Hepcidin Regulation in Malaria

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Statement of Originality: I, Natasha Spottiswoode, certify that the work presented in this thesis is my own. When information or resources are derived from other sources, this is indicated specifically within the manuscript.

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ABSTRACT

Epidemiological observations have linked increased host iron with malaria susceptibility. At the same time, blood-stage malaria infection is associated with potentially life-threatening anemia. To improve our understanding of these relationships, this work presents an examination of the mechanisms controlling the upregulation of the hormone hepcidin, the master regulator of iron metabolism, in malaria infection. Chapter 2 presents data from a mouse model of malaria infection which indicate that hepcidin upregulation in malaria infection is associated with increased activity of the sons of mothers against decapentaplegic (Smad) signaling pathway. Although the canonical Smad pathway activators, bone morphogenetic proteins (Bmp) are not increased at the message level following infection, activin B, which has been recently shown to increase hepcidin through the Smad signaling pathway in conditions of inflammation and infection, is upregulated in the livers of malaria-infected mice. Chapter 3 shows that both activin B and the closely related protein activin A upregulate hepcidin *in vitro* and *in vivo*. Chapter 3 also explores the effects of the activin-binding protein follistatin in both systems and in the same malaria-infected mouse model as presented in Chapter 2. The work presented in Chapter 4 extends these studies to human infections by demonstrating that activin A protein co-increases with hepcidin in human serum during malaria infection. Taken together, these findings are consistent with a novel role for activin proteins in controlling hepcidin upregulation in the context of malaria infection. This work may

form a basis for the development of novel therapeutics that speed recovery from malarial anemia by inhibiting activins' actions.

Chapter 5 examines the role of infected red blood cell-derived microparticles in the initial recognition of a *P. falciparum* malaria infection, and subsequent hepcidin upregulation. Microparticles stimulate production of cytokines from peripheral blood mononuclear cells (PBMC), which also upregulate activin A message in response to both microparticles and whole infected red blood cells. These data are consistent with a model in which malaria-derived stimuli such as microparticles trigger the systemic release of activin proteins, which then act on the liver to upregulate hepcidin.

Evidence has shown that cytokine levels at birth are related to malaria risk. In Chapter 6, hepcidin is measured in cord blood samples from participants in a large-scale clinical study in a malaria-endemic area, and shown to be elevated in cord blood from neonates with a clinical history of placental malaria. Cord blood hepcidin is also compared to birth levels of iron markers and other cytokines, and future clinical outcomes. Finally, the contributions of DNA methylation levels to cord hepcidin and cytokine levels are assessed by comparison of CpG methylation, at sites in genes encoding hepcidin and cytokines, to the serum concentrations of the genes' protein products. Several intriguing associations are noted which indicate a possible novel role for DNA methylation in the determination of birth cytokine and hepcidin levels. Chapter 7 synthesizes the data presented in this thesis, interprets the possible significance of the major findings, and offers suggestions for future work.

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

LIST OF ABBREVIATIONS

AP	Animal protocol
BMP	Bone morphogenetic protein
bp	Base pair
C	Day of challenge
cDNA	Complementary DNA
CHMI	Controlled human malaria infection
chPBS	Citrated heparinized PBS
CpG	Cytosine-phosphate-guanine site
CREB1	cAMP-reactive element binding protein 1
CREBBP	CREB1 binding protein
CRP	C-reactive protein
D	Daltons
dbSNP	SNP database
DoD	Day of diagnosis
EDTA	Ethylenediaminetetraacetic acid
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
EPO	Erythropoietin
ERFE	Erythroferrone
ERK1/2	Extracellular signal-related kinase ½
ESA	Erythropoiesis-stimulating agent
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FDR	False discovery rate
Fst	Follistatin
GCP	Good clinical practice
GDF	Growth differentiation factor
gDNA	Genomic DNA
GPI	Glycosylphosphatidylinositol
GWAS	Genome-wide association study
h	Hour(s)
Hb	Hemoglobin
HIF	Hypoxia induced factor
HIV	Human immunodeficiency virus
HJV	Hemojuvelin
HR	Hazard ratio
i.p.	Intraperitoneal
i.v.	Intravenous

IBP	Iron-binding protein
ID	Iron deficient
IFN	Interferon
IL	Interleukin
IQR	Inter-quartile range
iRBC	*P. falciparum*-infected red blood cell
IRE	Iron-responsive element
IRIDA	Iron refractory iron deficient anemia
JAK	Janus kinase
LDN	LDN-193189
LOD	Limit of detection
LPS	Bacterial lipopolysaccharide
MEM	Minimal essential media
min	Minute(s)
MOMS	Mother-Offspring Malaria Studies
mRNA	Messenger RNA
MyD88	Myeloid differentiation primary response 88
NCBI	National Center for Biotechnology Information
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NTS	Non-typhoidal salmonella
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells, excepting neutrophils
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
ppm	Parts per million
pSmad1/5/8	Phosphorylated Smad1/5/8
pSmad2/3	Phosphorylated Smad2/3
pStat3	Phosphorylated Stat3
PVDF	Polyvinylidene difluoride
qPCR	Quantitative PCR
qRT-PCR	Quantitative real-time PCR
RBC	Red blood cell
RNAi	RNA interference
SDS	Sodium dodecyl sulfate
SM	Severe malaria
SMA	Severe malarial anemia
Smad	Sons of mothers against decapentaplegic
SNP	Single nucleotide polymorphisms
Stat3	Signal transduction and activator of transcription 3
sTfR	Soluble transferrin receptor
STING	Stimulator of interferon genes
TBK1	Tank-binding kinase 1
TIBC	Total iron-binding capacity
TGFβ	Transforming growth factor β
TLR	Toll-like receptor

TNFα	Tumor necrosis factor α
TNF-RI	Tumor necrosis factor receptor 1
TNF-RII	Tumor necrosis factor receptor 2
TSS	Transcription start site
TWSG1	Twisted gastrulation factor 1
UIBC	Unsaturated iron-binding capacity
UPR	Unfolded protein response
uRBC	Uninfected red blood cell
UTR	Untranslated region
WHO	World Health Organization
ZPP	Zinc protoporphyrin
Δ	Change

CHAPTER 1: INTRODUCTION

1.1. Foreword

Among the many nutrients required for human survival, iron plays a unique role in determining disease susceptibility. This relationship is especially well studied in the case of malaria. Malaria is one of the world's deadliest and most geographically widespread human diseases, killing an estimated 600,000-1,000,000 people per year (Malaria World Report, 2013, [1]). The controversial relationships between malaria and iron have been the subject of widespread discussion and debate [2, 3]. While iron supplementation appears to be linked with increased malarial mortality, and iron deficiency with decreased malarial susceptibility, malaria infections are a major global cause of anemia [4], and measures taken to decrease malaria at a population level also decrease anemia [5].

The interactions between malaria and iron have only lately begun to be understood at the molecular level. Primarily, the discovery of the iron regulatory hormone hepcidin has given us new understanding of human iron physiology and pathophysiology. Hepcidin serves to block iron absorption from the diet and also to route iron in the body into macrophages and away from the serum. Hepcidin plays a complex but vital role in both the iron restriction that occurs during malaria infection, and in determination of baseline iron status and subsequent effects on disease susceptibility. Studying the molecular mechanisms that control hepcidin in the context of malaria may guide the development of much-needed novel treatments

for malaria-related anemia, and also inform our assessment of differing malaria risk between individuals.

1.2. Iron supplementation and malaria infection

Iron intake and malaria risk have been linked by multiple studies. Perhaps the first formal trial of the relationship between direct iron supplementation and malaria incidence was reported by Oppenheimer et al in 1984: infants at 2 months of age who were given intramuscular injections of iron dextran were found to have greater incidence of parasitemia and splenomegaly at 6 and 12 months of age than non-iron supplemented controls [6]. Injections of iron dextran to pregnant women were similarly found to be associated with increased perinatal malaria incidence, although this effect was only noted in the more vulnerable primiparae [7]. Researchers also noted flare-ups of malaria subsequent to increased iron consumption: symptomatic malaria peaked when poorly nourished Nigerian individuals were fed a hospital diet with improved iron content [8], or when iron-deficient Somali nomads were treated directly with oral iron [9]; this latter study also noted a marked increase in incidence of symptomatic bacterial infections post-iron treatment. The same researchers also observed that a pronounced difference in diet (consumption of fish, which is iron-rich, in a community that primarily subsists on iron-poor milk) was associated with greater frequency of symptomatic malaria and other infections [10].

The first large-scale randomized trial of iron supplementation in an area with very high malaria transmission was performed on the African island of Pemba in 2006 (the "Pemba trial") [11]. This trial was stopped prematurely when trial monitoring boards found a link between the supplementation of children with iron (folic acid was also included) and subsequent malaria infection and mortality [11]. This finding supported the previous smaller-scale studies that had described iron consumption-malaria links, and presented the international community with a knotty problem: given the evidence, could iron supplementation be administered safely in malaria-endemic areas?

Several studies that have followed the Pemba trial have produced results that appeared to contradict this finding, showing no association between supplementation and malaria risk [12, 13]. However, many of the studies that followed the Pemba trial were smaller in scale, located in areas without high transmission rates, and/or introduced measures to combat malaria, such as bednets or intermittent preventative chemoprophylaxis, which may have masked any increase in malaria susceptibility [14, 15]. One study purported to demonstrate a protective effect of iron supplementation on malaria parasitemia incidence [16], although this conclusion must be tempered by the observations that in this trial, hospital admissions *increased* significantly in the iron-supplemented group, and also that iron administration did not effectively increase hemoglobin levels, making any changes hard to interpret [17] .

Two Cochrane reviews have since been published in attempts to reconcile the apparently disparate findings of the Pemba study and subsequent trials [2, 3].

Both reviews concluded that iron supplementation did not increase the risk of malaria infection in children when "regular malaria surveillance and treatment services" were provided. However, many areas of the world have inadequate malaria surveillance and treatment, coupled with high levels of iron deficiency and anemia.

Moreover, the effects of iron supplementation on host-pathogen interactions are likely not limited to malaria susceptibility. Administration of micronutrient powders, which included iron, was associated with increases in diarrhea in several studies [18, 19], although not others [20, 21]. In a study specifically examining the effects of iron-fortified biscuits on gut microbiota, potentially harmful species were shown to increase with iron treatment [22], offering a possible mechanism for the increased diarrheal incidence noted in several trials. A meta-analysis of intravenous iron therapy for the reduction of allogenic red blood cell transfusion noted an increase in all infections associated with intravenous iron use [23]. In short, iron administration may have effects on pathogen susceptibility beyond malaria. Improved clinical research aimed at finding a safe and effective way to permit iron supplementation must be complemented by basic research aimed at improving our understanding of the relationships between iron, malaria, and other pathogens.

1.3. Iron deficiency is associated with protection from malaria infection

While iron supplementation appears to increase malaria risk, non-iatrogenic variations in the iron levels of susceptible hosts may modulate the frequency and clinical severity of malaria infections. Gwamaka et al (2012) collected detailed data

from a large cohort of Tanzanian children (birth – 3 years). In this vulnerable population, iron deficiency at healthy aparasitemic visits was strongly associated with decreased future risk of parasitemia and severe malaria [24]. Iron deficiency in this study was defined by low ferritin (<30 ng/mL) in individuals with low C-reactive protein (CRP). A higher ferritin cutoff (>70 ng/mL) was used to define individuals with higher CRP as iron deficient. Plasma ferritin, which binds and stores potentially harmful free iron, is regulated in healthy individuals primarily by the post-transcriptional iron-binding protein (IRP)/iron-responsive element (IRE) system (reviewed in [25]), and therefore reflects iron stores. However, ferritin is also increased at the transcriptional and post-transcriptional levels by various cytokines associated with inflammation and/or infection (reviewed in [26]). Hence, the ferritin levels considered to be representative of iron deficiency are higher when inflammation is present.

A further study in a slightly older cohort of Kenyan children (8 months – 8 years) found that iron repletion (defined as ferritin ≥12 ng/mL, with transferrin saturation ≥10%, children with high CRP excluded), was predictive of clinical malaria episodes in the year following measurement [27]. Separately, Jonker and colleagues noted a similar effect in Malawian children (6 months - 5 years): iron deficient children (defined as plasma ferritin <30 ng/mL), had a lower incidence of clinical malaria the subsequent year [28].

Studies performed in pregnant women also may indicate an association between iron deficiency and protection from clinical malaria, although somewhat fewer data are available. Two cross-sectional studies have found that at the time of

delivery, placental malaria was associated with iron replete status [29, 30]. A limitation of this approach is that all currently used measures of iron status, such as ferritin, can be distorted by inflammation and infection, and thus cross-sectional studies may be of limited utility in understanding this relationship. Only one study has so far attempted to examine the predictive values of iron status on future placental or peripheral parasitemia in pregnant women [31]. This study measured iron status by examining zinc protoporphyrin (ZPP) levels. ZPP is formed when protoporphyrin 1X is unable to bind an iron atom to form heme due to a lack of iron availability; instead, it binds a zinc atom, creating ZPP. Hence, ZPP levels are one indicator of iron deficiency [32]. ZPP is also upregulated in infectious context, although notably less so than ferritin [33]. At both the first antenatal visit and at delivery, levels of ZPP indicative of iron repletion were associated with parasitemia; however, analyzing these data for ZPP as a predictive measure was complicated by the elevation of ZPP by concurrent parasitemia. The authors concluded that in this population, with a high incidence of parasitemia and associated inflammation, ZPP alone is not a valid measure of iron status, a concern that also could be applied to pediatric populations. Although the available data suggests a relationship, further studies that recruit women early in pregnancy, stratify carefully by gravidity and malaria transmission intensity, and follow their outcomes closely are required to definitively answer the question of whether iron status in women can predict malaria risk as in pediatric populations.

1.4. How the malaria parasite benefits from iron

A mechanistic insight into the contributions of host iron status and iron supplementation to malaria susceptibility has been gained from *in vitro* studies of malaria infection in red blood cells taken from uninfected malaria-naive iron deficient or iron replete individuals [34]. *P. falciparum* parasites were found to less successfully invade and mature within red blood cells from iron deficient donors, an effect that was reversed when the iron deficient donors were supplemented with oral iron. In addition, younger red blood cells were more permissive to parasite growth; iron supplementation of an iron-deficient host increases the number of reticulocytes and young red blood cells. The effects of iron supplementation on the age of circulating red blood cells have been previously proposed as particularly important in determining susceptibility to *P. vivax*, which lives preferentially in young reticulocytes: an inverse association has been noted between severe anemia (in which reticulocyte levels are lowered) and *P. vivax* infection [35].

In an attempt to find a pharmaceutical agent to treat malaria infection without the potentially harmful effects of long-term iron deficiency, experiments have been conducted with iron-chelating agents such as desferrioxamine in malaria models. Iron chelators have been shown to restrict malaria growth in vitro [36], in murine models of malaria infection [37, 38], and in malaria-infected primates [39] (reviewed in [40]). In humans, preliminary work on the use of the iron chelators desferrioxamine, or the orally administered deferiprone, as an adjunct to standard antimalarial therapy seemed promising [41]. However, after further studies showed no clear benefit from administration of chelators, and one seemed to hint at a slight

increase in mortality in the trial group treated with desferrioxamine [42], a Cochrane review recommended that trials testing iron chelators for malaria treatment be discontinued [43].

