasion rates for each blood sample, the percent of new events was compared to the starting concentration of schizonts under each condition, then expressed relative to new events occurring in RBCs from healthy iron replete controls.

Flow cytometry analysis. Flow cytometry was performed onsite at MRCG in Keneba using a BD Accuri C6 flow cytometer. Channels and probes used included: SYBR Green I, FITC, and Alexa Fluor 488 (488nm excitation with a 530/30nm bandpass emission filter, detector FL1); PE (488nm excitation with a 585/40nm bandpass emission filter, detector FL2); and CellTrace Far Red DDAO and Alexa Fluor 647 (640nm excitation with a 675/25nm bandpass emission filter, detector FL4). Detector gain setting changes and compensation were not necessary with this configuration. Accuri C6 data was collected and analyzed with Accuri software (BD Accuri CSampler Analysis Software). Linear amplification of forward scatter was used to set event threshold in order to exclude cell debris, microparticles and doublets. For all experiments, samples were diluted to 0.001-0.002% hematocrit and ≥ 100,000 total events were collected.

Statistics. All experiments were done in triplicate. Growth rates, invasion assays, and hematological data were compared by two-tailed Student's *t*-test, one-way ANOVA, and/or 95% CI values using GraphPad Prism 5.

Multivariate modelling. We employed linear regression to estimate the effect of hematological characteristics on *in vitro* parasite growth rates. First, bivariate associations and their respective 95% CI were calculated between growth rates and hematological and patient characteristics at Day 0. We then used multivariate linear regression – specifically, we estimated the effect of hemoglobin and hemoglobin genotype on growth rate along with their respective 95% CI. We used directed acyclic graphs to identify potential confounders and controlled for them in our modelling approach (31). An *a priori* alpha of 0.05 was used to determine statistical significance. All statistical analyses were performed using R software (RStudio Version 0.99.902).

Ethics approval. The trial from which children were recruited was approved by the MRCG Scientific Coordinating and MRCG/Gambian Government Joint Ethics Committee (SCC 1357) and the UNC IRB (#13-3068). Participants were given a full description of the study in their native language and provided written signed consent.

Role of the funding source. None of the funding sources had a role in study design, data collection or interpretation, writing of the manuscript, or the decision to submit for publication. The

corresponding author had full access to all the data included in the study and assumed final responsibility for the decision to publish; all authors reviewed the report and agreed to submit for publication.

5.4 RESULTS

5.4.1 *P.falciparum in vitro* growth rates are reduced based on level of anemia in pregnant Gambian women.

We assessed parasite growth levels in RBCs taken from pregnant Gambian women at the time of study commencement prior to iron supplementation (Day 0). We consistently find reduced parasite growth compared to growth in RBCs from non-anemic, non-pregnant donors. Though not all of our study subjects are anemic (defined as Hgb <11g/dl), mean blood and iron parameter data amongst our population (see Table 5.1) do demonstrate a high level of anemia, and in particular iron deficiency anemia (IDA). This is not surprising considering past reports the WHO estimates anemia prevalence to be greater than 50% in pregnant women in the developing world and is reported as especially high in The Gambia (75% as reported by others) (32). It is difficult to assess anemia, and again, particularly IDA, in the pregnant woman (32). However our enrollment data indicate that our study population of pregnant Gambian women have much lower than average values for many common iron indices (MCH, MCHC, Hgb, Hct, ferritin, sTfR, for example; see Table 5.1). Growth rates in RBCs from anemic women are significantly lower than those in non-anemic women (Supplemental Figure S5.1A), noting 37.1% are anemic (Hgb<11g/dl) at baseline. In attempt to better understand the effect of anemia on parasite growth rates, we stratified RBCs by donor hemoglobin status at Day 0, and found growth rates were progressively lower in the lower hemoglobin groups (mean growth rate (GR) compared to control GR of 100%: Hgb<10.0g/dl 54.22%, Hgb 10.0-10.9 g/dl 68.84%, Hgb 11.0-11.9 77.62%, Hgb >12.0g/dl 87.67%) (Figure 5.1A). Means are significantly different by one-way ANOVA ($p<0.001$). To further examine the impact of iron deficiency on parasite growth given several common methods for defining iron deficiency and the difficulties of doing so with high levels of background inflammation, we additionally stratified anemic individuals using several different definitions of IDA (Figure 5.1B). We did not find any significant differences in mean parasite growth between classifications of IDA.

To further understand influences of parasite growth in RBCs from our study population, we performed bivariate and multivariate linear regression analyses to examine the impact of hematological,

iron, and inflammatory variables. We assessed the influence of several key factors known or assumed to influence anemia and/or parasite growth (Table 5.2). Hemoglobin, hemoglobin genotype, MCV, transferrin saturation, and hepcidin (log values) all significantly influenced parasite growth in RBCs from pregnant women drawn prior to iron supplementation, whereas CRP, ferritin, the sTfR:log ferritin ratio, and gestational age did not. It should be noted that the only hemoglobin genotype we were able to measure differences between was normal (AA) and sickle-trait (AS) due to absence of any other genetic hemoglobin variations in our population. The growth rate changes were 8.9% increase for every g/dl increase in Hgb, 24.8% decrease from AA to AS, and 0.9% increase for every 1fl increase in MCV. We confirmed the effect of hemoglobin on parasite growth was independent of hemoglobin genotype (Table 5.2), as well as other significant variables such as MCV, hepcidin, transferrin saturation, RDW, sTfR:log ferritin ratio, CRP, and gestational age (data not shown). We further standardized the impact of the significantly-associated variables to reflect percent GR change for every one standard deviation in the variable of interest, in order to better compare effect sizes between variables. Using this method, Hgb has the largest effect on parasite growth, changing 11.4% with every SD from the mean. Considering the distribution for Hgb ranges from 8g/dl to 12.5g/dl with mean of 11.3g/dl and SD of 1.28, this indicates a possible spread of nearly 35-40% GR within our population based on hemoglobin. Sickle-cell trait notably also has a large impact on parasite growth (no adjustment for SD so the same 24.8% reduction) (see Supplemental Figure S5.1B for visualization of the difference in growth rate comparing AA and AS individuals). Overall, this linear regression data is highly consistent with the dataset we reported in Chapter 4, and furthermore encompasses pregnant women who are non-anemic, thus allowing us to fully see the effects of hemoglobin on parasite growth in a broader population.

5.4.2 RBCs from anemic pregnant women are resistant to invasion by *P.falciparum*, but invasion deficits in anemic RBCs are not attributable to PfRh invasion ligands nor parasite attachment.

We proceeded to assess parasite invasion in RBCs from pregnant women at baseline, knowing growth rates in these RBCs were reduced compared to RBCs from non-anemic, non-pregnant controls. We have extensively proved parasite invasion is reduced into iron-deficient and anemic RBCs elsewhere, finding invasion deficits to be a large factor contributing to overall reduced growth in anemic RBCs (26, 33) (and Chapter 4). Experiments here examined new invasion events in target RBCs of interest (from

anemic individuals or non-anemic controls) after incubation with purified late-stage schizonts. Results are normalized to invasion into the non-anemic control RBCs (set at 1.0); mean invasion rates for FCR3-FMG and 3D7 are 0.60 and 0.74, respectively (Figure 5.3A).

Having confirmed invasion deficiency in RBCs from anemic pregnant women at baseline, we next began to examine possible mechanisms for reduced invasion into RBCs form anemic donors. First, we examined whether specific *P.falciparum* PfRh invasion ligands contributed mechanistically to the differential invasion into healthy non-anemic versus anemic RBCs. The hypothesis was that PfRh parasite ligands, from the reticulocyte binding like homolog family (one of 2 families of proteins encompassing parasite invasion ligands), may help the parasite "detect" the healthiest RBCs through putative ATP binding domains. Homolog domains in *P.yoelii* PfRh proteins were speculated to do just that, after merozoites initiate irreversible contact with RBCs which causes local release of ATP (34, 35). (The ATP release phenomenon is known to occur more in young vs. aged RBCs(36) and we hypothesized it would occur more in non-anemic versus anemic RBCs as well.) *P.falciparum's* multiple invasion ligands represent redundant invasion pathways the parasite can utilize to enter the RBC (reviewed in (37)). We thus utilized transgenic parasite strains (ΔPfRh1, ΔPfRh2a, and ΔPfRh2b) to test in our RBC barcoding assay, to determine if invasion susceptibility index differences between anemic and control RBCs shifted significantly in the absence of any given invasion ligand. Our RBC barcoding invasion assay involves differentially labeling two populations of RBCs (here control non-anemic and test anemic RBCs) to easily detect and compare invasion rates into each cell type in the same well, providing results in the form of a "Susceptibility Index" (SI), an SI of 1.0 indicating no invasion difference and an SI<1.0 indicating reduced invasion compared to the control RBC population. Invasion experiments for the transgenic strains were performed in parallel with the genetic parent strain 3D7, in the same 15 anemic donors for each parasite strain and SI values for the transgenic strains were compared to SI values for parent strain 3D7. Mean SI values were all below 1.0 confirming reduced invasion into the anemic RBCs, however there were no significant differences in invasion rates into anemic RBCs between the parent and transgenic parasite strains (Figure 5.3B), suggesting individual PfRh invasion ligands do not contribute to the significant difference in invasion of control vs. anemic RBCs.

To further assess mechanisms which may contribute to differential parasite invasion of RBCs from anemic versus non-anemic donors, we next examined whether merozoite attachment was affected during invasion of anemic RBCs. To do so, we used control and randomly selected anemic pregnant women RBC samples at baseline and seeded infection wells in parallel with late-stage schizonts in the presence and absence of cytochalesin D, which prevents invasion steps beyond attachment. Thus we could differentiate invasion and attachment events by flow cytometry analysis of the different conditions (assay developed by (30)). Despite observing expected invasion differences between non-anemic and anemic donors (SI mean of 0.62, 95% CI 0.49-0.76), we found *P.falciparum* merozoites attach to RBCs from anemic Gambian children equally as well as they attach to RBCs from non-anemic donors (mean attachment rate of 0.97 with 95% CI 0.86-1.08 for anemic RBCs) (Figure 5.3C). This finding indicates mechanical properties of anemic RBC membranes may be a large factor influencing reduced invasion into anemic RBCs, which is consistent with recent literature reporting detailed live cell imaging of invasion events indicating that localized RBC deformation is a very important step in parasite invasion (38). It is known that anemia, especially IDA, can affect RBC membrane properties and deformability (39, 40).

5.4.3 *P.falciparum* growth rates increase in RBCs from pregnant women undergoing iron supplementation.

In order to assess malaria susceptibility in individuals taking iron supplements, we performed *in vitro* growth assays in RBCs drawn from pregnant women before (Day 0), during (Day 14 and Day 49), and after (Day 84) 12 weeks of iron supplementation. *In vitro* growth rates served as a proxy measurement for malaria susceptibility because it is not ethical to provide iron supplementation without extensive anti-malarial countermeasures, hence using *in vitro* growth assays allows us to still capture a pure representation of malaria susceptibility in a large number of people for whom we also have extensively characterized in terms of RBC, iron, and inflammatory parameters. Note that the women in our trial were visited biweekly for health check-ups and received weekly rapid diagnostic test screening for malaria, and only two incidences of positive RDTs occurred within our study population over the 12 week iron supplementation period as the prevalence of malaria is low in The Gambia. Growth rates were always normalized to growth in RBCs from non-anemic, non-pregnant donors (set at 100%). Much like we have previously observed (see Chapter 4), we found that parasite growth rates were lower than non-

anemic controls at Day 0 (75.59%, 95% CI 70.63-80.55%), but increased markedly at Day 14 (148.5%, 95% CI 119.7-177.3%) and also remained higher than control levels at Day 49 (113.6%, 95% CI 99.16-128.1%), finally falling back to baseline-like values by Day 84 (65.33%, 95% CI 58.61-68.46%) (Figure 5.4A). One-way ANOVA reveals significant differences between the means ($p<0.0001$). We also examined growth rate changes in RBCs from those individuals for whom we were able to obtain RBCs from at each time point (n=44) and confirmed the same patterns of increased susceptibility over and above control levels of 100% at Day 14 and Day 49 (Supplemental Figure S5.2A). While the majority of individual's growth rate increases peak at Day 14, some do peak at Day 49, which is understandable, as slight differences in gestational age and other physiological factors may influence peak changes in RBC properties and population dynamics in the pregnant women studied here.