The development of antimalarial drugs that target the parasite's access to iron might meet with more success if we had a better understanding of how *Plasmodium* parasites acquire iron during their various life stages. *Plasmodium* parasites exhibit a complex life cycle. Briefly, humans are infected through the bite of a mosquito that carries parasites in its salivary glands. At that stage, the parasites are termed sporozoites. Sporozoites that gain access to a human host rapidly travel to the liver, where they grow and asexually reproduce, emerging ~1 week later into the blood stream as merozoites. Merozoites invade human red blood cells, inside which they mature into trophozoites and then schizonts. At the schizont stage, the red cell bursts open and releases many more infectious merozoites, and the asexual blood cycle continues. Blood-stage parasites may also differentiate into sexual male or female gametocyte forms, which can be taken up by another mosquito. In the mosquito, gametocytes mature into gametes, and eventually combine to form zygotes, which develop into ookinetes, and finally into sporozoites that migrate to the mosquito's salivary glands, ready to begin the process anew.

Blood-stage parasites have been theorized to acquire iron from serum transferrin [44], from iron produced during the breakdown of hemoglobin (Hb), or from a free pool of intracellular iron [45], but this important issue remains relatively unresolved. Very little work has been done that investigates the acquisition of iron by the obligate liver or mosquito life stages of malaria infection; these continue to be

fruitful areas for future research. For example, identification of *Plasmodium* encoded iron transporters would both increase our understanding of the mechanism by which different life-stages obtain their iron, and provide new drug targets aimed at inhibiting parasite growth.

Iron repletion may also have effects on parasite growth through different mechanisms than direct utilization by the parasite or red blood cell composition. A full synopsis is beyond the scope of this work, but iron has many effects on the immune system [46]. In malaria specifically, it has been suggested that parasitized red blood cells from iron deficient hosts may be more efficiently phagocytized by white blood cells, based on evidence from a murine model [47].

1.5. Anemia as a hallmark of malaria

Malaria infections are typified by sometimes life-threatening anemia. The mechanisms involved in the pathogenesis of malarial anemia are not fully understood, but include the increased clearance of infected and uninfected red blood cells, dyserythropoiesis as a consequence of cytokine upregulation, and inadequate absorption of oral iron (reviewed in [48]). On a global level, malaria infections are a major contributor to the worldwide burden of anemia [4] and measures taken to decrease malaria at the population level frequently decrease anemia prevalence [5]. Advances in basic iron biology have recently improved our comprehension of the iron perturbations that occur in malaria.

1.6. Human iron control: the hormone hepcidin.

Systemic mammalian iron metabolism is controlled at the level of iron absorbance from the diet and iron recycling through macrophages. Approximately 1 mg of iron is absorbed from the diet every day, roughly equivalent to the iron that is lost daily in poorly regulated activities such as sweating, any bleeding, and the sloughing off of enterocytes. At the same time, the approximately 30 mg of iron already in the body is constantly being recycled as macrophages phagocytize senescent or damaged red blood cells, digest the heme, and export the iron back into the circulation.

Both the export of iron across the basolateral membrane of enterocytes and the recycling of iron through macrophages are dependent on the same protein: ferroportin, the sole currently identified mammalian iron export protein [49-51]. Restriction of ferroportin activity therefore leads both to a failure to absorb iron from the diet, and a relocalization of iron from serum into macrophages.

Hepcidin, discovered a decade ago by three groups working independently [52-54], is a 25-amino acid protein that binds to ferroportin and causes it to be internalized and degraded [55]. The net effect of hepcidin is therefore to decrease serum iron levels, both routing iron away from pathogens that exploit circulating iron, and rendering the host anemic by restricting iron availability to the erythron (Schematic of hepcidin's actions shown in Figure 1.1).

Figure 1.1. Diagram of hepcidin's actions on iron flow. Schematic showing hepcidin's actions on iron metabolism. Iron is absorbed through the duodenum and circulates in the serum on transferrin. Most iron is utilized for erythropoiesis in the bone marrow. When red blood cells senesce, they are phagocytized by macrophages in the liver and spleen and the iron from the breakdown of heme is recycled back into circulation. Both absorption and recycling of iron require the iron export protein ferroportin; hepcidin causes the internalization and degradation of ferroportin. Figure reproduced from Spottiswoode et al (2012) [15].

Evidence from both animal models and from human genetic lesions suggests that the hepcidin-ferroportin interaction is functionally non-redundant. Mice underexpressing hepcidin are severely iron-overloaded [56], mimicking the human genetic disorder hereditary hemochromatosis, which (in rare cases) is caused by genetic lesions in the hepcidin gene itself [57], in genes that encode hepcidin-regulatory factors [58], or by mutations in ferroportin that render it resistant to hepcidin control [59]. Conversely, mice that overexpress hepcidin are fatally anemic [60], while patients with mutations that lead to chronic hepcidin overexpression suffer from iron-refractory iron deficiency anemia (IRIDA) [61].

1.7. Hepcidin is upregulated in uncomplicated malaria

Hepcidin is upregulated in hosts infected by bacterial, fungal, and viral pathogens [62]. Multiple studies have found that hepcidin is upregulated in malaria infection in symptomatic and asymptomatic natural human infections [63-65], in experimentally controlled human malaria infections (CHMI) [66], and in murine models of malaria infection [67, 68]. Resolution of infection leads to normalization of hepcidin levels [65, 66]. Hepcidin's upregulation in malaria has several important consequences. Firstly, the upregulation of hepcidin routes iron in the body away

from the serum and into macrophages, likely contributing to the dyserythropoiesis and anemia that typify malaria infections.

Additionally, hepcidin upregulation directly blocks dietary iron absorption: patients with asymptomatic malaria infection [69] or post-malarial anemia [70, 71] poorly incorporate orally administered iron into their red blood cells. This finding is paralleled by the effects of hepcidin on recovery from anemia following other infections: in a rodent model of anemia of inflammation, hepcidin levels were found to be significantly negatively associated with the response to erythropoiesis-stimulating agents (ESA), and inhibition of hepcidin improved the erythropoietic response to ESA [72]. Increased hepcidin levels are therefore likely to notably slow recovery from post-malarial anemia. These changes are likely relevant at the population level: in areas with seasonal malaria transmission, hepcidin upregulation has been proposed as a contributor to the increased frequency and severity of iron deficiency that occurs at the end of the malaria season [73].

Furthermore, hepcidin has been recently shown to play a crucial role in determining the multiplicity of infections within a single host. The obligate liver stage of the malaria parasite requires iron: hepcidin peptide injection or hepcidin overexpression by transgene or viral vector can reduce parasite survival at the crucial hepatic bottleneck [67]. The hepcidin upregulation initiated by one blood-stage infection thereby blocks the establishment of a second infection [67].

The physiological redistribution of iron as a consequence of hepcidin upregulation may also have a significant effect on host susceptibility to other pathogens. In a blood-stage malaria infection, raised hepcidin is expected to

contribute to increased macrophage iron levels, as does increased erythrophagocytosis. This increase in bioavailable macrophage iron may benefit pathogens that exploit the macrophage niche [74]. In particular, hepcidin upregulation may help to explain the association between malaria infections and susceptibility to non-typhoid salmonella (NTS). The epidemiological link between malaria and NTS is well-established [75]. Iron has been implicated in the contribution of malaria to NTS susceptibility through increases in both free heme and heme-oxygenase expression [76]. By routing iron to accumulate in macrophages, the hepcidin response to malaria may also render the host more vulnerable to NTS directly [74]. Careful studies of the role of hepcidin in coinfection models are required to investigate this clinically important hypothesis.

1.8. Hepcidin upregulation mechanisms

Hepcidin levels increase homeostatically in high iron conditions [52] and in response to inflammation and infection [77]. Two well-characterized pathways exist that upregulate hepcidin. Firstly, increased liver iron is thought to lead to upregulation of bone morphogenetic protein (BMP)6 [78] which upregulates hepcidin by binding to BMP Type 1 and Type 2 receptors in concert with hemojuvelin (HJV, also known as HFE2) [79]. This binding triggers the phosphorylation of sons of mothers against decapentaplegic (SMAD)1, SMAD5, and/or SMAD8, which then join with the common mediator SMAD4 and bind together to a well-studied site on the hepcidin promoter to increase hepcidin transcription [80]. This pathway will be subsequently referred to as the SMAD signaling pathway,

except in Chapter 3, where the effects of SMAD2/3 signaling are also discussed: in that chapter SMAD1/5/8 or SMAD2/3 signaling will be specified.

BMP proteins are members of the transforming growth factor beta (TGFβ) superfamily, which also includes the growth differentiation factor (GDF) and activin proteins. $Bmp6^{-/-}$ mice exhibit massive iron overload [81, 82], and as stated above, Bnp6 is induced by iron. Other BMPs have also been shown to upregulate hepcidin *in vitro,* including BMP2, BMP4, BMP5, BMP7 and BMP9 (shown in Figure 1.2, [79, 83, 84]), and Bmp2 injection into mice has been shown to cause hepatic hepcidin increase in one study [84], but the physiological contributions of BMP proteins to hepcidin regulation, beyond BMP6, are not completely clear.

The protein matriptase-2 (encoded by the gene *TMPRSS6*) is a negative regulator of hepcidin: it cleaves HJV and therefore interferes with hepcidin regulation by BMPs [85, 86]. Mutations in *TMPRSS6* can lead to inappropriately high hepcidin levels, resulting in IRIDA [87, 88].

Hepcidin is also upregulated in conditions of inflammation and infection. The cytokine interleukin (IL)-6 upregulates hepcidin [89, 90] by binding to its receptor and triggering the janus kinase (JAK)- signal transduction and activator of transcription 3 (STAT3) pathway [91, 92]. Related protein IL-22 may have a similar effect to IL-6 [62]. This pathway shall be hereafter referred to as the STAT3 signaling pathway.

In general, hepcidin upregulation by the SMAD signaling pathway has been seen as homeostatic, the STAT3 signaling pathway a response to pathogens or inflammation. However, recent cross-talk between these two pathways has been

demonstrated by the identification of another TGFβ superfamily member, activin B, which is upregulated by inflammation in mice, but itself upregulates hepcidin via SMAD signaling independently of IL-6 [93]. The closely related protein activin A has also been noted to have some effects on hepcidin upregulation in one hepatoma cell line [84]. A schematic of the TGFβ family members known to affect hepcidin is depicted in Figure 1.2. Finally, evidence suggests that an intact SMAD signaling pathway is required for effective STAT3 signaling: the type 1 BMP receptor Alk3 is required for IL-6-modulated hepcidin upregulation *in vivo* [94], and a type 1 BMP receptor inhibitor can block IL-6 mediated hepcidin upregulation *in vitro* [95].

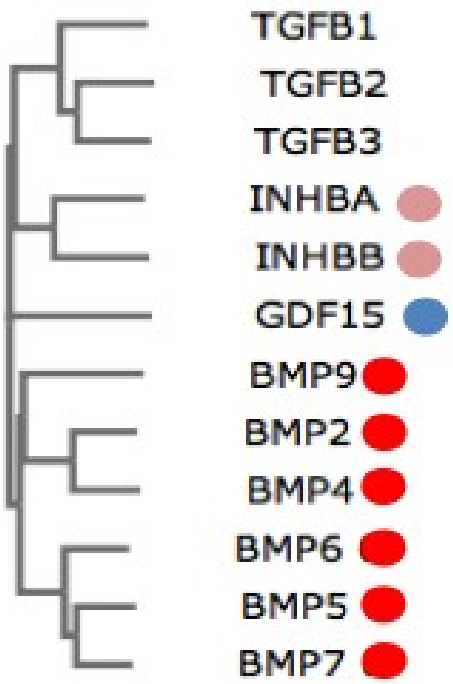

Figure 1.2. Known roles of TGFβ-family members in hepatic hepcidin control. Simplified phylogram of the TGFβ protein family tree. Proteins indicated with red circle have been shown to upregulate hepcidin in hepatoma cell lines or *in vivo*, including many members of the BMP family. Activin B protein and its homolog activin A only have shown to upregulate hepcidin in a single hepatoma cell line each,

both are shown with pink circles. GDF15 (blue circle) may suppress hepcidin, although this is controversial. Not all proteins in superfamily are shown. Phylogenetic tree generated with Clustal Omega[96], protein sequences are from NCBI.

Other pathways towards hepcidin upregulation exist, but are less well understood. A parallel pathway for homeostatic regulation of hepcidin through transferrin receptor 1 and, possibly, transferrin receptor 2 appears to be dependent on phosphorylation of the intracellular protein extracellular signal- related kinase 1/2 (ERK1/2). HFE, mutations in which are commonly associated with the hereditary iron overload disease hemochromatosis, is also likely involved in this pathway [97-99].

A final pathway of hepcidin induction is the unfolded protein response (UPR), triggered by endoplasmic reticulum stress by incorrectly folded proteins [100]. The UPR likely affects hepcidin synthesis through the CCAAT/enhancer binding protein a (C/EBP), which has been shown to bind to the hepcidin promoter [100], or through the C-reactive protein CREBBP [101]. Some overlap between pathways is likely: a recent unbiased RNAi screen of hepcidin-affecting factors found that CREBBP may cooperate with the SMAD signaling pathway [102]. The work presented in this thesis focuses primarily on the well-established SMAD and STAT3 hepcidin upregulation pathways, but we remain aware that less-studied pathways may play a role.

1.9. Hepcidin upregulation likely requires systemic upregulation of one or more signaling molecules

Hepcidin is primarily produced in the liver, but pilot experiments by this lab have shown that hepatoma cells co-cultured with malaria-infected red blood cells do not upregulate hepcidin (A. Armitage, unpublished). However, serum from malaria-infected mice causes hepcidin upregulation in primary murine hepatocytes [67], and medium from co-cultured macrophages and malaria-infected red blood cells may trigger some hepcidin upregulation in hepatoma cells (N. Spottiswoode, unpublished). These data together suggest that hepcidin upregulation in blood-stage malaria infection is likely contingent upon the release of one or more cytokines into the serum by a population of non-parenchymal cells (model shown in Figure 1.3).

How circulating cells detect the presence of a malaria infection and initiate cytokine release is currently controversial (for more detailed analysis see Chapter 5). The work presented in this thesis firstly attempts to delineate the intrahepatic pathways involved in hepcidin upregulation in malaria, and then to identify the infected red blood cell moiety that is detected by non- parenchymal cells, thus initiating the response to infection that results in hepcidin upregulation.

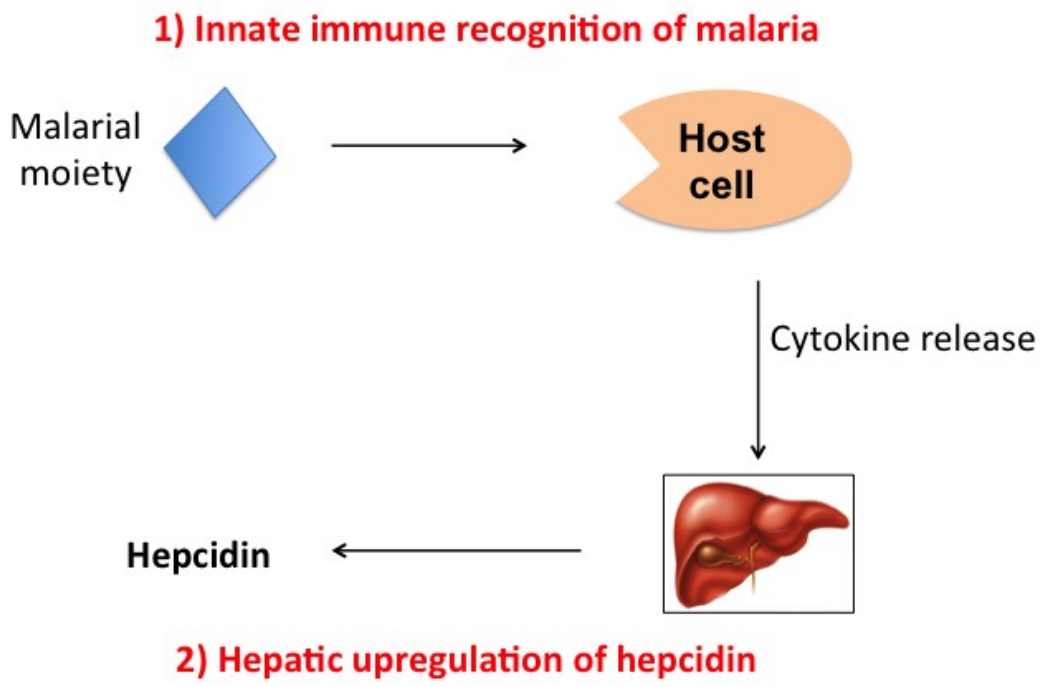

Figure 1.3. Two-step hepcidin upregulation model. The presence of infected red blood cells must be detected (step 1) by non-parenchymal host cells, which then trigger the release of one or more systemic cytokines. Cytokines are detected by the liver and upregulate hepcidin through one of the known intracellular hepcidin upregulation pathways (step 2).

1.10. Hepcidin suppression in severe malaria

While hepcidin upregulation has been noted in most studies of uncomplicated malaria, in certain severe malaria syndromes, hepcidin suppression may occur. Two studies have demonstrated that children with malaria and severe anemia (Hb <50 g/L) have lower hepcidin levels than children with uncomplicated malaria [103, 104], one also noted lowered hepcidin levels in children with cerebral malaria [103]. A study examining children presenting with severe anemia (over 50% of whom also had concurrent malaria parasitemia) found that a majority of the

measured hepcidin values were below the study's detection limits [105]. Taking the available evidence together, these studies clearly indicate that in severe malarial anemia, a signaling pathway that suppresses hepcidin can override the activation pathway associated with parasitemia.

Hypoxia-inducible transcription factors (HIF) were initially proposed as hepcidin suppressors [106], and more recent work has also proposed a role for platelet derived growth factor (PDGF)-BB as a hypoxia-stimulated hepcidin suppressor [107]. However, it has also been demonstrated that hypoxic hepcidin suppression is dependent upon downstream erythropoiesis [108]. Erythropoietin (EPO) administration suppresses hepcidin levels in both mouse models [109] and humans [110]; this effect can be inhibited by erythropoiesis-blocking agents [111]. These data together indicate that hepcidin is likely not suppressed directly by hypoxia or EPO itself, but by an erythropoiesis-dependent factor.

Initial candidates for this erythropoiesis-dependent factor included TGFβ superfamily member GDF15 (shown in Figure 1.2), and twisted gastrulation protein 1 (TWSG1) [112, 113]. GDF15 is increased in maturing erythroblasts, is higher in the serum from individuals with β-thalassemia, and suppresses hepcidin *in vitro* [113]. However, a later study examining $Gdf15^{-/-}$ mice showed similar hepcidin suppression as in normal mice following phlebotomy, indicating that GDF15 is likely not required for hepcidin suppression in erythropoiesis following acute blood loss [114]. Other work comparing GDF15 and hepcidin in patients with different etiologies of anemia [115], and examining both proteins in patients with increased erythropoiesis following stem cell transplantation [116], have similarly cast doubt

on the significance of GDF15 in hepcidin suppression. The role of TWSG1 is less well-studied, but in a mouse model of increased erythropoietic activity and hepcidin suppression, the expression of Twsg1 was not altered [108], again leading to uncertainty as to the physiological relevance of TWSG1.

Recently, however, a protein has been identified as a strong candidate for the factor that suppresses hepcidin in the context of increased erythropoiesis. The newly termed erythroferrone (ERFE) is produced by erythroblasts in the bone marrow and other organs in response to the need for increased erythropoietic activity, and suppresses hepcidin effectively [117]. ERFE may be involved in hepcidin suppression in severe malarial anemia. However, a hallmark of severe malarial anemia is inappropriately low erythropoietic activity for the degree of anemia, which may be caused by the effects of parasite products and cytokines such as TNFα [118, 119]. Even with this effect, however, the increased erythropoietic drive in severe malarial anemia plausibly may upregulate ERFE to some degree and thus suppress hepcidin. The work presented within this thesis focuses on identification of the mechanisms underlying hepcidin upregulation in uncomplicated malaria infection, but future work will parse the role of ERFE in hepcidin control in severe malarial anemia.

1.11. Assessing hepcidin at birth as a marker for malaria risk

In addition to the regulation of hepcidin in a malaria infection, hepcidin regulation plays a unique role in healthy individuals by controlling host iron status, which in turn affects malaria susceptibility (see sections 1.2-1.3). Recent work in a

cohort of young children (birth to 3 years of age) in Muheza, Tanzania has shown that levels of different cytokines in cord blood samples are predictive of later peripheral cytokine levels in healthy children, and of future risks of malaria parasitemia and severe malaria syndromes in early childhood [120]. These cord levels themselves are likely dependent on some combination of child genetic and epigenetic factors as well as the unique intrauterine environment.

Relatively few studies have examined hepcidin in cord blood samples. Two studies have found that cord blood hepcidin was high at birth and correlated with cord indices of iron status but not with maternal hepcidin [121, 122]. Hepcidin in cord blood is not significantly different in full-term intrauterine growth restricted infants [123] but appears to be positively associated with gestational age [124]. The sole study on cord hepcidin in a malaria-endemic area found that neither placental malaria nor maternal anemia was associated with changes in cord hepcidin [125], but this study was small in scope and limited to primigravidae. An analysis of the variation in cord hepcidin in populations in a malaria-endemic area, and associations with past placental malaria or with future susceptibility to iron deficiency or to malaria, is presented in this thesis.

Finally, the underlying mechanisms behind differing cord cytokine levels are not well understood. While genome-wide association studies (GWAS) have identified many single nucleotide polymorphisms (SNPs) associated with malaria risk (reviewed in [126] among others), relatively little research has been devoted to examining contributions of the epigenome to clinical outcomes in the context of infectious disease risk. Mechanisms of epigenetic variation include histone

methylation, histone acetylation, or DNA methylation at CpG (cytosine-phosphate-guanine) genome sites. These epigenetic alterations can be inherited or acquired before birth or during life (reviewed in [127]). At the time of writing, DNA methylation is the best-studied type of epigenetic variation. A limited number of studies show that variation in CpG methylation at cytokine-encoding loci can be associated with clinical outcomes [128], or cytokine levels in peripheral blood [129, 130]. New advances in epigenetic tools [131] are increasing our capacity to measure epigenetic changes, and thereby attempt to explain this component of disease susceptibility. The work presented in this thesis includes a first comparison of DNA methylation variation with cytokine and hepcidin levels in cord blood.

1.12. Summary

Host iron status plays a significant role in determining malaria susceptibility. At the same time, the pathogenesis of malaria includes notable iron-related pathology, primarily the development of potentially life-threatening anemia. As the master controller of iron metabolism, the hormone hepcidin plays a central role in these interactions. Hepcidin is known to be upregulated in uncomplicated malaria, however, at the commencement of this D.Phil, the mechanisms underlying this change had not been well studied either in infected humans or in animal models of malaria infection. Additionally, very incomplete data was available to identify the malaria-related moiety responsible for triggering the cytokines responsible for eventual hepcidin upregulation. Finally, the roles of 'steady state' hepcidin values in affecting or determining malaria susceptibility were generally unexplored.

The work in this thesis aims firstly to identify the proximate molecular mechanisms that control the upregulation of hepcidin in uncomplicated malaria. This project is addressed in Chapters 2, 3, and 4. In Chapter 2, hepcidin upregulation is examined in a murine model of malaria infection. Data is identified that suggest activin proteins may upregulate hepcidin through the Smad signaling pathway during infection. Chapter 3 examines the roles of activin proteins *in vitro* and *in vivo* and presents the results of experiments intended to block the effects of activins in a murine model of malaria infection. Chapter 4 details the changes in hepcidin, activin A protein, and other iron-related parameters that occur in human volunteers undergoing CHMI. Additionally, the upregulation of hepcidin is likely contingent on cytokine upregulation following the recognition of parasitized red blood cells. A secondary aim of this thesis was to identify the malaria-white blood cell interaction responsible for initiating cytokine upregulation, and eventually hepcidin. Chapter 5 explores the roles of membrane-bound vesicles from infected red blood cells in the recognition of malaria parasites and subsequent upregulation of hepcidin. Finally, the third aim of this thesis was to describe cord hepcidin and relate it to later iron metabolism phenotype and malaria susceptibility. Chapter 6 describes the hepcidin protein levels in cord blood from neonates enrolled in a longitudinal cohort study in a malaria-endemic area and the association of these levels with placental malaria history, other cytokines, iron markers, and future clinical outcomes. Chapter 6 also identifies key epigenetic variations that are associated with birth levels of hepcidin and cytokines. Chapter 7 summarizes findings and presents suggestions for future work.

CHAPTER 2: HEPCIDIN UPREGULATION IN A MURINE MODEL OF MALARIA INFECTION IS LIKELY CONTROLLED VIA THE SMAD SIGNALING PATHWAY

2.1. Introduction

Hepcidin is upregulated in symptomatic and asymptomatic natural human infections [63-65], in human volunteers experimentally infected in vaccine trials [66], and in murine models of malaria infection [67, 68]. The upregulation of hepcidin in malaria has multiple important consequences: including iron delocalization in infection, the prevention of iron absorption from dietary sources, and the modulation of coinfection susceptibility. Despite sustained interest in hepcidin's role in malaria infection, the molecular mechanisms of hepcidin upregulation in malaria infection have not yet been fully characterized.

Currently, there exist two well-studied pathways that act to upregulate hepcidin at the transcription level: the primarily homeostatic Smad pathway, which is induced by Bmp proteins, and the inflammatory Stat3 pathway that is thought to be primarily activated by IL-6 (both fully described in the Introduction). In mouse models of acute fungal and viral infections, hepcidin upregulation appears to be controlled via the Stat3 signaling pathway [62]. However, a possible role for Smad signaling in hepcidin upregulation in an inflammatory context was demonstrated by a recent study that found that activin B (a TGFβ superfamily member and therefore structurally related to Bmp proteins), was upregulated by bacterial

lipopolysaccharide (LPS) administration in mice and upregulated hepcidin via Smad phosphorylation *in vitro* [93].

Previous human and murine studies have so far not definitively shown which pathway(s) are responsible for hepcidin upregulation in malaria infection. Many studies that examine hepcidin in malaria have focused on measuring IL-6, the best-known agonist of the Stat3 pathway: IL-6 increases with hepcidin in malaria-infected humans [28, 103, 104], and in one murine experiment [68]. However, in another human study, urinary IL-6 and hepcidin showed no significant association in a multiple stepwise linear regression [65]. In mice, the prevention of a second liver-stage infection during malaria blood-stage infection, which is thought to be modulated by hepcidin, was maintained in mice treated with anti-IL-6 monoclonal antibodies [67]. A study *in vitro* found that peripheral blood mononuclear cells (PBMC), when co-cultured with *P. falciparum*-infected red blood cells, upregulated hepcidin expression without appreciable IL-6 increases [132], but the contribution of PBMC to total serum hepcidin is unknown. To summarize: the role of IL-6 and the Stat3 signaling pathway remains controversial.

This study compares hepcidin expression in a mouse model of malaria to indicators for the Stat3 and Smad signaling pathways. Expression of the Smad-responsive gene inhibitor of DNA binding 1 (*Id1*) is closely correlated with hepcidin expression in a mouse model of malaria infection, while Stat3 indicators are not. Furthermore, although canonical Smad signaling agonists, Bmp proteins, are not increased at the message level, activin B message is upregulated, indicating a novel possible involvement of activin B in hepcidin control in malaria infection.

2.2. Materials and Methods

Mouse husbandry and sporozoite infections

Male Balb/c mice (6-8 weeks of age, Harlan UK) were maintained with *ad libitum* access to standard mouse chow, Harlan-Teklad 2018SX (Fe^{2+} content ~200 ppm) and were housed in specific pathogen-free conditions. *Plasmodium berghei* ANKA strain sporozoites were obtained from the salivary glands of *Anopheles stephensi* mosquitoes 21 days after feeding on blood containing infectious *P. berghei* gametocytes. Mice were infected intravenously (i.v.) via the tail vein with 10^3 sporozoites in 200 µL RPMI, as previously described [133]. Uninfected mice were given control injections of 200 µL RPMI i.v. Mice were culled and samples harvested at 2, 4, 6, or 8 days post-infection. All murine malaria infection experiments were performed in accordance with the terms of the UK Animals (Scientific Procedures) Act Project Licence (PPL 30/2414) and were approved by the University of Oxford Animal Care and Ethical Review Committee.

Mouse sample harvest and storage

Mice were given a terminal anesthetic injection and blood was extracted via cardiac puncture. Serum was isolated using BD microtainers (Bunzl Healthcare, London, UK) as previously described [62]. Death was confirmed by cervical dislocation, and spleens, livers, and right hind legs were harvested.

Murine livers and spleens were removed and dissected into approximately 2 mm^3 pieces that were preserved in RNAlater (Qiagen, Crawley, UK) for future RNA extraction. Larger liver explants (approximately 4 mm^3) were snap-frozen in liquid nitrogen for Western blot analysis. Bone marrow was aspirated from the tibia of each right hind leg and immediately lysed in 350 µL RLT buffer (Qiagen). Marrow samples were homogenized using QIAshredders (Qiagen) prior to storage at -20°C before the completion of RNA extraction.

Parasitemia count

Blood-stage infection was monitored by thin smear microscopy following Giemsa stain. Number of parasitized red blood cells were counted in a minimum of 500 uninfected red blood cells/mouse. Parasitemia was calculated as follows: % parasitemia = (infected red blood cells)/(infected red blood cells + uninfected red blood cells)*100%.

Murine tissue RNA extraction

RNA was extracted from spleen, liver, or bone marrow tissue using the Qiagen RNeasy Mini Kit, Animal Cells Spin Protocol, according to kit instructions. Spleen or liver samples were first homogenized using a TissueRuptor in 600 µL RLT Buffer, then 350 µL of the resulting mixture was used for further extraction.

Western blotting

~10 mg explants of frozen murine livers were lysed on wet ice using a TissueRuptor in a lysis buffer as in previous studies [134]. Buffer contained 50 mM Tris-HCl pH 8, 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA) pH 8, 1% NP-40, and inhibitors of proteases and phosphatases (all Sigma). Protein concentration was assessed using the Thermo Scientific Pierce Protein Assay (Fischer Scientific), and lysates diluted to 20 µg protein/10 µL solution with 1/3 volume bromophenol blue loading buffer. Lysates were then run through 12% sodium dodecyl sulfate (SDS) separating gel and blotted onto activated polyvinylidene difluoride (PVDF) membranes. Size comparison was provided by Biorad precision plus All Blue ladder (Bio-Rad).