5.4.4 Pregnant women undergoing iron supplementation exhibit increased levels of young RBCs in circulation.

To evaluate the RBC population age structure of pregnant women undergoing daily iron supplementation, we measured levels of CD71-positive reticulocytes in RBCs drawn at baseline (Day 0), during (Day 14 and Day 49) and after (Day 84) iron supplementation. Paralleling changes in parasite growth rates, CD71 levels (measured relative to non-anemic, non-pregnant control blood donors normalized to 1.0) increased significantly from Day 0 (mean 2.67, 95% CI 2.40-2.91) to Day 14 (mean 4.06, 95% CI 3.65-4.46), remaining higher at Day 49 (mean 3.22, 95% CI 2.77-3.68), before normalizing to baseline-like levels at Day 84 (mean 1.95, 95% CI 1.75-2.15). One-way ANOVA indicates differences between the means are significant ($p<0.0001$). It is not surprising CD71 levels in pregnant women are on the whole higher than non-anemic, non-pregnant control donors, as pregnant women have an expanding blood volume with increased levels of erythropoietin. When we limit our examination to those pregnant women for whom we have CD71 values from all four time points (n=60), we see the same pattern of increases in young reticulocytes at Day 14 in particular, remaining somewhat higher at Day 49 as well (Supplemental Figure S5.2B).

We further examined markers on RBCs the same pregnant women undergoing iron supplementation over time (n=5), in order to attempt to better understand changes in RBCs which may contribute to increased parasite growth rates following iron supplementation. All surface markers

examined either have been implicated as parasite invasion ligands (Glycophorin A, CD35, CD55, CD147, and sialic acid) and/or have been reported to be reflective of RBC age and membrane integrity (the aforementioned molecules, and additionally CD47 and C3b). Compared to relative surface expression levels in a non-anemic, non-pregnant control donor, we again found significant increases in markers from Day 0 to Day 14 ($p<0.001$) as reflected by GPA (a sialoglycoprotein which is a known invasion receptor of *P.falciparum* as well as known to be increased in young RBCs(41)), CD47 (an anti-phagocytic marker known to be increased on young RBCs (42)), CD35 (complement receptor 1 also known as an invasion receptor for *P.falciparum* and possibly higher on younger/healthier RBC populations (43)), CD147 (basigin, a known essential RBC receptor for *P.falciparum* invasion (44)), CD55 (decay accelerating factor, another RBC receptor involved in *P. falciparum* invasion and regulation of complement levels on the RBC surface (43)), and C3b levels (part of the complement system, we hypothesized deposition on the cell surface to be reflective of increased RBC time in circulation or a less healthy RBC (43)). We also observed a significant increase ($p<0.01$) from Day 0 to Day 14 in sialic acid residues (known to be involved in *P.falciparum* RBC invasion as well as thought to be increased on younger and healthier RBC populations (42)) (Supplemental Figure S5.3). Some proteins measured also remain elevated at Day 49 and beyond, though the biggest changes are consistently measured between Day 0 and Day 14. This data parallels the increases in circulating reticulocytes, as well as changes observed in RBC populations from anemic children undergoing iron supplementation (see Chapter 4). Shifts in relative expression of these RBC markers that we observed are highly indicative of an overall increase in young, healthy RBCs in circulation, a shift that can be observed quite quickly in pregnant women already subject to physiological drive to increase RBC volume and then additionally supplemented with iron.

5.5 DISCUSSION

Pregnancy encompasses one of the most complicated periods of health maintenance for women, particularly women in the developing world. Most obviously, delivery itself can involve many risks, especially in the absence of good health facilities and qualified attendants. Another large area of concern is the increased nutritional needs of the pregnant woman, an issue that can compound delivery related risks, as well as overall pregnancy-related health. Anemia, and iron deficiency anemia in particular, has long been known to impact large percentages of pregnant women globally, and can in turn affect fetal

growth and development (7, 16). Furthermore, pregnancy is a period of time where women in malaria endemic areas become highly susceptible to malaria infection due to the parasite's ability to specifically home to the placenta – complications of which can also carry severe outcomes for maternal and fetal health (1).

Because iron supplementation has been shown to positively impact hemoglobin and other markers of anemia and iron deficiency in pregnant women, and to potentially increase fetal growth (16, 24), this strategy is an oft-cited WHO recommendation for prenatal care (15). However, because of the problematic relationship between iron and malaria susceptibility, these recommendations are not necessarily straight-forward to adopt. Several studies have found iron deficiency reduces malaria infection and malaria susceptibility can increase with iron supplementation (11–13, 21, 45–52)**,** though in the case of pregnant women, this is particularly hard to study because of changing hematological parameters which complicate standard measurements for anemia and iron deficiency. Regardless of unknown hematological standards, this is a critical topic to study, as many women are very likely to receive iron during pregnancy, and the effects on increased malaria susceptibility may be very real. This is difficult to study, however, as clinical trials giving iron supplements will be ethically obligated to provide extensive anti-malarial assistance.

We thus attempted to systematically measure malaria susceptibility in pregnant women undergoing iron supplementation via *in vitro* parasite growth rates measured in RBCs from women at Days 0, 14, 49, and 84 throughout a 12 week supplementation period. At baseline, we found significantly reduced parasite growth rates compared to growth in RBCs from non-pregnant, non-anemic control donors. Not all of our subjects were anemic (as defined by Hgb<11g/dl), but based on analyses with the entire population of subjects, growth rates directly correlated with hemoglobin levels, as well as further indicators of iron status such as MCV. Sickle-cell trait also significantly correlated with a reduction in parasite growth in RBCs drawn from pregnant women at baseline.

Iron supplementation significantly increased parasite growth rates in RBCs drawn from study subjects at Day 14, over and above growth levels seen in non-anemic, non-pregnant control RBCs. Parasite growth rates remained slightly elevated above control growth levels at Day 49, before eventually reducing to approximately baseline status. These patterns in growth rate changes were exactly paralleled

by patterns in measurements of young RBCs in circulation, namely CD71-positive reticulocytes, but also other markers of RBC age such as glycophorin A and CD47, among others. While it is true that pregnant women naturally have elevated levels of erythropoietin driving a blood volume and RBC mass expansion, there is reason to believe the iron supplements given to our population of pregnant women increased hemoglobin and young RBC output over and above background normal levels, considering that hemoglobin rates did not decrease in our population (though they are known to decrease in normal pregnancy without iron supplementation (discussed in (8)).

While classifications of anemia are difficult, as well as quantifying the increase in young RBCs and changing blood and iron parameters in our subjects due to iron supplementation versus normal physiology of pregnancy, the malaria data is real and speaks for itself: parasite growth rates are reduced in pregnant women amongst populations where anemia and iron deficiency are common, and growth rates increase significantly almost immediately following iron supplementation. Other significant limitations to our research certainly exist, namely that we are using *in vitro* parasite growth data in attempt to paint a broader picture of clinical malaria susceptibility in the real world. However, this parasite growth data using RBCs from a maternal population undergoing iron supplementation is highly consistent with all of our previous *in vitro* work (26). The data presented here are also exactly consistent with the vast amount of data we produced in a similar study examining *in vitro* parasite growth rates in RBCs from anemic Gambian children undergoing iron supplementation (see Chapter 4). Furthermore, all of our data combined fits clinically reported observations and provides one logical explanation for the relationship between iron deficiency, iron supplementation, and malaria susceptibility.

While all of the data presented in this chapter remains very much a work in progress in terms of analyses and presentation, initial findings would certainly suggest we have yet more evidence to give reason to advocate for temporary malaria prophylaxis whenever iron supplements are distributed in malaria endemic areas. Pregnant women in many malaria endemic areas do receive IPTp (Intermittent Preventative Treatment for malaria in pregnancy) (53); our evidence may thus strengthen the need for stringent IPTp guidelines for routine antenatal care that includes iron supplementation.

5.5.1 Work in Progress

We would like to emphasize to the reader that all the research presented in this chapter remains a work in progress in terms of data analysis and presentation. Currently, we are utilizing an unofficial dataset from the associated “Hepcidin and Iron in Pregnancy” iron supplementation trial, as the trial database is yet to be locked by the PI and data manager. We fully recognize we will need to revise some analyses, as well as investigate alternative methods and data presentation styles. However, we remain confident that the preliminary data presented here provides an accurate general overview of the results we will be able to convey from this study.

5.6 TABLES AND FIGURES

Table 5.1: Blood, inflammatory, and iron parameters of donors whose RBCs were used for parasite growth assays before (Day 0), during (Days 14 and 49), and after (Day 84) iron treatment. Donors were enrolled (Day 0) between 14-22 weeks of gestation. Tests were performed in MRC Keneba laboratories using a Medonic M20M GP and Cobas Integra 400 plus, or in the field using a HemoCue 301. Values in the Normal Range column are the normal or healthy range for each parameter for 2nd and 3rd trimesters of pregnancy, as defined by standard guidelines (54), if known. Numerical values reflect the mean value of all individuals and values in parentheses indicate standard deviation (SD). Note control non-anemic, non-pregnant donors had an average hemoglobin of 14.13 g/dl (SD 0.85). The WHO defines anemia in pregnant women as <11g/dL (severe <7g/dL, mild 7-9.9g/dL, moderate 10-10.9g/dL) (55).

Variable	Normal 2nd Trimester	Normal 3rd Trimester	Day 0 n=327	Day 14 n=82	Day 49 n=112	Day 84 n=115
Red Blood Cell (x10^6 per µl)	2.81–4.49	2.71–4.43	3.76 (0.52)	3.75 (0.72)	3.74 (0.53)	3.72 (0.40)
White Blood Cell (x10^9 per l)	5.6–14.8	5.9–16.9	7.59 (2.18)	8.16 (2.24)	7.63 92.33)	8.00 (1.85)
Hemoglobin (g per dl)	9.7–14.8	9.5–15.0	10.86 (1.45)	10.93 (1.54)	10.90 (0.94)	11.07 (1.00)
Hematocrit (%)	30.0–39.0	28.0–40.0	29.68 (4.12)	30.48 (4.88)	30.57 (3.95)	30.44 (2.92)
Mean Corpuscular Volume (fl)	82–97	81–99	79.30 (6.62)	80.68 (10.65)	82.12 (6.85)	82.18 (6.09)
Mean Corpuscular Hemoglobin (pg per cell)	30–33	29–32	29.10 (2.89)	29.50 (3.02)	29.64 (2.83)	29.95 (2.35)
Mean Corpuscular Hemoglobin Concentration (g per dl)			36.65 (1.45)	39.97 (1.17)	36.06 (1.03)	36.43 (1.06)
Red Cell Distribution Width (%)	13.4–13.6	12.7-15.3	13.88 (1.64)	14.72 (2.43)	14.70 (3.00)	13.78 (2.61)
Platelet Count (x10^9 per l)	155–409	146–429	253.18 (73.99)	238.02 (57.72)	232.04 (66.01)	231.23 (62.64)
Iron Total (µ mol per l)	7.9-31.9	5.4-34.6	14.58 (7.90)	23.74 (12.90)	22.79 (10.82)	32.41 (13.67)
Transferrin (g per l)	2.20–4.41	2.88–5.30	3.35 (0.67)	3.11 (0.69)	3.14 (0.62)	3.10 (0.66)
Transferrin Saturation (%)	10–44	5–37	18.6 (12.6)	38.0 (18.3)	35.1 (16.9)	49.1 (20.7)
Ferritin (ng per ml)	2–230	0–116	33.90 (35.24)	40.60 (23.86)	44.04 (32.16)	41.38 (23.58)
Alpha 1 Anti-glycoprotein (g per l)			0.65 (0.26)	0.55 (0.17)	0.42 (0.19)	0.44 (0.16)
C Reactive Protein (mg per dl)	0.04–2.03	0.04–0.81	5.93 (13.28)	4.80 (4.48)	4.69 (5.28)	4.33 (3.48)
Soluble Transferrin Receptor (nmol per l)			4.32 (2.48)	4.20 (4.43)	3.78 (1.30)	3.42 (1.05)
Soluble Transferrin Receptor: log Ferritin Index			3.85 (14.80)	2.85 (2.13)	2.55 (1.09)	2.40 (1.48)
Hepcidin (ng per ml)			4.57 (6.45)	8.46 (8.21)	10.22 (8.05)	9.93 (8.08)