Membranes were blocked in Odyssey blocking buffer (LI-COR Biosciences) for 1 h and incubated overnight with a combination of two primary antibodies: mouse anti–β-actin (1:10,000, antibody #AC15, Sigma-Aldrich) and either rabbit anti-phosphorylated Stat3 (pStat3, 1:1,000, antibody #D3A7, Cell Signaling); or rabbit anti-Stat3 (1:3,000, antibody #79D7, Cell Signaling). Membranes were washed 3x in phosphate buffered saline (PBS) with 0.1% Tween, then incubated for 1 h with two secondary antibodies (680 donkey anti-mouse Red at 1:20,000 and 800 goat anti-rabbit green at 1:15,000; both LI-COR Biosciences) in 50% Odyssey/50% PBS buffer with 0.01% SDS and 0.1% Tween. Membranes were washed 3x in PBS with 0.1% Tween and 1x in PBS only, then dried prior to examination with a LI-COR Biosciences instrument. Quantitative measurements of

band intensity were obtained using LI-COR software. Each pStat3 or Stat3 band was normalized to its internal β-actin control. Each pStat3 or Stat3/β-actin value from an infected mouse was normalized to the average from liver lysates from 3 uninfected age and sex-matched mice sacrificed at the same day and time and run on the same gel.

RNA quantification, cDNA synthesis, and quantitative real-time PCR (qRT-PCR)

A Nanodrop ND-1000 Spectrophotometer (Wilmington, DE, USA) was used to quantify RNA concentrations. RNA was reverse transcribed to cDNA using the High Capacity RNA-to-cDNA kit (Applied Biosystems) according to the manufacturer's instructions. qRT-PCR reactions were performed on an Applied Biosciences 7500 Fast Real-Time PCR System machine with cDNA at a final concentration of 1-5ng/µL, using Taqman Gene Expression Mastermix and inventoried Taqman Gene Expression Assays (all Applied Biosystems) as previously described [62]. All qRT-PCR reactions were run in technical duplicate. All murine qRT-PCR values are analyzed relative to the housekeeping gene hypoxanthine phosphoribosyltransferase-1 (*Hprt)*; human qRT-PCR values are analyzed relative to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH).* Genes were analyzed relative to the correct housekeeping gene using the $2^{\Delta Ct}$ method as previously described [62], and technical duplicates were averaged.

Statistical analysis, data processing, and data point exclusion

Unless otherwise stated, outliers in mouse experiments were identified and excluded using the ROUT test [135] with a false discovery rate (FDR) of 0.1%. All data processing was performed in Excel. All statistical analyses were performed, and all graphs generated, in GraphPad Prism (GraphPad Software, Inc., La Jolla, CA).

2.3. Results

Hepcidin upregulation in a mouse model of blood-stage malaria is associated with increased Smad pathway signaling

Male Balb/c mice were infected with 10^3 *Plasmodium berghei* ANKA sporozoites. In this animal model of malaria infection, parasites travel to the liver, replicate there for approximately 48 hours, and then emerge into the blood as merozoites, which invade red blood cells and initiate a blood-stage infection. Time-course experiments were performed: infected and uninfected control mice were harvested 2, 4, 6, or 8 days post-infection (*n*= 3 independent experiments, 3 mice per group per experiment). Parasitemia increased to ~1% 6 days post-infection and 2-4% 8 days post-infection (Figure 2.1A).

Hepatic hepcidin expression increases on day 8 post-infection (Figure 2.1B, Dunn's multiple comparisons test, $p<0.01$ for days 2 or 6 vs. day 8) along with the increase in parasitemia above 1-2%. This is consistent with previous studies that show an increase in hepcidin message only when parasitemia rises above a certain threshold [67, 68].

Indicator gene expression was examined for the two major well-characterized hepcidin control pathways. To identify which of these pathways might have been activated, liver samples were analyzed for the expression of *Id1,* a canonical Smad signaling responsive gene [136], and two Stat3-responsive acute phase genes: fibrinogen α chain *(Fga,* previously used in [62]) and serum amyloid α 1 (*Saa-1*). *Id1* was clearly upregulated on day 8 post-infection (Figure 2.1C, Dunn's

multiple comparisons test, $p<0.001$ for days 2 or 4 vs. day 8) and significantly correlated with *Hamp1* expression (Figure 2.1D, Spearman's correlation, $p<0.01$, $r=0.45$). Moreover, when only the 9 mice harvested on day 8 were included in analysis, the correlation was still marginally significant despite reduced *n* ($p=0.05$,

r=0.68.)

Figure 2.1. Hepcidin expression increase in malaria-infected mice correlates with Smad signaling indicator gene *Id1*. Data in all graphs is combined from 3 independent experiments (*n*=3 mice per group per experiment, *n*=9 total). (A) Mouse parasitemia as percentage infected red blood cells. (B) *Hamp1* message in the liver increases on day 8 post-infection, as parasitemia rises above 1-2%. (C) Smad pathway indicator gene *Id1* increases on day 8 post-infection. A single high *Id1* measurement on day 2 post-infection was excluded. (D) *Id1* expression correlates significantly with hepcidin message. Statistical analyses in box and whisker plots are Dunn's multiple comparisons tests after Kruskal-Wallis test. ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. In correlation graph, each symbol denotes a single mouse and color of symbol indicates the day of sacrifice. Correlation analysis is Spearman's correlation test.

Conversely, *Fga* expression did not increase on day 8 post-infection (Figure 2.2A, Dunn's multiple comparisons test, all $p>0.05$) and did not correlate with hepcidin (Figure 2.2B, Spearman's correlation, $p>0.05$, $r= -0.23$). *Saa-1* increased on day 8 post-infection (Figure 2.2C, Dunn's multiple comparisons test, $p<0.001$ for days 2 or 4 vs. day 8), but also showed no correlation with hepcidin (Figure 2.2D, Spearman's correlation, $p>0.05$, $r=0.3$).

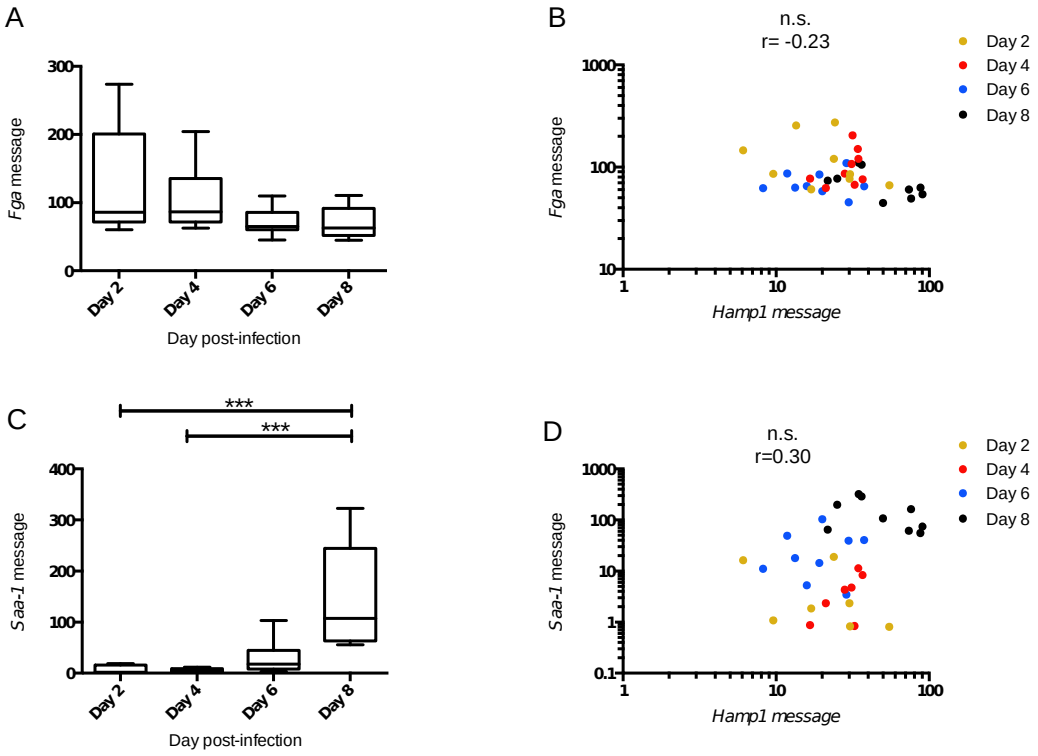

Figure 2.2. Stat3-responsive genes do not correlate with hepcidin. (A) *Fga* message does not increase on day 8 post-infection and does not correlate with hepcidin (B). (C) *Saa-1* message increases on day 8 post-infection (two very high outliers each on days 2 and 4 were excluded) but does not correlate with hepcidin (D). Statistical analyses in box and whisker plots are Dunn's multiple comparisons tests after Kruskal-Wallis test. *** $p<0.001$. In correlation graphs, each symbol denotes a single mouse and color of symbol indicates the day of sacrifice. All correlation analyses are Spearman's correlation tests.

The activity of the Stat3 pathway was further investigated by directly measuring pStat3 and total Stat3 protein by Western blot. Simultaneous multicolor imaging on a LI-COR Infrared imaging system was used to measure both β-actin, as a loading control, and pStat3 or Stat3 for each separate mouse sample in the same lane of the same gel. Stat3 or pStat3 was normalized to β-actin, and each (p)Stat3/β-actin ratio of an infected mouse was normalized to the (p)Stat3/β-actin ratio of liver lysates run on the same gel and obtained from uninfected mice harvested on the

same day. Figure 2.3 shows the full set of Western blots from a representative sporozoite experiment.

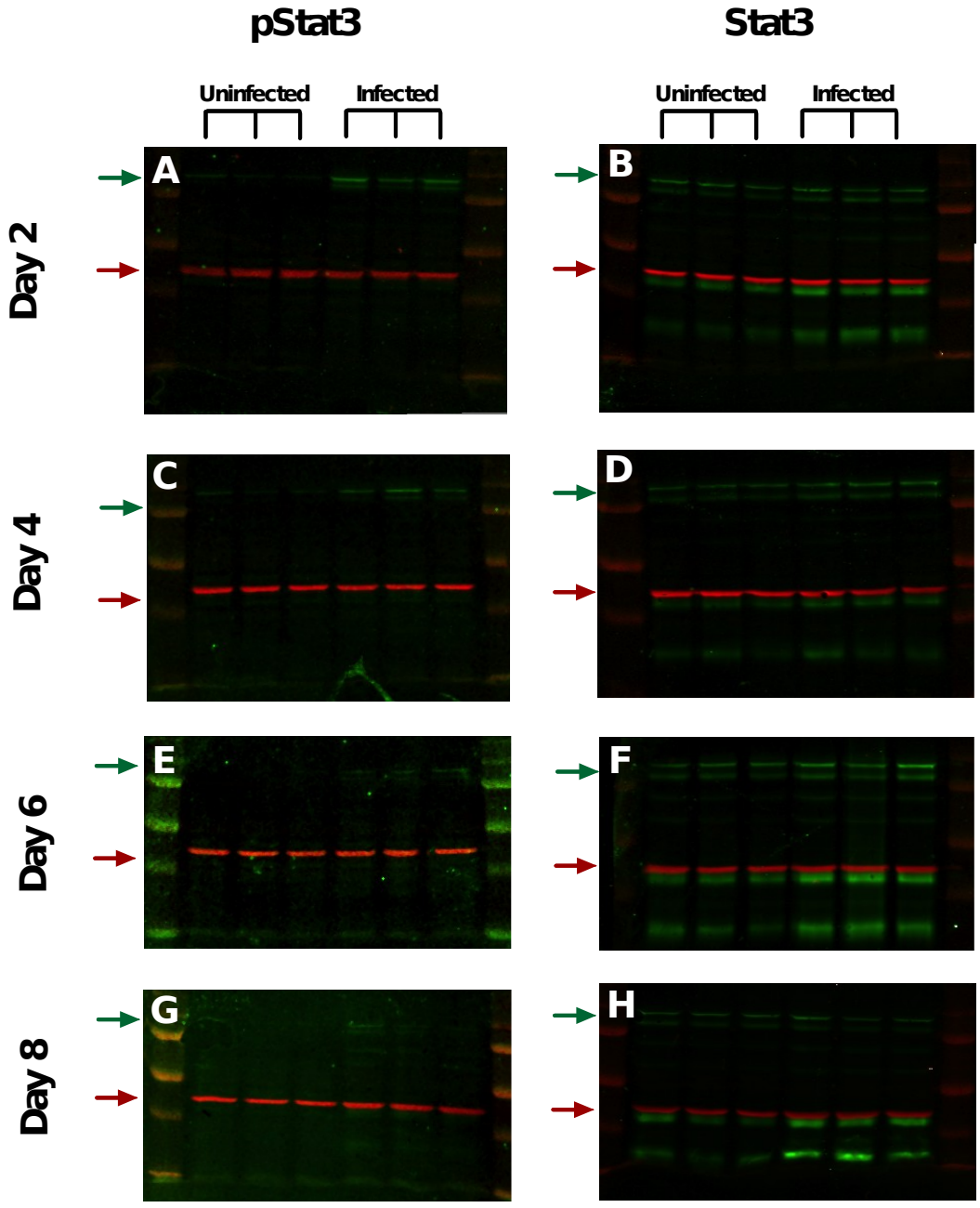

Figure 2.3. pStat3 and total Stat3 protein do not increase toward day 8 post-infection. pStat3 and Stat3 from mouse liver lysates were examined by Western blot. Images show all Western blots from a single representative sporozoite experiment. In all images, red bands at ~42 kilodaltons (kD) are β-actin, green bands at ~80 kD are pStat3 (left-hand images) or total Stat3 (right-hand images).

Some non-specific bands are visible; green arrows are at expected molecular weight of Stat3 or pStat3, red arrows are at expected molecular weight of β-actin. Ladders appear non-specifically stained by both secondary antibodies. Left three lanes of each blot are uninfected controls; right three lanes are liver lysates from infected mice.

pStat3 was not significantly upregulated at day 8 post-infection (Figure 2.4A, Dunn's multiple comparison test, all $p>0.05$), and again did not correlate with hepcidin (Figure 2.4B, Spearman correlation, $p>0.05$, $r= -0.10$). Total Stat3 protein showed a slight decrease towards day 8 post-infection (Figure 2.4C, Dunn's multiple comparison test, $p<0.05$ for day 4 vs. day 8), and was negatively correlated with hepcidin (Figure 2.4D, Spearman's correlation test, $p<0.01$, $r=-0.45$). Unexpectedly, pStat3 was not associated with *Fga* (Figure 2.4E, Spearman's test, $p>0.05$, $r=0.16$), but showed a significant association with *Saa-1* (Figure 2.4F, Spearman's test, $p<0.01$, $r=0.5$).

Taking the available evidence together, neither gene expression of Stat3 pathway-regulated factors, nor direct measurements of pStat3 showed any clear correlation with hepcidin expression. Indeed, when only the mice harvested on day 8 were included, there were trends towards negative correlations between *Hamp1* and *Fga* ($p=0.10$, $r= -0.6$), *Hamp1* and *Saa-1* ($p=0.25$, $r= -0.43$), and *Hamp1* and pStat3 ($p=0.08$, $r= -0.63$). Conversely, Smad signaling response gene *Id1* was clearly upregulated in concordance with hepcidin. As Bmp proteins are known to signal through Smad proteins, an increase in Bmp proteins seemed a plausible cause for the upregulation of both hepcidin and *Id1* in this model of blood-stage malaria.