Table 5.2. Effect of host hemoglobin, iron status, and other hematological characteristics on *in vitro P. falciparum* growth in RBCs from pregnant women (14-22 weeks gestation) at baseline. Growth rates (GR) were calculated relative to growth in healthy, non-anemic donors. Growth assays were performed in triplicate for each donor and the average value was used for linear regression modelling; multivariate analyses represent the estimated association for a given variable while controlling for potential confounders. Hgb genotype was evaluated solely based on AA vs. AS classification. For continuous variables, the β1 value represents the %GR change (x100) for every 1 unit increase in the primary variable. For categorical variables, the β1 value represents the %GR change (x100) based on yes-no genotype. For example, for Hgb AS, the %GR change is -24.8% relative to Hgb AA. Significant p values (<0.05) are bolded. The standardized %GR change for all continuous variables is calculated based on the SD for the exposure variable of interest (see Table 5.1) multiplied by β1 (x100%), to give the %GR change for every 1 SD change in the exposure variable; for Hgb genotype the adjusted %GR change is simply β1 (x100%).

Condition	B1 value	Lower CI	Upper CI	p value	Standardized % change
Bivariate analysis of measures affecting parasite growth					
Hgb (g/dL)	0.089	0.052	0.127	**<0.0001**	11.41
Hgb genotype (AA vs AS)	-0.248	-0.383	-0.114	**<0.0001**	-24.82
MCV (fL)	0.009	0.002	0.017	**0.017**	6.02
Ferritin (ng/ml)	<0.001	-0.001	0.002	0.895	0.35
Hepcidin (ng/ml)	0.006	-0.001	0.014	0.099	4.17
CRP (mg/dL)	0.001	-0.003	0.005	0.503	1.73
sTfR:log ferritin ratio	-0.001	-0.009	0.006	0.789	-1.51
Transferrin saturation (%)	0.687	0.297	1.078	**0.001**	8.66
Gestational age (weeks)	0.005	-0.004	0.014	0.262	3.04
RDW (%)	0.050	0.015	0.020	**0.001**	8.23
log hepcidin	0.086	0.022	0.150	**0.009**	6.77
Multivariate analysis of significant measures affecting parasite growth controlling for possible confounders					
Hgb affects parasite growth controlling for Hgb genotype	0.085	0.047	0.123	**<0.0001**	10.89

Figure 5.1: 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 Days 14, 49, and 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. For malaria assays, 2.5mL of venous blood was drawn directly into microvette tubes containing CPDA-1 (Sarstedt, Germany). Unavailable donors include safety exclusion (Hgb<7g/dl or positive malaria test (RDT positive)), those who delivered prematurely or aborted, or general loss to follow up (withdrawal and travel). Failure to collect blood from subjects (e.g. from phlebotomy failure, subject moved or withdrew, delivered, or became significantly ill) was 99.8% at Day 0, 95.2% at Day 14, 91.6% at Day 49, and 83.2% 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 S5.2).

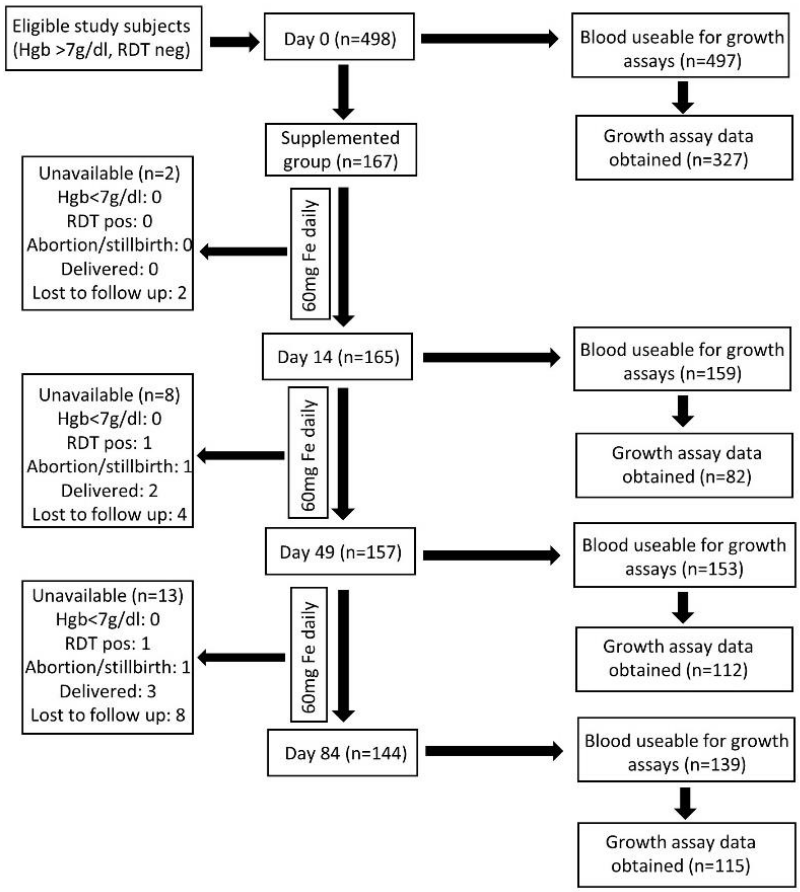


Figure 5.2: Parasite growth and invasion in RBCs from pregnant women (14-22 weeks gestation) at baseline. A) *P.falciparum* (strain FCR3-FMG) growth rates are proportional to hemoglobin concentration. Growth assays were performed in RBCs drawn from pregnant women at baseline (Day 0) and values are presented relative to growth in RBCs from non-anemic, non-pregnant donors. Each dot represents the mean result of triplicate growth assays from each donor and the error bars represent 95% CI. One-way ANOVA indicates the means are significantly different between Days ($p<0.05$); specifically, post-hoc analysis with Tukey's test indicates significant differences between Hgb levels <10 and 11.0-11.9 ($p<0.05$) or >12 ($p<0.001$), and between Hgb 10-10.9 and >12.0 ($p<0.05$). B) Parasite growth rates in RBCs from pregnant women categorized by different definitions of anemia at baseline. In analysis of parasite growth rates in RBCs from pregnant women at Day 0, we stratified anemic participants (Hgb<11g/dl) with growth rate data at Day 0 (n=124) using four different definitions to categorize the severity and type of iron deficiency in the presence or absence of inflammation: those with 1) hepcidin<2.5ng/ml (n=85); 2) ferritin<12ng/ml (n=39); 3) ferritin12-30ng/ml with CRP>5mg/ml (n=6); 4) hemoglobin increase of >0.5g/dl from baseline after 49d or 84d of daily iron supplementation (n=77); definitions 1-4 are not necessarily mutually exclusive nor do they ultimately encompass everyone with anemia. Growth rate values are presented relative to growth in RBCs from non-anemic, non-pregnant donors. Each dot represents the mean result of triplicate growth assays from each donor and the error bars represent 95% CI. Mean growth rate results (with 95%CI) are: hepcidin<2.5ng/ml=58.71% (49.08-68.34%); ferritin<12ng/ml=74.27% (58.90-89.64%); ferritin 12-30ng/ml with CRP>5mg/ml=51.73% (7.76-95.69%), ΔHgb>0.5g/dl=68.51% (58.46-78.55%). There are no significant differences between the means.