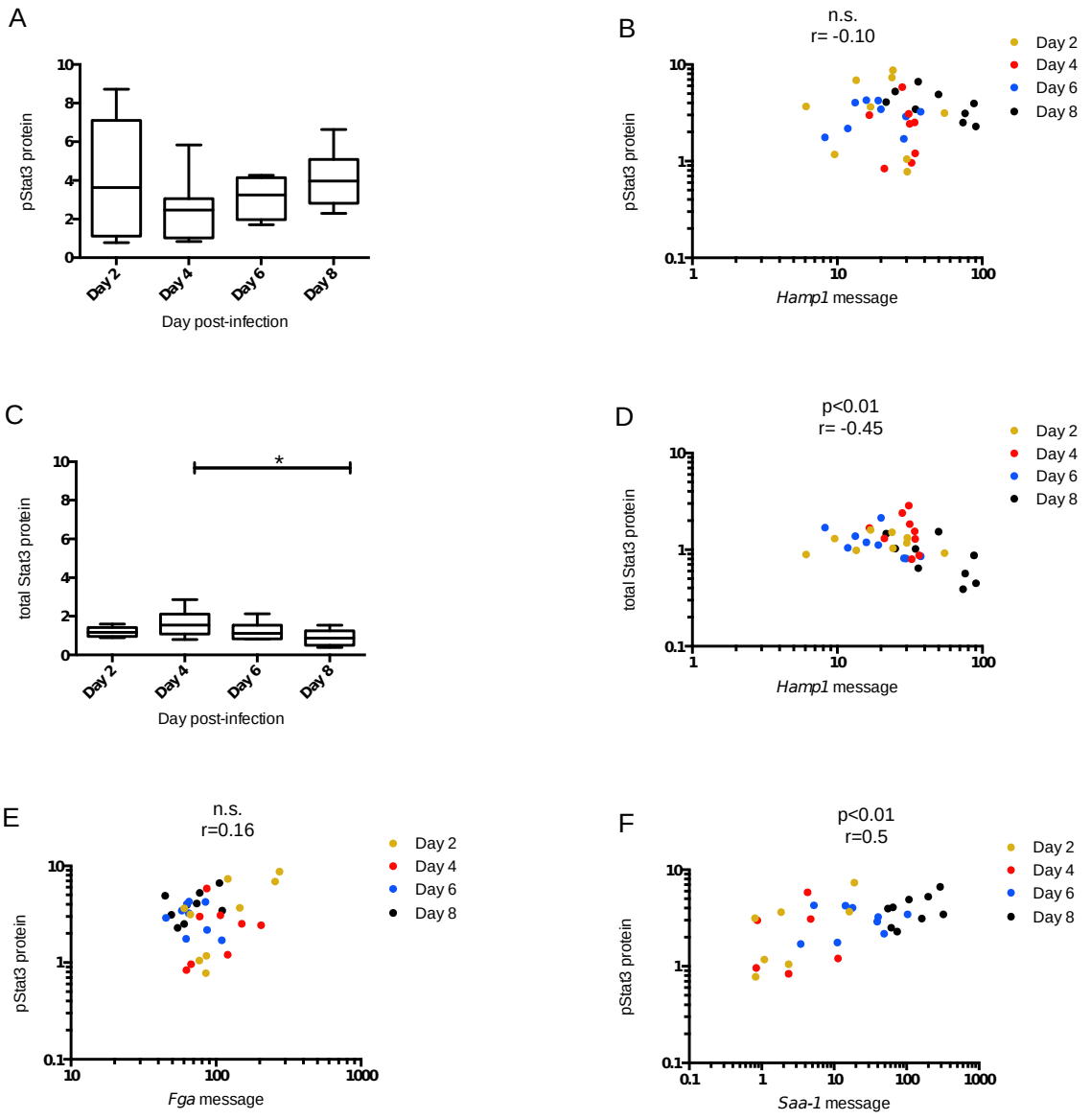

Figure 2.4. pStat3 is not associated with hepcidin expression. (A) pStat3 does not increase significantly on day 8, and does not correlate with hepcidin (B). (C) Total Stat3 protein is slightly but significantly decreased on day 8 compared to day 4 post-infection, and shows a negative correlation with hepcidin expression (D). pStat3 is not correlated with *Fga* expression (E) but does correlate with *Saa-1* expression (F). * p<0.05. In correlation graph, each symbol denotes a single mouse and color of symbol indicates the day of sacrifice. All correlation analyses are Spearman's correlation tests.

Activin B, not Bmp gene expression, increases in murine malaria infection

Bmp6 is the Bmp protein best known as a regulator of hepcidin: *Bmp6* knockout mice exhibit severe iron overload [81, 82], and blocking Bmp6 *in vivo* also decreases hepcidin and increases serum iron [82]. Changes in Bmp6 protein were therefore initially considered as a candidate for the modulation of hepcidin in our model of malaria infection. However, *Bmp6* mRNA was unexpectedly downregulated in hepatic tissue from infected mice as parasitemia increased (Figure 2.5A, Dunn's multiple comparison test, $p<0.05$ for day 2 vs. day 8 only). To extend these investigations, additional *Bmp* genes were examined in the liver and also in bone marrow and spleen samples in one experiment, as secreted factors from both tissues have been linked to hepcidin upregulation in different contexts [112, 137, 138].

Bmp6 (Figure 2.5B, Dunn's multiple comparisons tests, all $p>0.05$) and *Bmp2* (Figure 2.5C, Dunn's multiple comparisons tests, all $p>0.05$) were either unchanged or non-significantly decreased across all three tissues on day 8 of infection. *Bmp9* was not detectable in bone marrow or spleen and was not upregulated in the liver (Figure 2.5D, Dunn's multiple comparisons tests, $p>0.05$). Although hepcidin and *Id1* were both clearly upregulated in infection, it did not appear that the expression of *Bmp* genes was similarly increased.

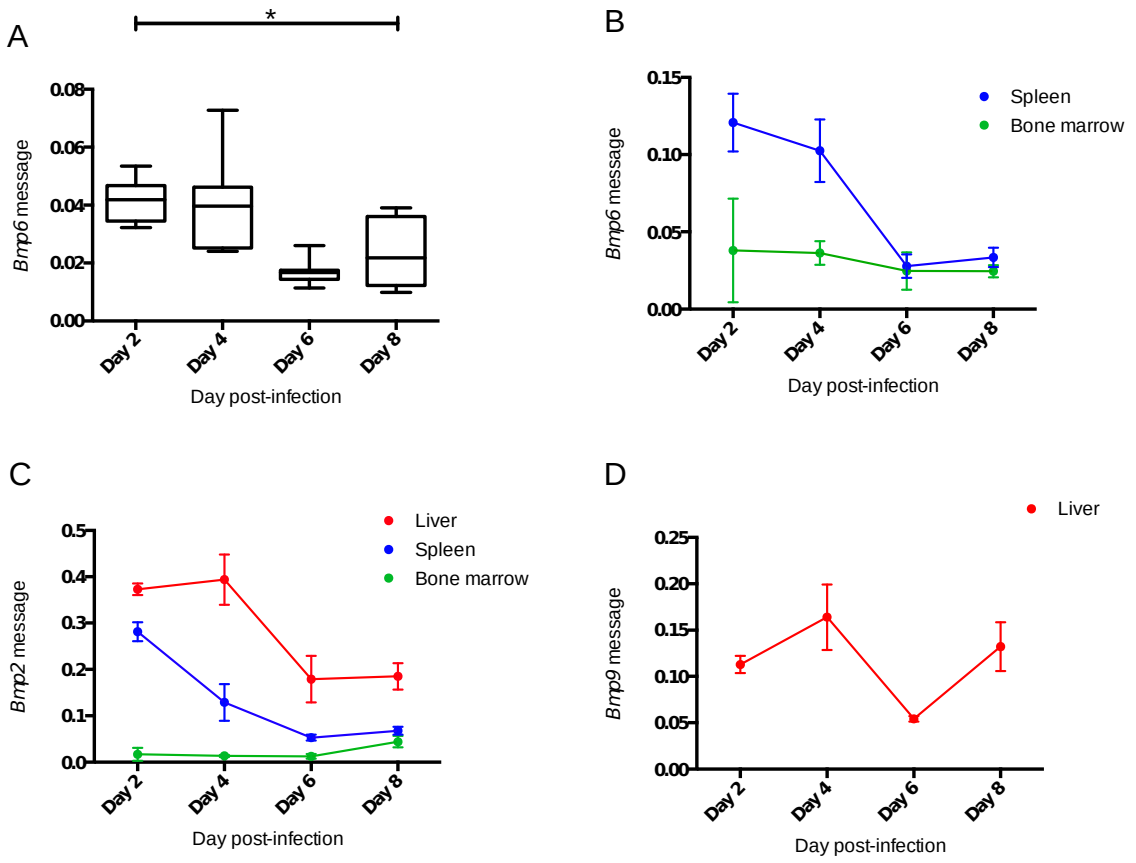

Figure 2.5. *Bmp* genes are not upregulated at the message level during malaria infection. (A) Liver *Bmp6* mRNA expression decreases on day 8 of infection (n=3 mice per day per experiment, n=9 total). (B-D) A representative experiment (n=3 mice per day) was examined further to see if other *Bmp* genes might increase in other candidate tissues. (B) *Bmp6* mRNA did not show any trend towards upregulation in spleen or bone marrow. (C) *Bmp2* did not increase in liver, spleen, or bone marrow. (D) *Bmp9* mRNA was undetectable in spleen and bone marrow and did not increase in liver. Statistical analyses in box and whisker plots are Dunn's multiple comparisons tests after Kruskal-Wallis test. * $p<0.05$.

A recent report has detailed the possible contribution of increased hepatic activin B protein to the upregulation of hepcidin via Smad signaling in an inflammatory context [93]. Activin A, a close homolog of activin B, is known to increase in animal and human sera following similar stimuli [139, 140]. Based on this evidence, hepatic expression of both activin proteins were measured. Activin B

mRNA (*Inhbb)* was increased significantly on day 8 post-infection (Figure 2.6A, Dunn's multiple comparisons tests, $p<0.01$ for days 2 or 4 vs. day 8). Activin A mRNA *(Inhba)* showed a decrease towards day 8 (Figure 2.6B, Dunn's multiple comparisons tests, $p<0.0001$ for days 2 or 4 vs. day 8). Activin B expression showed high inter-mouse variability, but exhibited a trend towards correlation with *Hamp1* (Figure 2.6C, Spearman's correlation, $p=0.13$, $r=0.26$), and correlated significantly with the Smad signaling response gene *Id1* (Figure 2.6C, Spearman's correlation, $p<0.01$, $r=0.45$). These results suggest that activin B might contribute to hepcidin upregulation in murine blood-stage malaria infection.

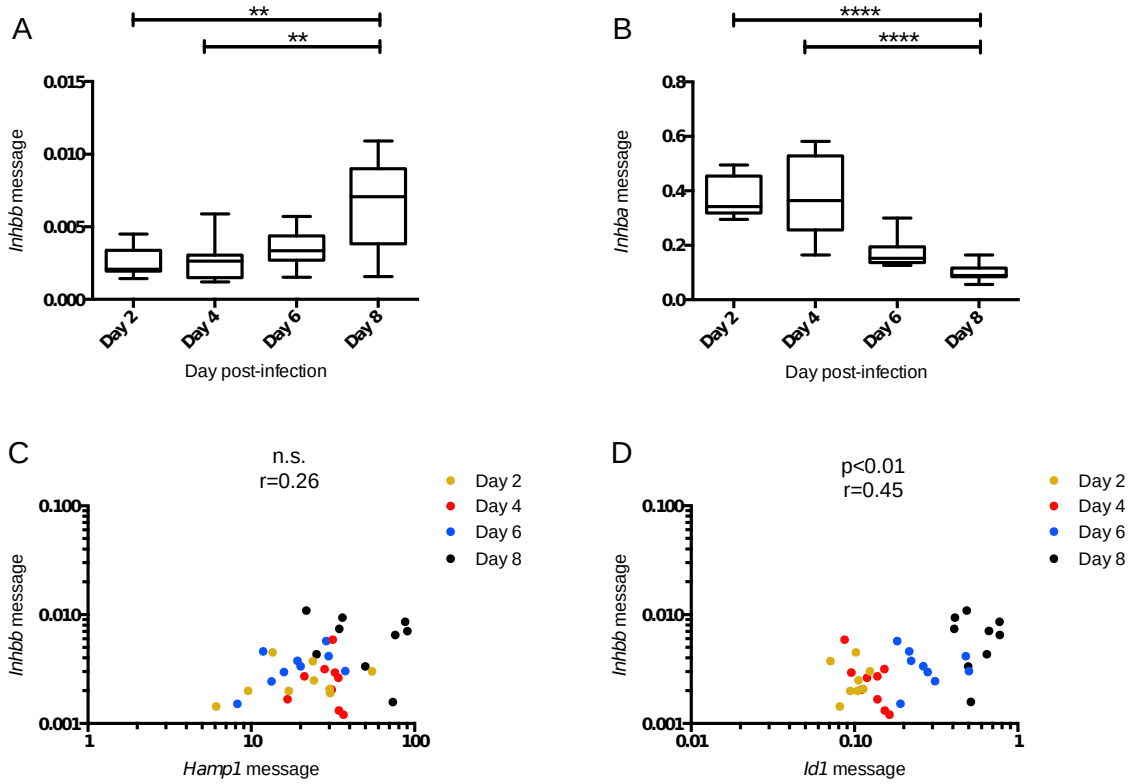

Figure 2.6. Activin B is upregulated in infection and correlates with *Id1*. (A) Liver *Inhbb* mRNA expression increases on day 8 of infection (*n*=3 mice per day per experiment, *n*=9 total). (B) *Inhba* expression was significantly decreased on day 8. (B). *Inhbb* expression shows a trend towards correlation with *Hamp1* (C), and correlates with *Id1* (D). Statistical analyses in box and whisker plots are Dunn's multiple comparisons tests after Kruskal-Wallis test. ** p<0.01, **** p<0.0001. In correlation graph, each symbol denotes a single mouse and color of symbol indicates the day of sacrifice. All correlation analyses are Spearman's correlation tests.

One striking finding in these experiments was the highly variable upregulation of Stat3 response gene (*Fga* and *Saa-1)* expression, and pStat3 increases, in infected mice 2 days post-infection. Two *Saa-1* gene expression values were classified as outliers using a stringent statistical test (ROUT test, 0.1% FDR)

and were therefore excluded from Figures 2 and 4, but their presence was re-examined in the context of variable day 2 *Fga* and pStat3 increases. At 48 hours post-infection, parasites are only just exiting the liver, and it is hard to conceive that they would elicit such a dramatic response at that time in a minority of mice. We hypothesized that the mosquito gland preparation used to infect the mice might itself be immunostimulatory.

Mice were therefore mock-infected (n= 3) with an equal volume of prepared uninfected mosquito salivary glands as were administered to infected mice (referred to hereafter as "mock-infected" mice). These mice were sacrificed along with uninfected and infected mice at 2 days post-infection. Figure 2.7 shows all uninfected and infected mice (n=9 per group, no outliers excluded) as compared to mock-infected mice (n=3). Stat3 signaling marker gene *Fga* trended towards an increase in mock-infected or infected mice (Figure 2.7A, Dunn's multiple comparisons test, all $p>0.05$), *Saa-1* was significantly increased in mock-infected mice but not infected mice (Figure 2.7B, Dunn's multiple comparisons test, $p<0.05$ for control vs. mock-infected mice). pStat3 showed a trend towards upregulation in both infected and mock-infected mice, although this was not significant (Figure 2.7C, Dunn's multiple comparisons test, all $p>0.05$). Unexpectedly, total Stat3 increased in mock-infected mice (Figure 2.7D, Dunn's multiple comparisons test, $p<0.05$ for control vs. mock-infected mice). Given these data, it seems likely that the observed variable increases in Stat3 signaling markers around day 2 post-infection are likely due to the immunostimulatory effect of mosquito salivary glands. Future studies that examine early-stage responses to malaria infection may need to consider the

implications of the inflammatory consequences of a commonly used infection method.

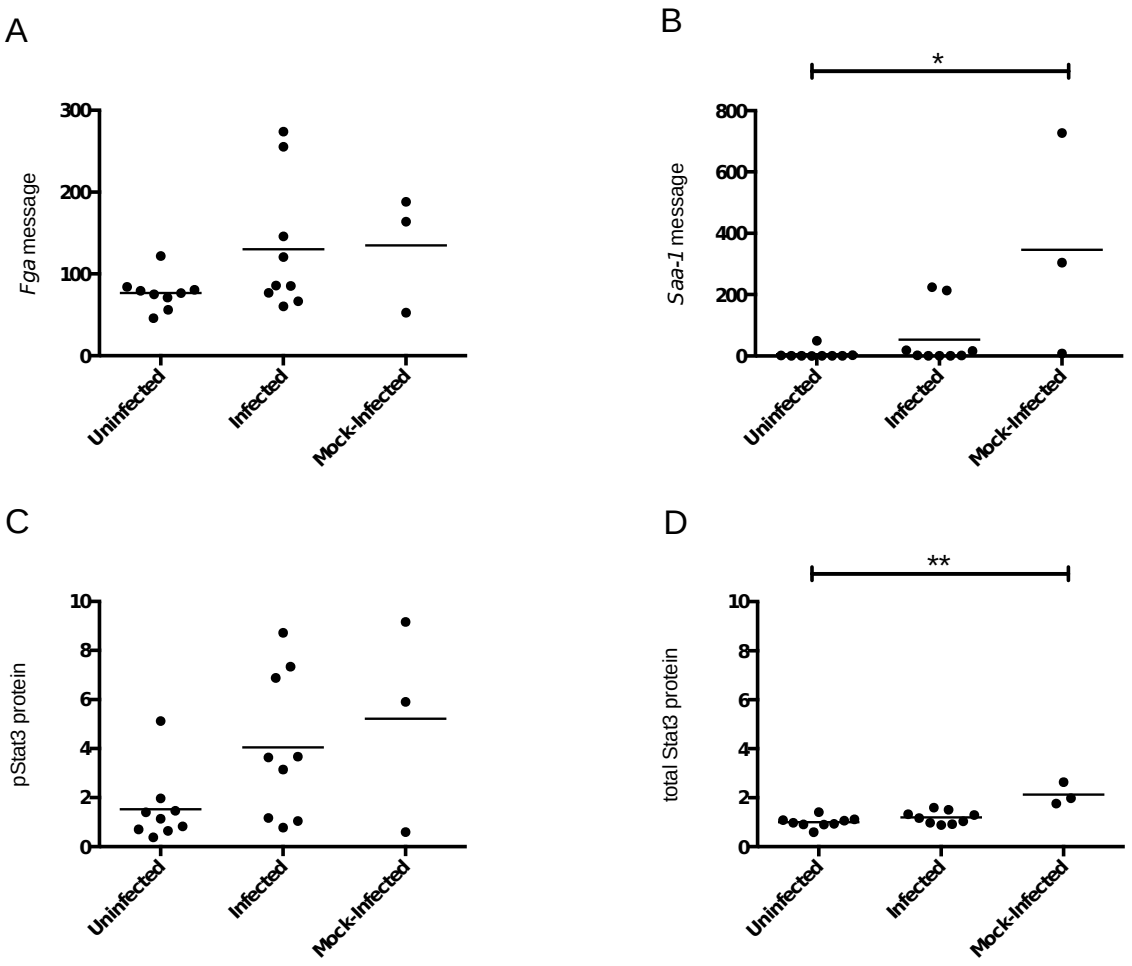

Figure 2.7. Mosquito salivary glands upregulate Stat3 pathway indicators. Mice were mock-infected and sacrificed 2 days post-infection for comparison with infected and uninfected mice (*n*=3 in mock-infected group, *n*=9 in infected and uninfected groups). Outlier exclusion was not performed. Indicator genes *Fga* (A) and *Saa-1* (B) are variably upregulated in mock-infected and infected mice. (C) Similarly, pStat3 shows a trend towards increase in both mock-infected mice and infected mice. Total Stat3 protein was increased in mock-infected mice over the uninfected controls (D). Statistical analyses in dot plots are Dunn's multiple comparisons tests after Kruskal-Wallis test. * $p<0.05$, ** $p<0.01$.

2.4. Discussion

These data demonstrate that in a murine model of malaria infection, hepcidin upregulation is correlated with the Smad signaling response gene *Id1*. Conversely, *Hamp1* expression was not correlated with the expression of the Stat3 pathway response genes *Fga* and *Saa-1*, nor with direct measurements of pStat3.

These findings are in contrast with a previous study of hepcidin upregulation in a mouse model of malaria infection, which found a close correlation between pStat3 levels and *Hamp1* that this study did not replicate [68]. Both our study and theirs utilized the same parasite strain (*P. berghei* ANKA) but different mouse strains, and different antibodies against pStat3; differences in parasite response according to mouse strain may potentially account for this discrepancy. Additionally, our results differ from one study of hepcidin control in a murine model of *Candida albicans* and acute influenza virus infections. In both models of infection, *Hamp1* expression was significantly correlated to *Fga*, while *Id1* remained static [62]. Nevertheless, the apparent control of hepcidin via Smad signaling in malaria infection is in indirect agreement with other studies. One murine study reported a message-level increase of *Id1* during malaria infection [67], a finding this work can corroborate. The same authors also noted that in mouse hepatocytes treated with sera from *P. berghei*-infected mice, hepcidin message increase was abrogated by the Bmp type 1 receptor inhibitor dorsomorphin (which also likely inhibits activin signaling, see Chapter 3), and only partially reduced by anti-IL-6 antibodies [67]. Indeed, anti-IL-6 antibodies administered systemically to malaria-infected mice were found to reduce but not eliminate the upregulation of hepcidin [68], and failed

to alter the hepcidin-mediated prevention of malaria superinfection [67]. Our data support the conclusion that hepcidin upregulation in this murine model of malaria infection is controlled primarily via Smad signaling, although this does not exclude the possibility that the Stat3 pathway plays a minor contributing role.

The co-upregulation of *Hamp1* and the Smad-response gene *Id1* led us to hypothesize that an increase in Bmp proteins might occur in malaria infection. However, all *Bmp* genes assayed in the tissues of infected mice were either static or moderately downregulated as parasitemia increased. This finding mirrors a report showing that in mice injected with LPS, Smad signaling indicators increased concurrently with *Hamp1*, yet *Bmp* genes' expression was not elevated [93]. These authors, however, noted a significant increase in hepatic *Inhbb* expression post-LPS injection (also in [139]). In this model of malaria infection, *Inhbb* was found to increase concurrently with hepcidin and correlate with *Id1*.

These new findings further echoed Besson-Fournier (2012) in that activin A message decreased as *Hamp1, Inhbb*, and parasitemia increased. However, as activin A hepatic message and serum protein levels are well known to be decoupled [139, 140] these data were difficult to interpret. Based on these findings, the role of activin A remained unclear, and required further investigation.

An interesting aspect of this study is the disparity between *Fga* expression, *Saa-1* expression and pStat3. Although pStat3 and *Saa-1* expression were significantly correlated, pStat3 was not correlated with *Fga* expression. It is possible that the difference between *Fga* and *Saa-1* expression may be accounted for by activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)

signaling. NF-κB plays a major role in controlling various acute phase proteins' expression: it is thought to complex with pStat3 to upregulate *Saa-1* [141]. However, induction of NF-κB signaling by IL-1β treatment inhibits the upregulation of γ-fibrinogen gene by Stat3 signaling [142, 143]. It is possible that α-fibrinogen chain *Fga* is similarly inhibited by NF-κB signaling. If so, then increased NF-κB signaling during blood-stage malaria may actually decrease *Fga* expression while augmenting *Saa-1*, thus explaining the varying relationships between our two measured acute phase genes and pStat3.

An additional unexpected finding was the mild decrease in Stat3 on day 8 post-infection, and its inverse correlation with hepcidin expression. It is possible that Stat3 is downregulated slightly during the increased parasitemia on day 8 as part of a mechanism to prevent over-signaling through phosphorylated Stat3, but this seems unlikely in light of the largely static pStat3 levels. This phenomenon is so far unexplained.

In brief, hepcidin increase at the message level in this murine malaria model is correlated with Smad signaling indicator gene *Id1,* not Stat3 signaling indicator genes or pStat3. Although *Bmp* genes do not increase, activin B is upregulated at the message level and correlates with *Id1*, and may thus represent a plausible controller of hepcidin via the Smad signaling pathway in this murine model of malaria infection.

Future studies should measure phosphorylated Smad1/5/8 (pSmad1/5/8) levels in mouse liver during malaria infection. Repeated attempts were made to do so in this study, but failed as a result of continuing technical difficulties. Both the LI-

COR system detailed above and traditional chemiluminescence methods enabled the visualization of several bands, some at the correct molecular weight for pSmad1/5/8, but the band intensity did not appear to change between mice in any experiment. As positive controls, to show that our system was truly able to detect Smad phosphorylation, LI-COR methods were used to measure pSMAD1/5/8 proteins from human hepatoma cells treated with BMP proteins directly. Detection of changes in human pSMAD1/5/8 was successful in that system but establishing a murine positive control of changes in pSmad1/5/8 in the liver proved more difficult. Liver samples were obtained from Bmp6 protein-treated mice and controls (kind gift from J. Babitt and lab) and from mice with hepcidin-resistant ferroportin mutations, which exhibit high levels of Bmp signaling as a result of chronic iron overload, and their littermate controls (kind gift from S. Altamura, Muckenthaler lab). *Id1* was upregulated in both groups (J. Babitt and S. Altamura, personal communications) and pSmad1/5/8 would therefore be expected to be elevated, as in *P. berghei*-infected mice. However, in neither case were we able to detect increased pSmad1/5/8. Upon extensive consultation with members of both labs, it was ascertained that although previously both groups had been able to effectively use the primary antibody (Cell Signaling antibody #9511), both were having difficulty with recent batches of the same antibody as applied to murine liver. Faced with these technical difficulties, we considered *Id1* as representative of Smad signaling.

As well as measuring pSmad1/5/8 in malaria infection, further studies should assay Id1 protein levels to verify changes noted at the message level. In

addition, serum activin A and B protein levels should be measured during infection, especially as activin A serum levels do not necessarily reflect hepatic activin A expression. Pilot experiments to measure activin A levels in murine serum using the R&D Activin A Human/Mouse/Rat ELISA kit (catalog number #DAC00B, further described in Chapter 4) failed to detect activin A protein in the serum of either infected or control mice. No activin B ELISA is currently commercially available for human or murine serum.

Further experiments should also focus on measuring hepcidin, *Id1*, Stat3 pathway markers, and activin message levels in other mouse-parasite combinations. The correlation between hepcidin message and indicators of Smad signaling observed in Balb/c mice infected with *P. berghei* ANKA may not necessarily extend to other mouse-parasite combinations.

Finally, at the time of writing, the evidence linking activins to hepcidin upregulation is relatively limited. Activin A has been shown to increase hepcidin transcription to a limited extent in one human hepatoma cell line, Hep3B [84]. Activin B protein's effects on hepcidin expression have also been tested on only one, different, hepatoma cell line (HepG2) [93]. A better characterization of the effects of activins on hepcidin *in vitro*, and the extension of these studies to *in vivo* models, is required as a basis for further exploration of the potential roles of activins in hepcidin control during malaria infection.

CHAPTER 3: THE EFFECTS OF ACTIVIN A, ACTIVIN B, AND THE ACTIVIN-BINDING PROTEIN FOLLISTATIN ON HEPCIDIN *IN VITRO* AND *IN VIVO*

3.1. Introduction

The data presented in Chapter 2 demonstrate that hepcidin upregulation in a mouse model of malaria infection is likely controlled via the Smad signaling pathway. Canonical Smad pathway agonists, Bmp proteins, were not increased at the message level in our model, but activin B, which has been recently shown to have a role in hepcidin control [93], was elevated at the message level. Relatively few studies have examined the effects of activins on hepcidin *in vitro* or *in vivo*; therefore, this chapter aims to directly investigate the effects on hepcidin of manipulating both activin B and the closely related protein activin A.

Activins were first described as stimulators of follicle-stimulating hormone (FSH) secretion by the pituitary gland, but since have been shown to be highly pleiotropic, with roles that include modulating erythropoiesis, inflammation and the immune response, glucose control, and recently, control of iron metabolism via hepcidin [93, 144-146]. The activin proteins are part of the TGFβ superfamily, and as such, are structurally related to BMP proteins. Mammals have four genes that encode activin β subunits: βa (*INHBA),* βb (*INHBB)*, βc (*INHBC),* and βe (*INHBE)*. These β subunits can form homodimers to create activin A, activin B, activin C, or

activin E, respectively, or can heterodimerize with each other. Additionally, they can heterodimerize with the larger and structurally distinct inhibin α subunit (*INHA)* to form inhibin proteins, which oppose some of the activins' endocrine actions [147]. Of the activin proteins, only activin A, and to a lesser extent activin B, have been well studied. The roles of activin C or E are currently unclear: murine knockouts of either or both genes fail to show an obvious phenotype with regard to liver function, liver regeneration, or FSH secretion [148].

Recent studies have shown that activin A is increased in inflammation and infection. Serum activin A is acutely elevated subsequent to LPS challenge in different animal models [139, 140, 149]. In humans, septicemia [150, 151], hepatitis C infection [152], and acute respiratory failure [153] are all associated with elevated activin A protein in serum. Interestingly, however, activin A message does not increase during any tissue measured in mice post-LPS challenge [140]. Instead, protein levels decrease in the bone marrow, possibly indicating that activin A protein stored in bone-marrow derived cells is released following stimulation. Bone-marrow derived neutrophils in particular are known to release whole activin A protein in response to stimulation by tumor necrosis factor α (TNFα) [154]. However, *de novo* production of activin A in response to toll-like receptor (TLR) ligands has been noted in circulating dendritic cells [155] and monocytes [156].

Based on early *in vitro* studies, activin B was thought to fulfill similar functions as activin A [157, 158]; more recent work in knockout mouse models has demonstrated that activin B is functionally distinct from activin A in some contexts [159-161]. Fewer data exist regarding the changes of activin B protein levels in

serum during infection or inflammation, in part because assays to measure it have only been developed very recently [153, 162]. A single report has indicated that activin B is elevated in acute respiratory failure along with activin A [153].

Several recent reports have provided varying evidence for a stimulatory effect of activin A or B on hepcidin *in vitro*. A study describing the effects of BMP proteins on hepcidin message in human Hep3B hepatoma cells also noted that activin A administration produced a relatively minor upregulation of hepcidin message [84]. Similarly, Besson-Fournier et al (2012) found that activin B protein upregulated hepcidin in human hepatoma HepG2 cells and primary murine hepatocytes with a concurrent increase in phosphorylated Smad1/5/8, likely through the BMP type 1 receptor Alk3 [93]. This is in contrast to canonical activin signaling: activins are thought to signal through phosphorylation of Smad2/3 after binding to the type 1 receptors Alk4/5/7 [163].