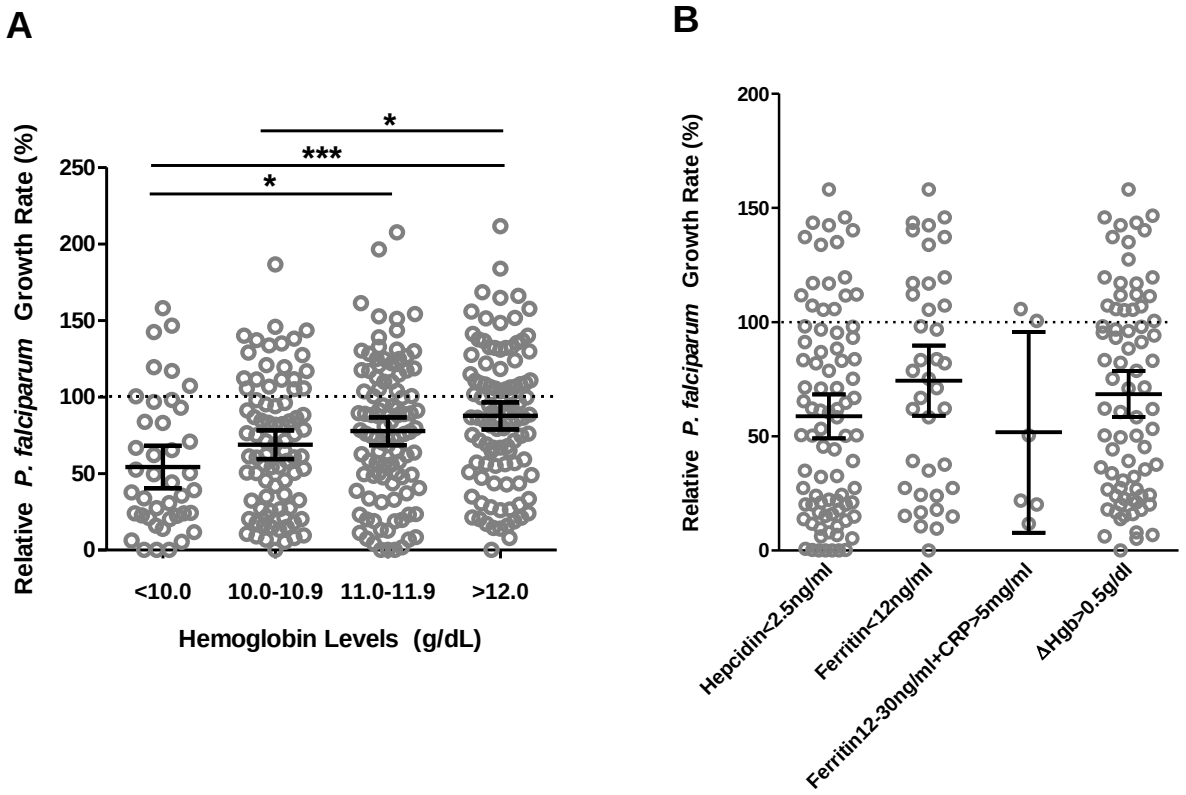


Figure 5.3: *P.falciparum* PfRh invasion ligands and attachment rates are not involved in mechanisms of differential invasion into RBCs from anemic versus non-anemic donors. A) Comparison of invasion into RBCs from anemic pregnant women and non-anemic, non-pregnant donors using *P.falciparum* laboratory strains. Invasion experiments for RBCs from all anemic donors (drawn at Day 0) were performed independently and each experiment was performed in duplicate. Data show the mean relative invasion using RBCs from 4 donors for strain 3D7 and 3 for FCR3-FMG. The relative invasion was determined calculating the percent of ring-stage infected RBCs after incubating purified schizonts from culture with target RBCs from donors, taking into account percent infected RBCs at the experiment start time in each target RBC experimental set-up. All values are normalized to 1.0 being the invasion rate for the control non-anemic, non-pregnant donor in each experiment. The bars represents the 95% CI. Both strains 3D7 and FCR3-FMG give relative invasion values significantly decreased from the control value of 1.0: 3D7 mean 0.74 (95% CI 0.60-0.87); FCR3 mean 0.60 (95% CI 0.44-0.76). B) Competitive invasion of subject and control RBCs using *P. falciparum* parent strain 3D7 and transgenic strains missing individual PfRh invasion ligands. Invasion experiments for RBCs from all test subject donors were performed independently and each experiment was performed in triplicate. Data show the mean SI using RBCs from 15 pregnant test donors for each strain. The SI defines the relative susceptibility to invasion of two different types of RBCs, control and test subject. The marker represents the SI point estimate and the bar represents the 95% CI. An SI of 1.0 indicates no difference in parasite invasion of two RBC populations. 95% CI are shown; there are no statistically significant differences in SI values from the transgenic strains versus the parent strain 3D7. C) *P. falciparum* merozoite invasion into and attachment to RBCs from anemic pregnant women (Hgb<11g/dl) compared to RBCs from non-anemic, non-pregnant donors. Purified schizonts were incubated with control or subject RBCs in parallel for 3-7hrs to allow for schizont rupture. Subsequent fixation and staining with DNA dye allows for detection of either new invasion events or attachment events, which are distinguishable from seeded schizont-infected RBCs by flow detection of DNA content. Attachment was specifically assayed with inclusion of cytochalesin D during schizont incubation to prevent further invasion. New parasite events relative to starting seeding parasitemia were compared between subject and control RBCs (with rates in subject RBCs normalized to those in control RBCs within each experiment, in order to allow for inter-experiment comparisons). Results are shown from 2 experiments with RBCs from 3 control and 5 subject donors each, assayed in triplicate. Error bars represent 95% CIs. There is a significant difference in merozoite invasion in subject RBCs compared to control RBCs (mean RBC invasion value of 0.63 for subject relative to controls, *p<0.001 by student's t-test), but no difference in merozoite attachment (mean RBC attachment value of 0.85 for subjects relative to controls).

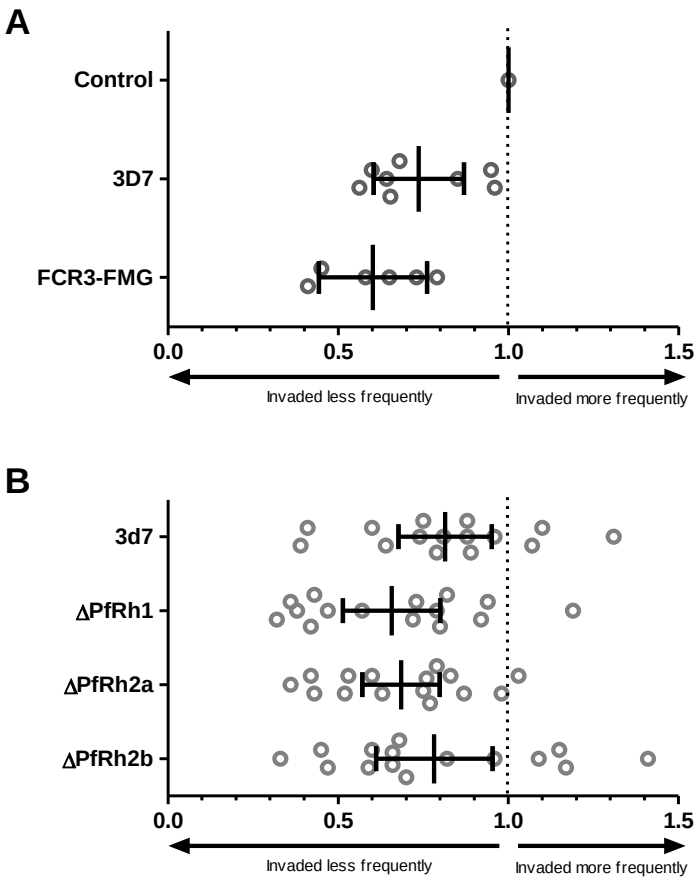
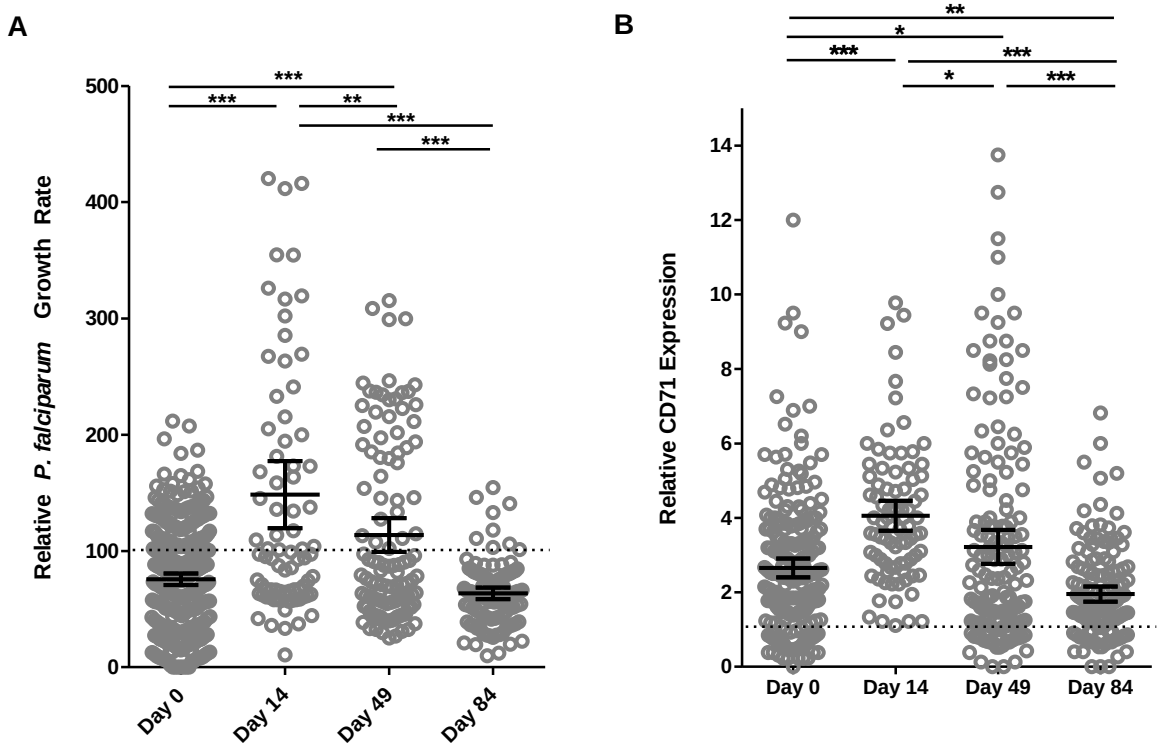

A
Control
3D7
FCR3-FMG
0.0
0.5
1.0
1.5
Invaded less frequently
Invaded more frequently
B
3d7
ΔPfRh1
ΔPfRh2a
ΔPfRh2b
0.0
0.5
1.0
1.5
Invaded less frequently
Invaded more frequently
C
Relative Attachment or Invasion (%)
1.5
1.0
0.5
0.0
Control RBCs
Subject RBCs
*
No Tx
(Invasion)
Cyt D
(Attachment)

Figure 5.4: Malaria susceptibility increases transiently during iron supplementation and pregnant women receiving iron supplements have increased numbers of young RBCs. A) *P.falciparum in vitro* growth rates in RBCs from pregnant women increase over time with iron supplementation (60mg iron daily for 84 d). Parasite growth assays were conducted in RBCs from pregnant women at Day 0, Day 14, Day 49, and Day 84 using strain FCR3-FMG. Growth rates are reported relative to growth in RBCs from non-anemic, non-pregnant donors. Each dot represents the mean of triplicate assays and error bars represent the 95% CI. Differences between growth rates at the different time points were significant ($p<0.0001$ by one-way ANOVA); specifically, post-hoc analysis with Tukey's test indicates significant differences between Day 0 and Day 14 (***$p<0.001$), between Day 0 and Day 49 (***$p<0.001$), between Day 14 and Day 49 (**$p<0.01$), between Day 14 and Day 84 (***$p<0.001$), and between Day 49 and Day 84 (***$p<0.001$). The only non-significant differences were between Day 0 and Day 84. n=327 pregnant women at Day 0, n=82 pregnant women at Day 14, n=112 pregnant women at Day 49, and n=115 pregnant women at Day 84. B) Levels of CD71 positive RBCs increase over time in pregnant women undergoing iron supplementation. Percent CD71-positive RBCs was measured by flow cytometry analysis of CD71 surface expression. Error bars represent the 95% CI; one-way repeated measures ANOVA indicates the means are significantly different between Days ($p<0.0001$); post-hoc analysis with Tukey's test indicates significant differences between Day 0 and Day 14 (***$p<0.001$), between Day 0 and Day 49 (*$p<0.05$), between Day 0 and Day 84 (**$p<0.01$), between Day 14 and Day 49 (*$p<0.05$), between Day 14 and Day 84 (***$p<0.001$), and between Day 49 and Day 84 (***$p<0.001$). n=209 pregnant women at Day 0, n=83 pregnant women at Day 14, n=152 pregnant women at Day 49, and n=142 pregnant women at Day 84.



5.7 SUPPLMENTAL TABLES AND FIGURES

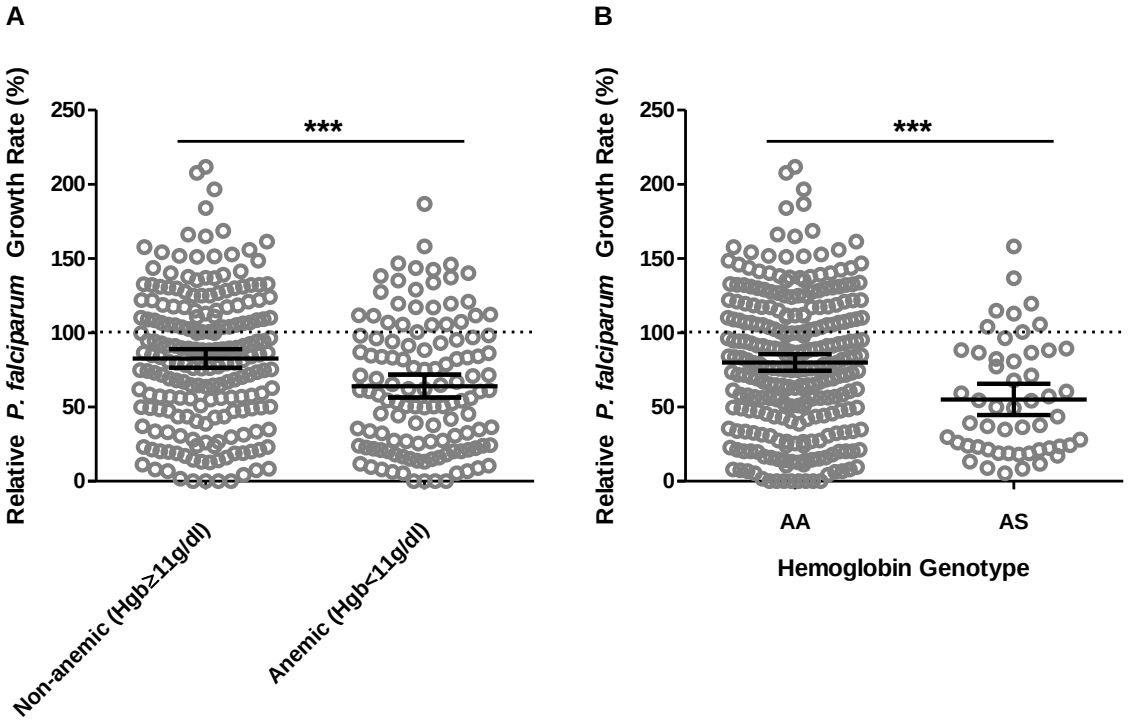
Supplemental Table S5.1: Description of subjects at baseline. Hemoglobin genotyping was performed by electrophoresis. *A portion of subjects (% of total) were unable to be tested for hemoglobin genotype so are not included in the denominator for other genotypes (which denote % of those tested).