Besson-Fournier et al (2012) also implicated activin B in hepcidin upregulation *in vivo,* showing that BMP-responsive Smad1/5/8 were phosphorylated in the livers of LPS-injected mice concurrently with hepcidin upregulation, hinting at control of hepcidin through Smad signaling pathway following inflammatory stimulus [93, 164]. No *Bmp* genes were upregulated, but *Inhbb* message was powerfully increased, leading the authors to hypothesize that activin B might be responsible for hepcidin upregulation under those circumstances. A second study has also described hepatic *Inhbb* increases following LPS injection [139]. These findings mirror what was observed in a murine model of malaria infection (Chapter 1): *Id1* co-increases with *Hamp1* in malaria infection, indicating

Hamp1 upregulation through the Smad signaling pathway, *Inhbb* is upregulated while *Bmp* genes are not. This parallel suggested a similar potential role for activin B in malaria-infected mice.

Inhba was decreased at the message level in livers of infected mice (Chapter 1), a finding that was also observed in LPS-treated mice [93]. However, as *Inhba* expression in tissue is decoupled from its levels in serum [139, 140] it was necessary to investigate further a possible role for activin A in hepcidin upregulation during malaria infection, as well as activin B.

Administration of the follistatin protein has been considered as a potential tool to alter the effects of activins *in vivo*. Follistatin is an autocrine glycoprotein that binds activin proteins extracellularly [165, 166], possibly binding activin A with slightly greater affinity than activin B [167]. Follistatin levels rise in animal serum following inflammatory and infectious challenge, which may be a negative feedback mechanism to prevent excessive effects from raised activins [139, 168, 169]. Indeed, one study showed that exogenous follistatin administration decreased mouse mortality following LPS injection [139], raising hopes that follistatin might one day be used to reduce mortality in sepsis. In a single subsequent report, however, mice infected with gram-negative bacteria *Escherichia coli* K1 showed no detectable improvement following treatment with exogenous follistatin [170].

Because follistatin is required for embryonic development, knockouts are neonatal lethal [171], but a follistatin haploinsufficent mouse has been produced which is lacking one functional copy of the *Fst* gene ($Fst^{-/+}$) [172]. As well as binding circulating activins, follistatin also serves as an autocrine regulator of the muscle

protein myostatin, which is a negative regulator of muscle mass [173]. As such, follistatin-haploinsufficient mice are characterized by reduced skeletal muscle size while follistatin-overexpressing mice have markedly overdeveloped musculature [174]. Follistatin mutants have not, however, yet been examined for alterations in basal hepcidin expression or iron status.

The data presented below demonstrate that both activin A and B produce an increase in *HAMP* and the SMAD-responsive gene *ID1 in vitro*, indicating upregulation of hepcidin through the SMAD signaling pathway. *In vivo,* hepcidin message was significantly upregulated at the message level in mice injected with activin A or activin B. This chapter also characterizes the effects of the activin-binding protein follistatin on hepcidin *in vitro* and *in vivo,* and describes an experiment that attempted to block the upregulation of hepcidin in a malaria infection by follistatin administration. Although this latter experiment suffered from technical difficulties, it remains the first attempt at characterizing the effects of follistatin administration during malaria infection.

3.2. Materials and Methods

Hepatoma cell culture and protein treatment

HepG2 human hepatoma cells (ECACC) were cultured in minimal essential medium (MEM, Sigma, alpha modification) supplemented with 10% fetal calf serum (FCS, PAA), 2mM glutamine, 100U/mL penicillin, and 0.1mg/mL streptomycin (all Sigma). Cells were plated in a 12-well plate at $2*10^5$ cells/mL, 1 mL/well. Cells were allowed to adhere overnight, then starved for 5 h with MEM with 0.1% FCS prior to activin protein treatment. Cells were treated with activin A (50 ng/mL), activin B (50 ng/mL) or BMP9 as a positive control (100 ng/mL) for 4 h, except in one time-course experiment, in which cells were harvested after 1, 4, 8, 12, or 24 h.

For activin-blocking experiments, LDN-193189 (LDN, 100 nM, Axon Medchem), follistatin-288 (R&D, 1, 3, or 9 nM) or follistatin-315 (a generous gift from Babitt and Schneyer laboratories, 1, 3, or 9 nM) were added to activin-conditioned media and incubated for 30 minutes (min) at 37° C prior to administration to cells. Cells were treated for 4 h. In the LDN experiment, activins were used at 50 ng/mL, and BMP9 at 100 ng/mL. In the follistatin experiments, activins and BMP6 were both used at 1 nM, so that molar ratios of 1:1, 1:3, and 1:9 could be attained.

For RNA extraction, cells were lysed in the wells using RLT buffer, and homogenized by passage through Qiashredder columns (Qiagen, UK). RNA was extracted using a RNeasy Mini Kit (Qiagen UK). All experiments were performed in biological duplicate.

Mouse husbandry information

With the exception of the generation of follistatin-haploinsufficient mice (*Fst*$^{-/+}$) and littermates (described in Figure 3.4), all murine experiments presented in this chapter were performed at the National Institute of Allergy and Infectious Diseases (NIAID)/NIH in accordance with the guidelines of NIAID/NIH Institutional Animal Care and Use Committees. Animal protocols (AP) specifically described in this chapter were registered as AP 5417_00, 5417_02, 5417_03, 5417_04, 5417_05, 5417_07, and 5430_01. All mice utilized at LMIV were male Balb/c strain of 6-8 weeks of age. In all experiments unless otherwise indicated, mice were maintained with *ad libitum* access to water and to standard mouse chow, Harlan-Teklad 2018SX (Fe^{2+} content ~200 ppm).

Livers and sera from follistatin haploinsufficent mice and littermates were a kind gift from the Matzuk laboratory. Mice were 6-8 weeks old and had been fed standard mouse chow. Matzuk laboratory mice were male and on a C57Bl/6 background.

Mouse activin protein treatment

Male Balb/c mice (6-8 weeks of age, NCI Mouse Repository) were maintained with *ad libitum* access to Harlan-Teklad diet TD.09127 (minimal Fe^{2+} content, ~2-4 ppm) for 14 days prior to activin protein treatment to lower baseline hepcidin expression, as previously performed [175]. Mice were injected i.p. with 2 µg

commercially available recombinant human/mouse/rat activin A or mouse activin B protein (both R&D) in 200 µL PBS. Control mice received a 200 µL PBS injection i.p. Mice were culled and samples harvested 4 h post-injection.

Mouse follistatin protein titration and timecourse

For a titration experiment, 2-10 µg follistatin-315 was injected i.p. into standard-fed mice in 200 µL PBS. Controls received 200 µL PBS only. Mice were sacrificed 4 h later and organs harvested. For the timecourse, mice were injected i.p. with 10 µg follistatin-315 in 200 µL PBS or 200 µL PBS, then were harvested 4, 12, and 24 h post-injection.

Follistatin treatment during sporozoite infection

Mice were infected with 10^3 *Plasmodium berghei* ANKA strain sporozoites, suspended in 200 µL RPMI i.v. via the tail vein (as previously described in Chapter 2). At 6 days post-infection, one group of mice were injected with 25 µg follistatin-315 in 200 µL PBS i.p., control infected mice received 200 µL PBS i.p. Injections were continued every 12 h for a total of 4 injections (100 µg follistatin-315 total) per mouse. Mice were harvested at 8 days post-infection, 12 h subsequent to the last follistatin injection. Follistatin-315 had been previously tested for *in vitro* efficacy.

Serum iron measurements

Serum iron and unsaturated iron binding capacity (UIBC) of mouse sera was obtained using a commercially available kit (Pointe Scientific), according to the manufacturer's protocol, modified to use volumes suitable for a 96-well clear-plate (6% of that recommended in the protocol). The assay was read on an Infinite M200 Pro Tecan microplate reader at 560 nm. Total iron binding capacity (TIBC) was calculated by serum iron + UIBC. % Transferrin saturation was calculated by: (serum iron/TIBC) x 100%.

Previously described methods

Parasitemia counts, murine sample harvest and storage, murine tissue RNA extraction, RNA quantification, cDNA synthesis, and qRT-PCR were all performed as detailed in Chapter 2.

3.3. Results

Activins upregulate hepcidin in vitro and in vivo

In human HepG2 hepatoma cells, *HAMP* was significantly upregulated 4 h following treatment with activin B protein, and showed a trend towards upregulation following activin A treatment (Figure 3.1A, Dunn's multiple comparisons test, $p<0.01$ for activin B vs. control only). These results are in agreement with previous studies [84, 93]. Both proteins induced a trend towards upregulation of *ID1* mRNA (Figure 3.1B, Dunn's multiple comparisons test, $p>0.05$ for all comparisons). Both activin proteins produced the most pronounced changes in gene expression between 1-8 h post-treatment (Figures 1C-D). The mechanism of activin action was explored by pre-treating cells with the BMP type 1 receptor inhibitor molecule LDN, an optimized dorsomorphin derivative [176]. Pre-treatment with LDN reduced upregulation of both *HAMP* (Figure 3.1E) and *ID1* (Figure 3.1F) approximately to control levels, although LDN also reduced baseline levels of *HAMP* and *ID1* without activin treatment. Our data are consistent with the hypothesis that activin A and B proteins can act at least in part through the LDN-sensitive BMP type 1 receptors Alk2 or Alk3, as has been previously suggested [93].

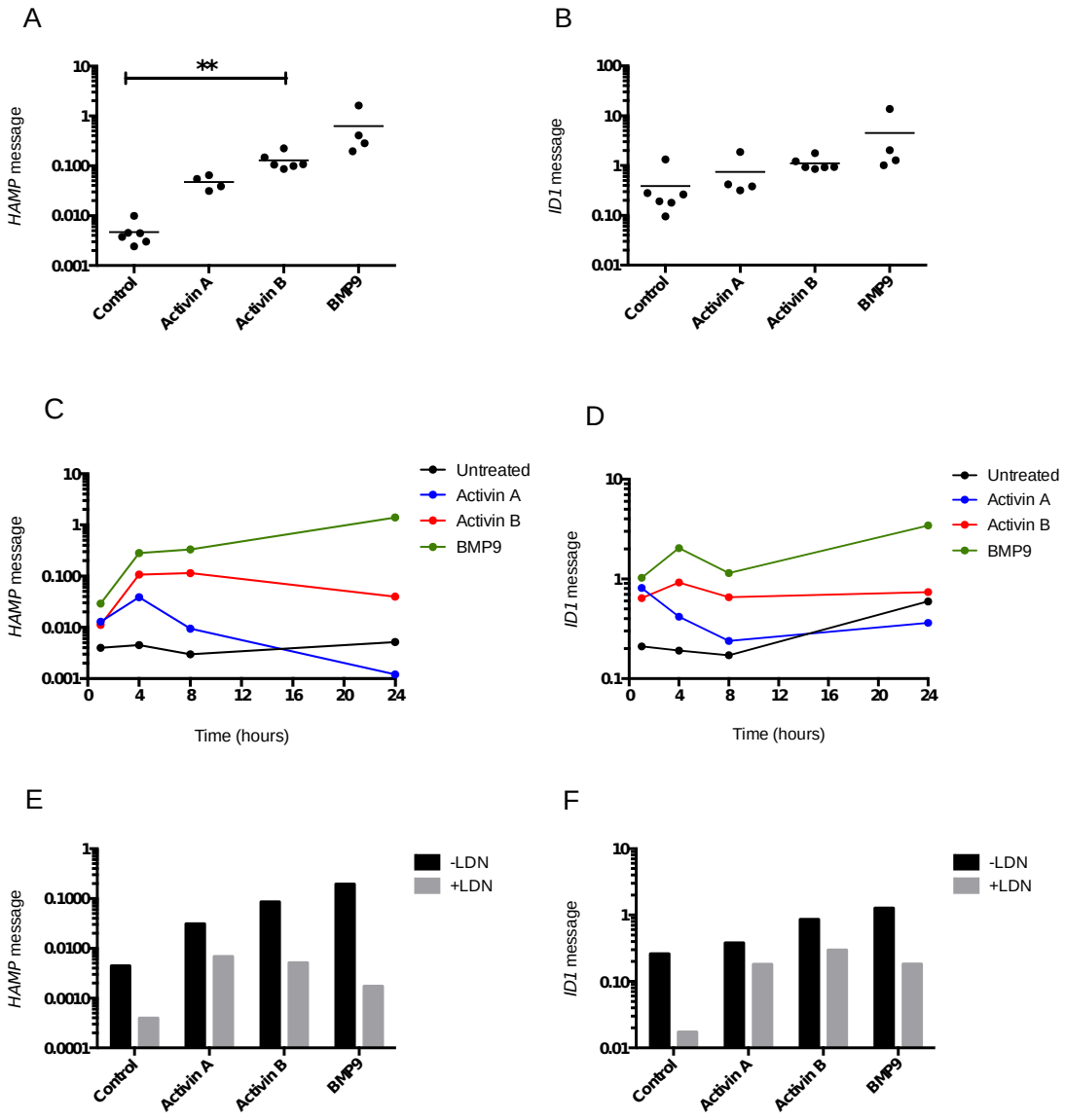

Figure 3.1. Activin A and activin B proteins upregulate hepcidin message *in vitro*. (A-B) Activin A or B protein administration to hepatoma cells results in upregulation of *HAMP* (A) and a trend towards an increase in *ID1* (B). BMP9 is included as a positive control. Each point shown depicts the average of two biological duplicates from an independent experiment (n=4-6, not all conditions run in each experiment). To better characterize the kinetics of the activin response, HepG2 cells were treated as in (A-B) and harvested at timepoints ranging from 1 to 24 h post-treatment; maximal upregulation of *HAMP* (C) and *ID1* (D) was between 1-8 h post-treatment. To determine whether activins act via BMP receptors, LDN was added 30 minutes before activin or BMP9 application for 4 h. LDN inhibited *HAMP* (E) and *ID1* upregulation (F) by both activin proteins and by BMP9, but also decreased gene expression baseline. Statistical measures in dot plots (A-B) are Dunn's multiple comparison's tests after Kruskal-Wallis test. ** $p<0.01$.