Characteristic		**All participants (n=498)**	**Those to receive 60 mg iron daily (n=167)**
Age, years, mean (SD)		27.1 (5.8)	27.0 (5.9)
Gestational age at enrollment, weeks, mean (SD)		19.9 (6.1)	19.4 (6.8)
Hemoglobin genotype, n (%)			
	AA	400 (82.3)	129 (79.6)
	AS	86 (17.7)	33 (20.4)
	SS	0	0
	AC	0	0
	SC	0	0
	Untested*	12 (2.4)	5 (3.0)

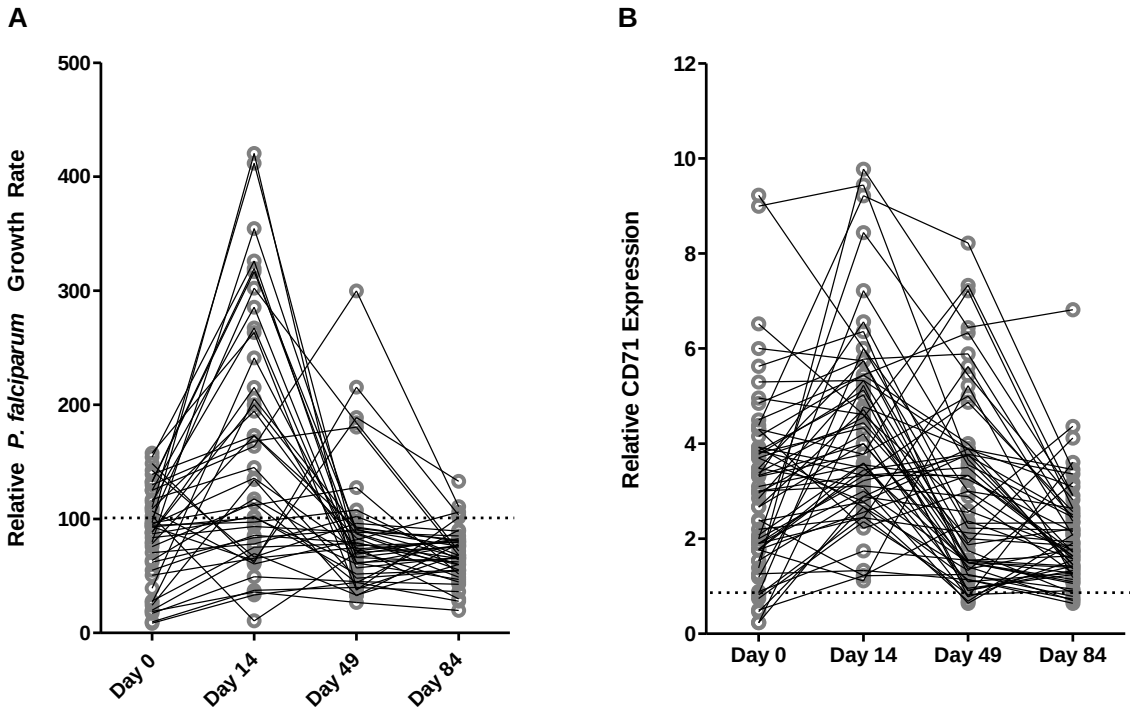
Supplemental Table S5.2: Blood, inflammatory, and iron parameters of study participants with reportable parasite growth rate data versus those without. Numerical values reflect the mean value of all individuals of a particular category and time point, and values in parentheses are the SD.

Variable	**Day 0 w/ GR** n=327	**Day 0 no GR** n=172	**Day 14 w/ GR** n=82	**Day 14 no GR** n=83	**Day 49 w/ GR** n=112	**Day 49 no GR** n=45	**Day 84 w/ GR** n=115	**Day 84 no GR** n=29
Red Blood Cell (x10^6 per µl)	3.76 (0.52)	3.73 (0.48)	3.75 (0.72)	3.55 (0.36)	3.74 (0.53)	3.56 (0.36)	3.72 (0.40)	3.55 (0.43)
White Blood Cell (x10^9 per l)	7.59 (2.18)	7.14 (1.89)	8.16 (2.24)	7.39 (1.85)	7.63 92.33)	7.22 (1.89)	8.00 (1.85)	8.63 (2.47)
Hemoglobin (g per dl)	10.86 (1.45)	10.61 (1.32)	10.93 (1.54)	10.44 (0.96)	10.90 (0.94)	10.72 (1.06)	11.07 (1.00)	10.74 (1.22)
Hematocrit (%)	29.68 (4.12)	29.65 (3.62)	30.48 (4.88)	28.49 (2.61)	30.57 (3.95)	29.23 (3.05)	30.44 (2.92)	29.74 (3.52)
Mean Corpuscular Volume (fl)	79.30 (6.62)	78.89 (7.44)	80.68 (10.65)	80.56 (6.51)	82.12 (6.85)	82.26 (5.12)	82.18 (6.09)	84.19 (7.93)
Mean Corpuscular Hemoglobin (pg per cell)	29.10 (2.89)	28.71 (3.13)	29.50 (3.02)	29.62 (2.83)	29.64 (2.83)	30.25 (2.12)	29.95 (2.35)	30.48 (3.03)
Mean Corpuscular Hgb Concentration (g per dl)	36.65 (1.45)	36.33 (0.96)	39.97 (1.17)	36.72 (1.11)	36.06 (1.03)	36.77 (1.06)	36.43 (1.06)	36.2 (0.80)
Red Cell Distribution Width (%)	13.88 (1.64)	13.51 (1.68)	14.72 (2.43)	14.00 (1.89)	14.70 (3.00)	13.70 (1.83)	13.78 (2.61)	13.45 (1.04)
Platelet Count (x10^9 per l)	253.18 (73.99)	262.72 (67.88)	238.02 (57.72)	268.86 (72.54)	232.04 (66.01)	232.76 (71.75)	231.23 (62.64)	231.46 (65.21)
Iron Total (µ mol per l)	14.58 (7.90)	15.73 (6.97)	23.74 (12.90)	22.14 (12.68)	22.79 (10.82)	16.92 (7.78)	32.41 (13.67)	34.01 (12.06)
Transferrin (g per l)	3.35 (0.67)	3.23 (0.60)	3.11 (0.69)	2.92 (0.70)	3.14 (0.62)	2.47 (0.43)	3.10 (0.66)	3.32 (0.50)
Transferrin Saturation (%)	018.6 (12.6)	23.8 (12.7)	38.0 (18.3)	34.7 (18.1)	35.1 (16.9)	27.7 (12.6)	49.1 (20.7)	48.6 (18.1)
Ferritin (ng per ml)	33.90 (35.24)	36.35 (50.01)	40.60 (23.86)	50.02 (42.18)	44.04 (32.16)	41.24 (38.33)	41.38 (23.58)	38.88 (20.17)
Alpha 1 Anti-glycoprotein (g per l)	0.65 (0.26)	0.68 (0.27)	0.55 (0.17)	0.57 (0.18)	0.42 (0.19)	0.39 (0.17)	0.44 (0.16)	0.50 (0.37)
C Reactive Protein (mg per dl)	5.93 (13.28)	5.95 (11.89)	4.80 (4.48)	5.96 (7.78)	4.69 (5.28)	4.62 (4.99)	4.33 (3.48)	5.12 (5.41)
Soluble Transferrin Receptor (nmol per l)	4.32 (2.48)	4.58 (2.06)	4.20 (4.43)	3.99 (1.63)	3.78 (1.30)	3.18 (1.06)	3.42 (1.05)	3.38 (0.96)
Soluble Transferrin Receptor: log Ferritin Index	3.85 (14.80)	3.56 (8.91)	2.85 (2.13)	2.62 (1.37)	2.55 (1.09)	2.28 (1.08)	2.40 (1.48)	2.26 (0.92)
Hepcidin (ng per ml)	4.57 (6.45)	7.75 (8.87)	8.46 (8.21)	8.65 (8.33)	10.22 (8.05)	8.71 (7.98)	9.93 (8.08)	9.43 (6.70)

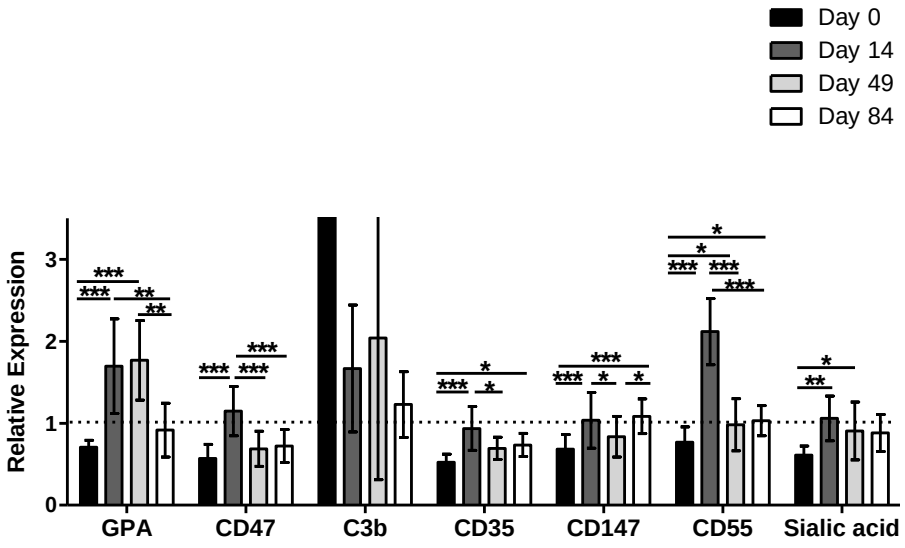
Supplemental Figure S5.1: Parasite growth in RBCs from different donor groups at baseline. A) Parasite growth rates in RBCs from pregnant women categorized by anemia status, non-anemic (Hgb≥11g/dl) and anemic (Hgb<11g/dl). Growth rate values are presented relative to growth in RBCs from non-anemic, non-pregnant donors. Each dot represents the mean result of triplicate growth assays from each donor and the error bars represent 95% CI. Mean growth rate results (with 95%CI) are: non-anemic 82.67% (76.38-88.96%) and anemic 64.01% (56.28-71.74%). Student's t-test indicates significant difference between the means (p<0.001). n=203 non-anemic and n=124 anemic donors. B) Parasite growth rates in RBCs from pregnant women categorized by hemoglobin genotype, normal (AA) and with the sickle-cell trait mutation (AS). Growth rate values are presented relative to growth in RBCs from non-anemic, non-pregnant donors. Each dot represents the mean result of triplicate growth assays from each donor and the error bars represent 95% CI. Mean growth rate results (with 95%CI) are: AA 79.84% (74.19-85.48%) and AS 55.02% (44.47-65.58%). Student's t-test indicates significant difference between the means (p<0.001). n=263 with AA genotype; n=52 with AS genotype.



Supplemental Figure S5.2: Changes in parasite growth and reticulocytosis in RBCs from pregnant women before, during, and after daily iron supplementation. A) Levels of parasite growth rates increase over time in pregnant women undergoing iron supplementation, as depicted by line graph in order to highlight changes for each individual that had data available at all time points (n=44 pregnant women with complete repeat growth measures at Day 0, 14, 49, and 84), with 88.6% having increased growth rate at Day 14 and 54.5% having increased growth rate at Day 49. One-way repeated measures ANOVA of growth rate values indicates the means are significantly different between Days ($p<0.0001$); post-hoc analysis with Tukey's test indicates significant differences between Day 0 and Day 14 means ($p<0.001$), between Day 14 and Day 49 ($p<0.001$), and between Day 14 and Day 84 ($p<0.001$), but no significance between Day 0 and Day 49 or Day 84, nor between Day 49 and Day 84 for those pregnant women with repeat measures. B) Levels of parasite growth rates increase over time in pregnant women undergoing iron supplementation, as depicted by line graph in order to highlight changes for each individual that had data available at all timepoints (n=60 pregnant women with complete repeat CD71 measures at Day 0, 14, 49, and 84), with 78.3% having increased growth rate at Day 14. One-way repeated measures ANOVA of growth rate values indicates the means are significantly different between Days ($p<0.0001$); post-hoc analysis with Tukey's test indicates significant differences between Day 0 and Day 14 means ($p<0.001$), between Day 14 and Day 49 ($p<0.001$), and between Day 14 and Day 84 ($p<0.001$), but no significance between Day 0 and Day 49 or Day 84, nor between Day 49 and Day 84 for those pregnant women with repeat measures.



Supplemental Figure S5.3: Surface markers of RBC age and integrity change in a pattern consistent with an increase in erythropoiesis in pregnant women undergoing iron supplementation (60mg daily). We measured GPA (an abundant sialoglycoprotein which contributes to RBC surface charge and is found at higher levels on younger RBCs (41)), CD47 (an anti-phagocytic marker which influences RBC senescence and is found in lower levels in RBCs that have been in circulation longer or are less healthy (42)), surface deposition of complement factor C3b (higher levels of which would correlate with increased RBC time in circulation, or less healthy RBC membranes (43)), and levels of *P.falciparum* merozoite receptors (CD35, CD147, CD55, and sialic acid residues). Note that GPA is also a merozoite receptor, and CD35 and CD55 involved in the complement system have also been described as reflecting RBC age (more abundant on younger/healthier RBCs (43)), as has sialic acid abundance (reduced on older RBCs) (42). CD147, known as basigin, is the only known essential *P.falciparum* invasion receptor (44). Data represent relative expression based on subject donor RBC MFI values (GPA, CD47, CD35, CD147, CD55, and sialic acid residues) or percent positive population values (C3b), compared to RBCs from a non-anemic, non-pregnant control donor not receiving iron supplementation (relative expression=1.0). RBCs from the same 6 donors were examined over time. Error bars represent the 95% CIs. If indicated, one-way repeated measures ANOVA with post-hoc Tukey's test analysis indicates significant difference between expression levels (*p<0.05, **p<0.01, ***p<0.001).



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CHAPTER SIX: DISCUSSION AND FUTURE DIRECTIONS

6.1 BRIEF SUMMARY OF WORK PRSENSED IN THIS DISSERTATION

The research undertaken in this dissertation was designed to expand upon epidemiological findings of reduced malaria infection in people with iron deficiency and potentially increased malaria susceptibility following iron supplementation, attempting to understand this relationship on a cellular level.

This was initiated with a study enrolling people from UNC hospitals who were iron deficient or iron replete and using their RBCs to conduct *in vitro* assays comparing various aspects of parasite growth (Chapter 2). We determined that parasite growth was significantly reduced in RBCs from iron deficient individuals, and we went on to characterize this reduced growth as involving both reduced invasion into as well as replication within iron deficient RBCs, but not altered parasite maturation. In RBCs from iron deficient and iron replete donors who received iron supplementation, there were no differences. We further characterized increased parasite growth and invasion in young RBCs and put forth a model hypothesizing that RBC population dynamics influenced malaria susceptibility, not just simple iron withholding as is the case with many other pathogens such as bacteria. In our model, presence of iron deficient RBCs limits infection potential due to their reduced susceptibility to parasite infection, and when someone receives iron they begin to erythropoiese at elevated rates, such that they have a much higher level of young RBCs in circulation which are highly susceptible to malaria infection. Above-average numbers of young RBCs and replacement of protective iron deficient RBCs would thus constitute a period of increased malaria risk before finally, RBC populations would normalize as would infection susceptibility.

In anticipation of testing this model using RBCs from relevant populations in malaria endemic areas, we next sought to develop a method for preserving valuable RBCs to allow for prolonged usage in *in vitro* malaria experiments (Chapter 3), as established malaria culture protocols require freshly drawn RBCs (utilized within 2-4 weeks from blood draw). We first characterized growth, invasion, and replication

within RBCs stored over time, confirming significant reductions in parasite growth and invasion in RBCs stored longer than 2 weeks. We tested several different RBC storage medias, but did not find any improved RBC viability. However, we did find that biopreservation of RBCs in liquid nitrogen provided a means for preserving RBCs for prolonged periods without any detriment to *in vitro* parasite growth indices.

We next conducted a field study partnering with clinical trial research of iron supplementation in anemic Gambian children aged 6-24 months (Chapter 4). We attempted to better characterize the degree of protection from iron deficiency as well as changes in RBC population dynamics following iron supplementation that influence parasite growth. This work was all conducted using *in vitro* malaria culture as a proxy measure of malaria susceptibility, both because clinical malaria incidence is very low in The Gambia currently, and because this method provided a better means of systematically measuring malaria infection potential without biases from anti-malarial preventative measures (provided to trial participants in accordance with ethical safety precautions). We determined that RBCs from anemic children significantly reduced parasite growth and invasion, and that parasite growth rates specifically correlated with hemoglobin concentration within our study population. In fact, within our population of anemic children, the parasite growth reduction was on par with or greater than growth reduction observed in RBCs from those children with sickle-trait. Following iron supplementation, we observed significant increases in parasite growth and invasion in subject RBCs, over and above growth in non-anemic controls. These growth changes paralleled increases in circulating reticulocytes and other measures of young RBC populations following iron supplementation, fitting with our proposed model of how RBC population structures might influence overall infection susceptibility.

Briefly, regarding the data from this study, it should be mentioned why we have ultimately focused on anemia (as defined by hemoglobin values <11g/dl) and thus hemoglobin as the primary variable influencing parasite growth, as opposed to the degree of iron deficiency – especially when we have spent so much time throughout this dissertation discussing the importance of iron deficiency specifically. It is true anemia can have multiple causes beyond iron deficiency and the term anemia is less specific than iron deficiency anemia. There are a number of reasons for our shift towards using the term anemia. First, in our linear regression modeling examining which variables correlated with parasite growth rates, we did

not find any measurements relating to iron status were significant (i.e. ferritin, serum iron, soluble transferrin receptor (sTfR), hepcidin, transferrin saturation). We believe this is due to the second point – that it is notoriously difficult to measure iron deficiency in populations with high levels of inflammation, such as seen with children exposed to many infectious organisms and less sterile environments (as is common in developing countries). This influences accurate measurement of iron status because several markers of iron status are also acute phase proteins that rise with inflammation, thus falsely portraying the true iron status of an iron deficient individual simultaneously experiencing inflammation. Understanding this point, it is not surprising that we found no significant correlation of parasite growth rates with iron markers, because so many of the children in our study do have high levels of inflammation (specifically, 24.9% had CRP >5mg/dl at baseline and 61.5% had AGP levels >1 at baseline). Multiple definitions of iron deficiency anemia have been proposed to more accurately measure iron status in the presence of inflammation. We thoroughly examined the data our study population to enumerate the number of children fitting into several well-known or practical definitions of iron deficiency anemia (based on ferritin, CRP, hepcidin, and Hgb response to iron supplementation) and consistently found that approximately 60% of our population had iron deficiency based on these measurements (discussed in Chapter 4). However, taking into account other very relevant variables, nearly every child could be categorized as having iron deficiency anemia specifically. In particular, we found MCV to be below normal levels (<70fL) in 83.8% of the population at baseline and sTfR:log ferritin ratios to be >2 in every single participant (ratios above 2 being highly indicative of iron deficiency anemia as soluble transferrin receptor levels rise in the presence of iron deficiency and ferritin levels drop). Thus, we remain confident in saying that our study population of anemic children was in fact suffering from iron deficiency anemia. This discussion of measurements of iron deficiency anemia closely relates to the final point of why we focused on using the terms hemoglobin and anemia as impacting parasite growth rates in presentation of our results – because hemoglobin is a simple measurement which allows for very practical and easy translation to the field setting in malaria endemic areas where it would be desirable to predict malaria susceptibility and the impact of iron supplementation. Hemoglobin measurements can be made on-the-spot with relatively simple and inexpensive diagnostic tools, rather than the complex and expensive diagnostic machines required for measuring complete iron panels which are nearly entirely absent from

the developing world with the exception of research laboratories. Our results show that hemoglobin measurement alone is sufficient to predict malaria susceptibility, as measured by our in vitro growth data, thus providing a more practical option for researchers and clinicians who have continuously struggled to measure iron deficiency and understand the relationship between iron status markers and malaria susceptibility.

Finally, we also partnered with a clinical trial of iron supplementation in pregnant women, in order to investigate the same malaria-related safety questions in this population (Chapter 5). Not all participants were anemic, but we still found significantly reduced parasite growth in RBCs from study subjects at baseline, compared to growth in RBCs from non-anemic, non-pregnant donors. Again, parasite growth rates correlated significantly with hemoglobin concentration, and again, the growth reductions seen with anemia status were on par with growth reductions observed in RBCs from donors harboring the sickle-trait genotype. We attempted to further characterize mechanisms of reduced invasion into RBCs from anemic donors, but did not find the invasion deficit was due to either specific PfRh proteins being able to "identify" differences in iron replete versus iron deficient RBCs, nor to merozoite attachment differences. Yet again, we observed significantly elevated parasite growth rates in subject RBCs following iron supplementation with growth rate changes paralleling increases in young RBC populations in circulation. Analyses for the pregnant women iron supplementation study are ongoing, but preliminarily they clearly match our findings from the study involving anemic children undergoing iron supplementation and also fit our larger model describing the relationship between iron deficiency, iron supplementation, and malaria susceptibility.

6.2 WAYS FORWARD

Our research has contributed significantly to knowledge of factors driving parasite growth reductions in RBCs from people with anemia, as well as solidified the reality of clinical epidemiological work suggesting iron supplementation may put people at increased risk for malaria infection. Of course, there is still much to be learned and many possible pathways forward for the overarching research topic of iron and malaria.

6.2.1 Further investigating mechanisms of reduced invasion into iron deficient RBCs.

The reduced growth of *P.falciparum* in RBCs from iron deficient donors raises important questions: What molecular properties of IDA RBCs confer protection from malaria, and can these properties be exploited by medical interventions? Invasion is critically important to blood stage vaccine development and IDA provides a rare opportunity to characterize a strong natural difference in invasion phenotype. The merozoite is one of the rare parasite stages directly exposed to the host immune system, and if unable to invade a RBC, merozoite viability quickly diminishes (1). Therefore, the speed and ease with which the merozoite is able to identify and invade its target cell is critical to parasite survival, and determining the mechanisms responsible for reduced invasion of IDA RBCs could benefit understanding of parasite biology and counter-infection research.

We have begun probing both host and parasite factors which could impact reduced parasite invasion into iron deficient RBCs. RBC invasion by merozoites occurs via a series of well-orchestrated steps (2)**.** When a merozoite encounters an uninfected RBC, it will form a low affinity, reversible specific interaction, which triggers a deformation of the RBC that tumbles the merozoite over the surface and allows it to reorient such that its apical end is irreversibly attached to the RBC. Subsequently, a moving tight junction is formed between the parasite and host RBC which involves parasite proteins AMA1 and the RON complex. The junction moves from the merozoite apical to posterior end using a junction-associated actin-myosin motor to propel itself inside (3). Merozoite invasion ligands important for target cell recognition and junction formation signaling so far belong to two major families, the erythrocyte binding-like antigens (EBA) and the reticulocyte binding-like homologs (RBL or PfRh). *P.falciparum* has an extensive array of invasion ligands and can utilize a variety of host receptors to bind RBCs. The parasite is very adaptable: if a host receptor is absent from a RBC, the parasite may utilize an alternate receptor through differential EBA and PfRh binding pathways. Multiple physiological differences between IDA and IR RBCs could impact invasion. We initially focused on interrogating host receptors and parasite invasion ligands for determining mechanisms governing reduced anemic RBC invasion. We did this by characterizing levels of host receptors on RBC surfaces over time following iron supplementation, as well as examining whether invasion preference for IR RBCs relied on any of the PfRh proteins based on competitive invasion experiments with transgenic parasites missing the PfRh proteins. While we did find

levels of several host receptors increased on RBCs following iron supplementation, many of these receptors are also markers of RBC age. This makes it difficult to interpret whether their increased levels simply reflect increases in young RBCs which are known to be more amenable to parasite invasion, as opposed to increased levels of parasite receptors aiding invasion. It is also true young and iron replete RBCs are larger in size, which could contribute to differential receptor levels, although that is difficult to determine definitively with the methods we used just looking at overall relative expression levels. More precise measurements of receptor levels per cell as well as the impact of cell size could be further pursued. We did not find that absence of any PfRh ligand disrupted the parasite's ability to preferentially differentiate between iron deficient and iron replete RBCs, though this could be explored further through knockout strains missing the other invasion EBA ligands. We could also focus on interrogating invasion ligands and host receptors more through antibody inhibition invasion experiments.

However, we believe our results finding reduced invasion but not reduced attachment to RBCs from IDA donors points to mechanical membrane differences as playing a more important role in differential invasion of RBCs from IDA donors. It is well known that IDA RBCs have greater osmotic fragility and membrane rigidity and accelerated *in vivo* ageing (4–6). There is also growing evidence in the malaria literature that localized membrane deformability is a critical factor in merozoite invasion, with novel live cell imaging studies breaking down invasion into more discreet steps (7). These researchers found slight RBC deformation occurred following an initial weak interaction of merozoites with the RBC surface, followed by strong interactions of merozoite PfRh and EBA proteins with the RBC surface which in turn strongly deform the RBC. The authors interestingly hypothesized about "host cell selection through deformation which possibly helps embed the merozoite in the erythrocyte surface, leading to subsequent downstream events". Their interpretation of the data was that RBC deformation might be a large factor contributing to rapid selection of a host RBC to invade, as they observed that when a merozoite was unable to efficiently trigger echinocytosis of the target RBC, it often detached and attempted to invade a different RBC (7).

In relation to our system, it would be very interesting to compare extensive live cell imaging of invasion into RBCs from anemic donors versus non-anemic donors, in order to try to visualize which steps of invasion are impaired, the RBC deformation dynamics during invasion of anemic RBCs, and whether

merozoites do detach from anemic RBCs more often. We could additionally use confocal imaging to examine merozoite-RBC tight junction integrity in anemic RBCs through staining of parasite proteins involved in tight junction formation (such as RON4 and AMA-1), to see if junction proteins colocalize with RBC membranes, as opposed to attached merozoites which are not properly reoriented and forming junctions and thus still reversibly bound to RBCs. Furthermore, we could determine the impact on invasion of different levels of RBC deformability, quantifying deformability of RBCs from anemic and non-anemic donors through methods such as ektacytometry or microfluidic flow chambers, as well as altering deformability of non-anemic RBCs with cell-stiffening agents such as diamide (8). We could also try to alter intracellular ATP content to see if this impacted invasion, as intracellular ATP is also known to influence both parasite invasion and membrane deformability (8–11). To better understand other host membrane properties which might be altered in IDA RBCs beyond the known parasite invasion receptors we examined, mass spectrometry analysis of RBC membranes from anemic individuals before and after iron supplementation could also provide useful information. Finally, we could attempt to adapt the parasite to grow in IDA RBCs and see whether over time it can invade IDA RBCs as efficiently and examining adapted parasite protein expression for clues as to invasion pathways. This method has been successfully employed previously in studying sialic acid independent invasion, identifying parasite use of specific invasion proteins by causing upregulation of certain invasion pathways over time with adaptation to growth in RBCs treated to remove sialic acid residues (12). Overall, there are many methods which could be utilized to try to further characterize the mechanisms causing reduced invasion of RBCs into iron deficient donors. Further research into this arena may help elucidate natural mechanisms for inhibiting parasite pathogenesis which could be exploited by drug or vaccine development, or would help contribute to overall knowledge of parasite pathogenesis and biology.

6.2.2 Further investigating iron acquisition and utilization pathways in the falciparum malaria parasite.

Although we have focused on the effects of iron status on RBC physiology and RBC population structure affecting parasite invasion, growth, and overall malaria susceptibility, another direction forward would be to delve into understanding iron acquisition and utilization pathways in *P.falciparum*, as has been a more traditional aspect of nutritional immunity in the case of other pathogens like siderophoric

bacteria.

There is no question that iron is an essential nutrient for *P. falciparum*. Iron is required for parasite DNA synthesis, glycolysis, pyrimidine synthesis, heme synthesis, and electron transport. The parasite rapidly proliferates inside RBCs, multiplying 8-32 times in its 48hr life cycle. The host RBC does contain iron, but a majority is sequestered in heme which is incorporated into hemoglobin. While the parasite's iron source is unclear, most evidence indicates it obtains iron from the host – not from hemoglobin, but from another intra-erythrocytic pool, possibly labile ferrous iron and/or ferritin (13–15). The parasite efficiently metabolizes hemoglobin protein in its acidic digestive vacuole and uses the resultant amino acids for its own protein needs. However, the parasite cannot extract the iron molecule from heme: it lacks heme oxygenase activity and to our knowledge, no plasmodial iron storage proteins, siderophores, or chelators have been identified. As iron is critical to the parasite, much work has been done investigating iron chelating agents as possible anti-malarials. In fact, many iron chelating agents suppress parasite growth *in vitro* and in animal models (16, 17). Given the parasite's iron requirement, it was understandably initially believed that reduced iron availability was responsible for malaria protection associated with IDA. Although our data indicates iron withholding is not the mechanism by which IDA confers resistance to malaria, identifying parasite iron acquisition and utilization pathways could be of significant importance in understanding parasite pathogenesis and finding novel antimalarial drug targets.

Despite the parasite living in an intracellular niche where iron is a central component (as part of hemoglobin), it is still debatable exactly from where and how the parasite acquires iron – either from serum sources (e.g. transferrin or non-transferrin bound iron) or intracellularly (e.g. hemoglobin, ferritin, or a labile iron pool), or perhaps a combination. There is evidence to suggest transferrin associates with infected parasites, that labeled transferrin-bound iron is incorporated into parasite infected RBCs, and it has even been published that parasite transferrin receptors exist (15, 18–21), however other evidence has not found transferrin-bound iron to specifically associate with or be incorporated into infected RBCs and called into question the findings about transferrin receptors (22–24) (all reviewed in (25)). It has been difficult to parse out whether levels of serum iron affect parasite growth because *in vitro* the parasite must always live within a cell in which iron sources are available, though evidence to date has not found differences in parasite growth following iron depletion or addition to culture media

(reviewed in (25)). In terms of intracellular iron, there is no significant evidence that the parasite utilizes iron in heme, at least not via any traditional heme-oxygenase activity-like pathway (13, 26), and though there is some evidence the parasite does liberate some type of intracellular iron (as the bioavailable iron pool increases in maturing infected RBCs (15)), it is unclear where from or how. However, it is important to keep in perspective that given the RBC is one of the most iron-rich environments in the entire human body, the parasite would only need to acquire a small amount of heme-bound or ferritin-incorporated iron intracellularly for its needs (reviewed in (25)).

Given that several unknowns still exist when it comes to iron and the malaria-infected RBC, there are numerous opportunities to try to learn more. More work could be done to determine whether a transferrin receptor exists, or where exactly the parasite gets its iron sources from, through radiolabeling experiments, pull-down assays, and membrane analyses. More could be done to examine whether malaria development in iron deficient RBCs affects pathogenic properties of the parasite, such as parasite protein export to the RBC surface and endothelial cytoadherence, or phagocytosis susceptibility. To learn more about iron utilization pathways, we could employ RNA sequencing (RNA-Seq; also called whole transcriptome shotgun sequencing) to compare parasite development in iron deficient versus iron replete RBCs throughout the developmental cycle. Now that merozoite isolation is more feasible (bearing in mind the merozoite is extremely short-lived as the extra-cellular parasite stage), it could be possible to do further experiments examining invasion in an iron-depleted or saturated extracellular environment. It is also possible now to more easily develop knockout strains of *P.falciparum*, which could be utilized to test whether an identified heme-oxygenase homolog (not known to be active) is essential in the erythrocytic stage of infection, or to follow up on the role of any interesting proteins identified through RNA-Seq exploration of parasite development in iron deficient and iron replete RBCs. Clearly there are many avenues which can be pursued, and elucidating exactly from where and how the parasite utilizes host iron could lead to interesting developments understanding parasite pathogenesis, development, and growth, and possibly help with novel antimalarial development – this basic role of iron in intraerythrocytic malaria growth should not be confused with the role we have put forth for iron affecting RBC physiology and population dynamics in a way that significantly affects overall infection potential in a host.

6.2.3 Clinical needs: further understanding impact of anemia on malaria pathogenesis and implications for global health strategies.

Given the epidemiological evidence and now our strong confirmatory *in vitro* research showing reduced malaria infection in the face of iron deficiency anemia, as well as the increased susceptibility following iron supplementation, there are many public health issues which should be addressed in the near future. For one thing, our research adds to the evidence that antimalarial prophylaxis is necessary when giving iron supplements. This is in line with the most recent Cochrane reviews and World Health Organization guidelines stating that universal iron supplementation in malaria endemic areas is important and favorable as long as antimalarial public health measures exist (27–29) (such as health surveillance, health clinics available nearby, or other factors such as insecticide-treated bed net distribution). There is no question iron deficiency anemia is in and of itself a significant health burden for pregnant women and children, and iron supplementation is a simple and logical way to counter this in areas where nutritional deficiencies are common. However, our work would suggest that not only should standard public health measures be available to people undergoing iron supplementation, but that it would be best to include temporary provision of antimalarial chemotherapy in conjunction with iron supplementation campaigns, much like Intermittent Preventative Treatment (IPT) measures for antimalarial drug delivery in pregnancy and other high risk populations living in malaria endemic areas. IPT/temporary prophylaxis would be particularly advantageous given our *in vitro* findings that in RBCs from both pregnant women and children undergoing iron supplementation, there is a defined period of increased malaria susceptibility which eventually wanes to normal or pre-supplementation levels (Chapters 4 and 5). Of course there are further unknowns, such as the exact length of increased susceptibility (as we only had access to RBCs from one or two time points during a 12 week supplementation period), but our research at least strongly advocates for the need for a clinical trial studying iron supplementation in conjunction with antimalarial drug provision. We hope that our findings will provide useful justification for doing this research.

Aside from relating to safety of iron supplementation in malaria endemic areas, there are other significant ways in which understanding the impact of anemia on malaria pathogenesis might become an important public health issue, such as malaria drug sensitivity and transmission potential.

To begin with drug susceptibility, it is a well-known reality that the *P.falciparum* parasite has successfully developed drug resistance to every kind of antimalarial drug class used in the human population to date. With drug resistance now arising to even the most important first line artemisinin-based antimalarials (30), careful understanding of artemisinin activity and proper drug usage is essential, to avoid inadvertently worsening any growing drug resistance spread or independent emergence outside of Asia. As iron deficiency is common in populations amongst which malaria treatment is sought, and the mechanism of action of artemisinin deals with reactive oxygen species (ROS) which are known to exist at altered levels in iron deficient RBCs, examining artemisinin efficacy in iron deficient versus iron replete RBCs is important. Although the parasite possesses mechanisms that have been identified to work to maintain red-ox balance in the RBC where its digestion of hemoglobin increases ROS potential due to heme liberation (31), ROS do still rise in infected RBCs and many antimalarials further exploit ROS activity against the parasite (32). Early studies of the artemisinin mechanism of action showed that the compound interacted with heme and iron, and was hypothesized to involve cleavage of an endoperoxide ring which gave artemisinin oxidizing properties through formation of free radicals(33, 34). More recent work shows artemisinin activity to be promiscuous, and confirms that artemisinin activation relies on heme interactions (much more so than iron) that subsequently form reactive oxygen species (largely via oxidation of the iron associated with free heme), which in turn causes lots of downstream cascading damage to many RBC proteins and functions important to the parasite as well (35). Parasite-infected RBCs are thought to be more sensitive to artemisinin because of increased levels of free heme (both parasite-synthesized in early ring stages and digested from host hemoglobin in later trophozoite stages) which interact with artemisinin to initiate downstream ROS damage (35, 36). It might be assumed that iron deficient infected RBCs would have increased susceptibility to artemisinin activity because of the added levels of ROS already present in iron deficient RBCs. However, this may not necessarily be the case – what if there is less room for the artemisinin to act “over and above” the ROS already present in iron deficient RBC, or what if some pathways for ROS creation in the iron deficient RBC have already been exhausted? While recent studies did not find artemisinin activation to rely on iron interactions as opposed to heme (35), it is also true past evidence indicates iron chelators antagonize artemisinin activity (33), again giving a mechanism by which artemisinin activity might differ in the iron deficient RBC. While

reduced artemisinin activity in iron deficient RBCs may not be a likely or intuitive phenomenon, it is worth exploring further in order to rule out the possibility. Evaluating this can be initiated through simple *in vitro* drug sensitivity assays infecting RBCs from iron deficient and iron replete donors, using the standardized ring stage sensitivity assay for examining artemisinin efficacy.

Finally, the potential effect of iron deficiency on malaria transmission dynamics is very real and deserves further study. Malaria transmission depends on mosquito feeding of infected individuals which ingest sexual stage-infected RBCs, which then go on to further develop and sexually reproduce in the mosquito, eventually allowing for transmission to a new host. Induction of the sexual stage gametocyte is relatively rare compared to normal asexual parasite forms, and the life cycle is quite different as it takes 15 days of development within the RBC for gametocytes to reach maturity (reviewed in (37)). While exact mechanisms driving genetic switch to gametocyte fate is an ongoing area of research, clearly the number of mature gametocytes formed and how early they start to form within an infected individual, can play a big role in overall transmission potential. This is especially true as not all commonly used antimalarial drugs are active against the gametocyte stage infection. It is known that stressors can induce gametocytogenesis, which is not surprising from an evolutionary standpoint for the threatened parasite to try to ensure ongoing survival in another host. Such stressors can include drug treatment, lack of nutrients, or high infection numbers, which cause a portion of asexual stage parasites to undergo genetic programming to induce gametocyte gene expression upon division into merozoites, and these merozoites go on to infect new RBCs destined to develop into gametocytes (reviewed in (37)). Other stressors can include RBC mutations such as sickle-trait and thalassemias, with gametocyte formation reported to be elevated in these people (38–40). Similarly, it is highly plausible that gametocytogenesis will be more readily induced in an iron deficient person, in which parasites cannot invade RBCs or replicate as well. In fact, it would be a “perfect storm” if iron deficient individuals had fewer malaria symptoms and were able to control infection better (thus less frequently seeking drug treatment), but simultaneously had higher levels of gametocytes in their blood to increase overall malaria transmission potential. To study this, we could easily begin with *in vitro* gametocyte growth assays in iron deficient versus iron replete RBCs, assessing whether gametocytes start to form earlier, or at higher rates. Gametocyte cultures require human serum; we could also test whether serum from iron deficient versus iron replete individuals

differentially influences gametocytogenesis. Gametocytes are quantifiable by microscopy or more scalable methods such as flow cytometry using parasites with gametocyte stage proteins fluorescently tagged, or RT-PCR examination of gametocyte specific transcripts. It could be more difficult to enumerate gametocytes in blood smears from infected and uninfected individuals as gametocytes are rare in circulation until they reach maturity, but it is another possible method to use. Studies using gametocyte growth *in vitro* in RBCs from anemic and non-anemic individuals followed by membrane feeding techniques to infect mosquitos can help determine true transmission viability of gametocytes formed under different conditions. Following mosquito infection, midgut and salivary gland dissection can reveal if malaria oocysts and sporozoites properly developed, and there is also the ultimate test of controlled refeeding on humans to monitor infection potential. These are certainly more complicated techniques, but do admittedly more stringently measure the entire transmission cycle potential of the issue at question. Regardless of technique chosen, evaluating gametocytogenesis in the face of iron deficiency is a highly relevant question when it comes to malaria elimination campaigns and certainly deserves further study.

6.2.4 New research into human evolution against blood stage malaria infection.

Our research examining the relationship between anemia, iron, and malaria is now becoming highly relevant to the broader picture of understanding human RBC adaptation to thousands of years of malaria pressure. As discussed in Jallow *et al.*, only 2% of variation in severe malaria disease is attributable to the most well-known genetic alteration of sickle-cell trait, as compared to an estimated 25% total impact of genetics on severe malaria formation. Thus there is presumably much room for discovery and improved understanding of other genetic factors contributing to malaria susceptibility (41). Anemia is widespread throughout the developing world, iron deficiency being the most prevalent cause (42). Certainly, much of this is due to nutritional availability of iron sources. However, it is also intriguing to think about tendency towards anemia having evolved as a protective survival advantage in the presence of longstanding malaria pressure.

In fact, it is well-established that African American populations have lower hematocrit, hemoglobin, mean corpuscular volume (MCV), white blood cell (WBC) counts, and transferrin saturation (albeit higher serum ferritin) than their non-black American counterparts. This idea largely originated from the first American National Health and Nutrition Examination Survey (NHANES). This was originally

hypothesized to relate back to nutrition and/or economic status, but it was determined that the finding still holds true after controlling for these factors (discussed in (43)). The hematological discrepancies remained even after accounting for incidence of hemoglobinopathies and other RBC variations known to disproportionately affect African Americans (44) and correcting for outlying low transferrin saturation (45). Another study of population-matched black and non-black Americans found the difference in hemoglobin levels was not due to influence of different levels of iron, zinc, or copper, and also that MCV and mean corpuscular hemoglobin (MCH) was consistently lower in black populations (46). Perry and coworkers further confirmed with NHANES II that differences in hemoglobin were not due to differences in iron intake or other obvious iron-related factors, instead questioning whether there were innate differences in erythropoiesis or iron metabolism factors (47). It should be mentioned though, that others found in a study of military personnel that accounting for iron intake did reduce differences in hemoglobin somewhat(48). However, significant differences in hemoglobin levels and hematological parameters in black and non-black Americans despite controlling for confounders are persistently found in more recent studies using data from NHANES III(49). Regardless of cause, it is now indisputable that separate reference values are necessary for black and white populations in terms of hemoglobin levels and MCV, with blacks having consistently lower Hgb (on the order of 0.5-1.0 g/dL) and MCV (on the order of 3-6fL) across age-matched, income-matched, sex-matched individuals and also accounting for prevalence of genetic RBC disorders (44–47, 50).

Why did these difference arise? What if anemia is in fact one of the most widespread and influential contributors to malaria attenuation in the developing world? This idea was brought to our attention due to results in this dissertation (Chapter 4) and is a line of research that deserves further analysis and inquiry. If this were the case, it would be predicted that genetic variations leading to anemia or lower hemoglobin levels should exist, which will again have to be examined further. Known mutations do exist to cause, for example, iron refractory iron deficiency anemia (IRIDA). In IRIDA, mutations in the *TMPRSS6* gene (coding for a serine protease) affect pathways of the iron regulatory peptide hormone hepcidin, ultimately amounting to persistently elevated hepcidin levels (51, 52). High levels of hepcidin (normally induced by inflammation or high iron stores) prevent uptake of intestinal iron through degradation of the ferroportin transporter on the basal surface of duodenal cells, preventing dietary

absorbed iron from reaching the blood stream; it also increases macrophage uptake of serum iron and reduces erythropoiesis (53). A meta-analysis of the association of risk alleles in *TMPRSS6* across different ethnicities does find increased levels of certain risk alleles in Asians (significantly elevated) and African Americans (elevated but not significant) compared to Caucasians, but there was very limited data available for the African Americans so the topic could be examined further (54). Other recent research adds weight to the idea that hepcidin levels are in careful evolutionary equilibrium, indicating that higher levels of hepcidin at birth indeed correlate with increased risk for anemia but simultaneously reduce risk of malaria infection (55). Of course much of the anemia in malaria endemic areas can be explained by nutritional limitations, but more research into genetic causes for anemia and lower hemoglobin values in people of African descent could lead to further understanding of whether the anemia prevalence is at least in part historically related to malaria pressure.

6.3 CONCLUSIONS

Not only have humans evolved to increase survival against infection and severe malaria, but so too have malaria parasites evolved to counter human protective measures. The most obvious present-day example of this is drug resistance. There is now also clear evidence of *P.vivax* infection of duffy-negative individuals, which could possibly reflect new invasion ligand adaptation. *P.knowlesi*, traditionally infecting apes, now crosses over to cause human zoonotic infections at increasing rates (56). Plausibly, the parasite has even evolved ways to take advantage of human genetic adaptations, with evidence that gametocyte formation and thus transmission potential is higher in people with hemoglobinopathies (38–40). Another intriguing development is the finding that parasites are potentially responding to selective pressure from extensive rapid diagnostic testing (and subsequent drug treatment) to have lost the *hrp2* gene whose protein product is detected by most rapid diagnostic tests (57, 58).

Clearly, the evolutionary relationship between the malaria parasite and the human host is far from at an end point, and we have much more to be learned. Studying natural human evolutionary adaptation to malaria infection can provide much insight into parasite pathogenesis and help identify strategies to develop new antimalarial targets and vaccines. Research into human erythrocyte-related genetic adaptations to malaria in populations originating from malaria endemic areas can also help us identify and better understand human conditions and biological differences. This is certainly the case with

hemoglobinopathies in African American populations, and as proposed, now may also shed light on widespread prevalence of anemia in African populations and people of African descent.

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