To analyze whether activin proteins produced a similar effect *in vivo* to that noted *in vitro*, murine activin proteins were injected directly into mice. Mice were kept on a low-iron diet for two weeks prior to activin protein injection in order to lower *Hamp1* expression baseline and enable better detection of any changes. Hepatic *Hamp1* was variably but significantly upregulated in both activin A- and activin B-treated mice compared to controls (Figure 3.2A, Dunn's multiple comparisons tests, $p<0.05$ for both proteins). % Transferrin saturations did not change significantly over this brief period of time (Figure 3.2B, Dunn's multiple comparisons tests, $p>0.05$). *Id1* expression was also unchanged following activin protein injection (data not shown). These data indicate that activin proteins can upregulate hepcidin *in vivo* as well as *in vitro.*

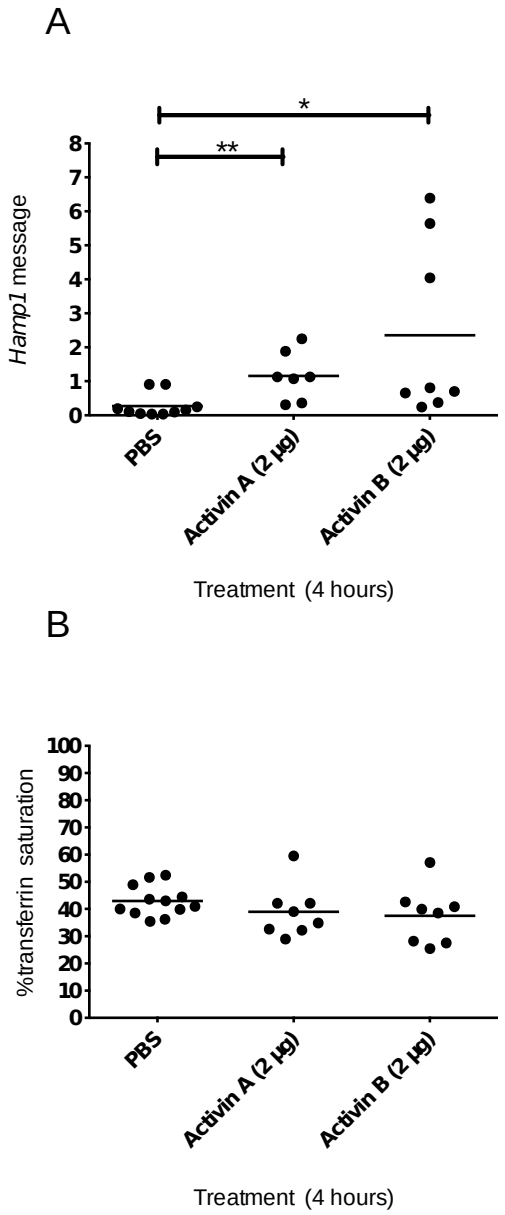

Figure 3.2. Activin A and B proteins upregulate hepcidin message *in vivo*. Iron-deprived mice were injected i.p. with activin A, activin B, or PBS only and sacrificed 4 h post-injection. Graphs depict two independent experiments combined (*n*=7-10 mice/group total). (A) *Hamp1* message was significantly increased in mice treated with either activin protein. Two mice with very high hepcidin measurements were excluded from the control group, one was excluded from the activin A-treated group. (B) % Transferrin saturation was not altered in activin-treated mice. Each dot represents values from a single mouse. Statistical analyses in dot plots are Dunn's multiple comparisons tests after Kruskal-Wallis test. *p<0.05, **p<0.01.

Hepcidin and the manipulation of activin-binding protein follistatin

Follistatin is a high-affinity activin binding protein [144, 166]. If activins contribute significantly to hepcidin control under homeostatic conditions, mice with constitutively lowered follistatin levels might be expected to exhibit lower hepcidin expression and increased serum iron parameters. Liver gene expression and serum % transferrin saturation were measured in follistatin-haploinsufficient mice as compared to their wild-type littermates (Figure 3.3). *Hamp1* and *Id1* expression were unchanged in $Fst^{-/+}$ mice (Figure 3.3A-B, both Mann-Whitney unpaired t tests, both $p>0.05$). The liver-expressed Smad2/3 responsive gene *Serpine1* was also not altered (Figure 3.3C, Mann-Whitney unpaired t test, $p>0.05$), indicating that canonical activin signaling likely is not affected in the liver in this model. % Transferrin saturation was also unchanged (Figure 3.3D, Mann-Whitney unpaired t test, $p>0.05$). As *Serpine1* was unchanged, these data did not provide sufficient information to ascertain whether this model is representative of increases in circulating activin proteins.

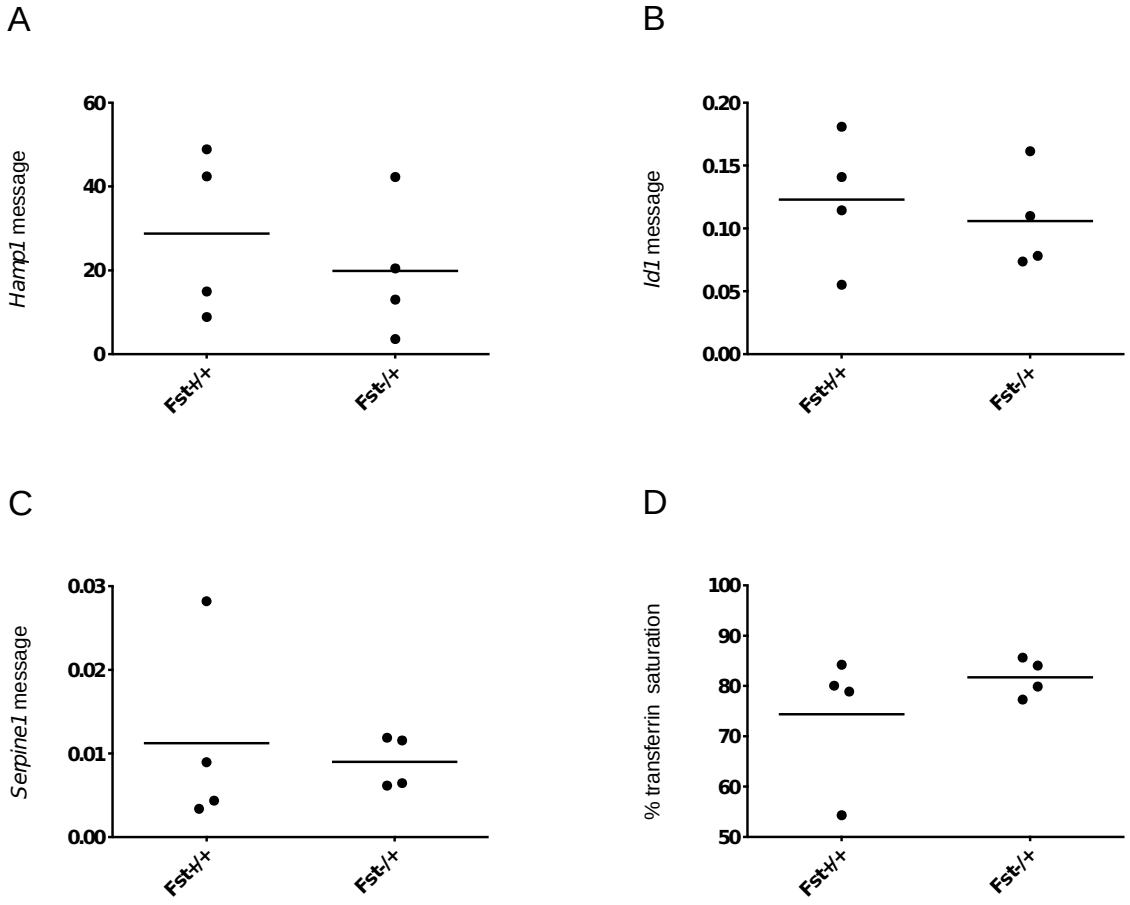

Figure 3.3. **Basal hepcidin expression is not altered in follistatin haploinsufficent mice.** Gene expression was measured in livers of follistatin haploinsufficient ($Fst^{-/+}$, n=4) mice and their wild-type littermates ($Fst^{+/+}$, n=4) and serum was analyzed for % transferrin saturation. *Hamp1* (A), *Id1* (B), and *Serpine1* (C) expression all remained unchanged between genotypes. % Transferrin saturation (D) also showed no change. Statistical comparisons in dot plots are unpaired Mann-Whitney tests.

As we were unable to probe the contributions of follistatin to hepcidin expression using a haploinsufficient mouse model, we explored the effects of treating wild-type mice with exogenous follistatin. Follistatin exhibits several isoforms, including follistatin-288, follistatin-305, and follistatin-315. Of these, follistatin-288 is thought to exist mostly in tissues and to be primarily responsible

for autocrine signaling, while the longest 315 isoform circulates and binds activin proteins systemically [177].

Most commercial sources of follistatin only produce the follistatin-288 protein isoform, but this protein isoform is thought to be quickly cleared from mouse circulation, likely serving as a poor inhibitor of serum activin activity (A. Schneyer, personal communication). We therefore utilized follistatin-315 protein produced by our collaborators K. Zumbrennan-Bullough, J. Babitt, and A. Schneyer. A series of experiments was performed to directly compare the efficacy of commercially available follistatin-288 with lab-produced follistatin-315. HepG2 cells were treated with activin A, activin B, or BMP6 (as a positive control), and increasing concentrations of either R&D follistatin-288 or Babitt/Schneyer lab follistatin-315. *HAMP*, *ID1*, and Smad2/3 response gene *SERPINE1* were measured.

Both isoforms of follistatin efficiently inhibited the upregulation of all three response genes by activin A or B administration (Figure 3.4 A-F). Neither isoform of follistatin, at any concentration, caused any changes to *HAMP*, *ID1*, or *SERPINE1* expression in untreated cells, which could be interpreted as an indication that activins do not contribute significantly to baseline hepcidin expression in cell culture. Follistatin-315 (right-hand graphs) appeared to be slightly more effective, mole for mole, in its inhibition of activin A than follistatin-288 (left-hand graphs). 1 nM Activin A's effects on all three genes were blocked with 3 nM follistatin-288; only 1 nM follistatin-315 appeared to be required for the same effect. Both follistatin isoforms inhibited the response to treatment with 1 nM activin B at 3-9 nM. The upregulation of *HAMP* and *ID1* by BMP6 (Figure 3.4A-D) was not appreciably

inhibited by either follistatin isoform, BMP6 did not upregulate *SERPINE1* (Figure 3.4E-F). In brief, both commercially available follistatin-288 and follistatin-315 obtained from our collaborators could effectively block hepcidin upregulation by activin proteins *in vitro.* Several batches of follistatin-315 were produced for the purposes of this project, and each was tested for *in vitro* effectiveness as in Figure 3.4 before use in mice (not shown).

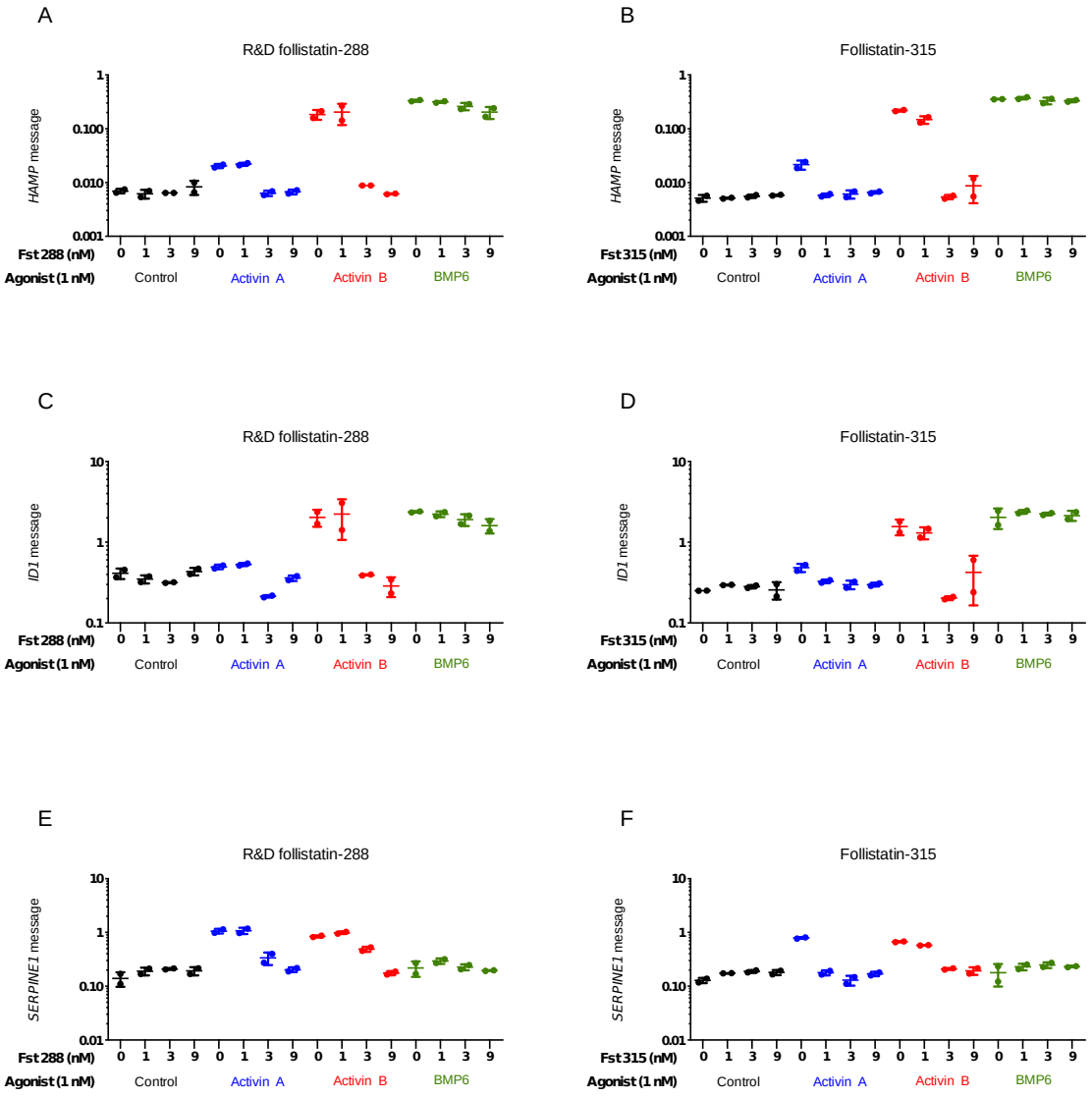

Figure 3.4. Activin-binding protein follistatin prevents the upregulation of *HAMP* and *ID1* in response to activin proteins *in vitro*. HepG2 cells were treated for 4 h with activin A, activin B, or BMP6 (all at 1 nM). Activin-treated media was pre-incubated with 0, 1, 3, or 9 nM of follistatin (Fst)-288 protein (left-hand graphs), or follistatin-315 protein (right-hand graphs). *HAMP* upregulation by activin A and B proteins (A-B) was blocked by both follistatin-288 and follistatin-315 at low molar ratios. Follistatin had no effect on *HAMP* expression in control cells that were not treated with activins. (C-D) *ID1* upregulation was similarly prevented by follistatin at low molecular ratios. *SERPINE1* is increased by both activin proteins but not by BMP6; the activin-mediated increase in *SERPINE1* is also prevented by follistatin treatment (E-F). Each dot represents a biological duplicate from a representative experiment.

After demonstrating that follistatin-315 was effective in the prevention of hepcidin upregulation by activins *in vitro*, we then sought to characterize the effects of administering follistatin-315 to mice. Mice were injected with 2, 4, or 10 µg follistatin-315 i.p. in a pilot titration experiment and sacrificed 4 h later. Follistatin injection had no clear effects on *Hamp1* (Figure 3.5A, Dunn's multiple comparisons test, $p>0.05$ for all comparisons) or *Id1* expression (Figure 3.5B, Dunn's multiple comparisons test, $p>0.05$ for all comparisons), but *Serpine1* was significantly decreased in the 10 µg follistatin-315 treated mice (Figure 3.5C, Dunn's multiple comparisons test, $p<0.05$ for 10 µg follistatin group compared to controls). % Transferrin saturation was not significantly altered in any group (Figure 3.5D, Dunn's multiple comparisons test, $p>0.05$ for all comparisons). These data indicate that follistatin-315 is at least partially efficacious in blocking the basal activity of activin proteins, as shown by the inhibition of *Serpine1.* However, outside the context of inflammation and infection, activins may not contribute significantly enough to hepcidin control for their inhibition to modulate hepatic hepcidin expression or % transferrin saturation.

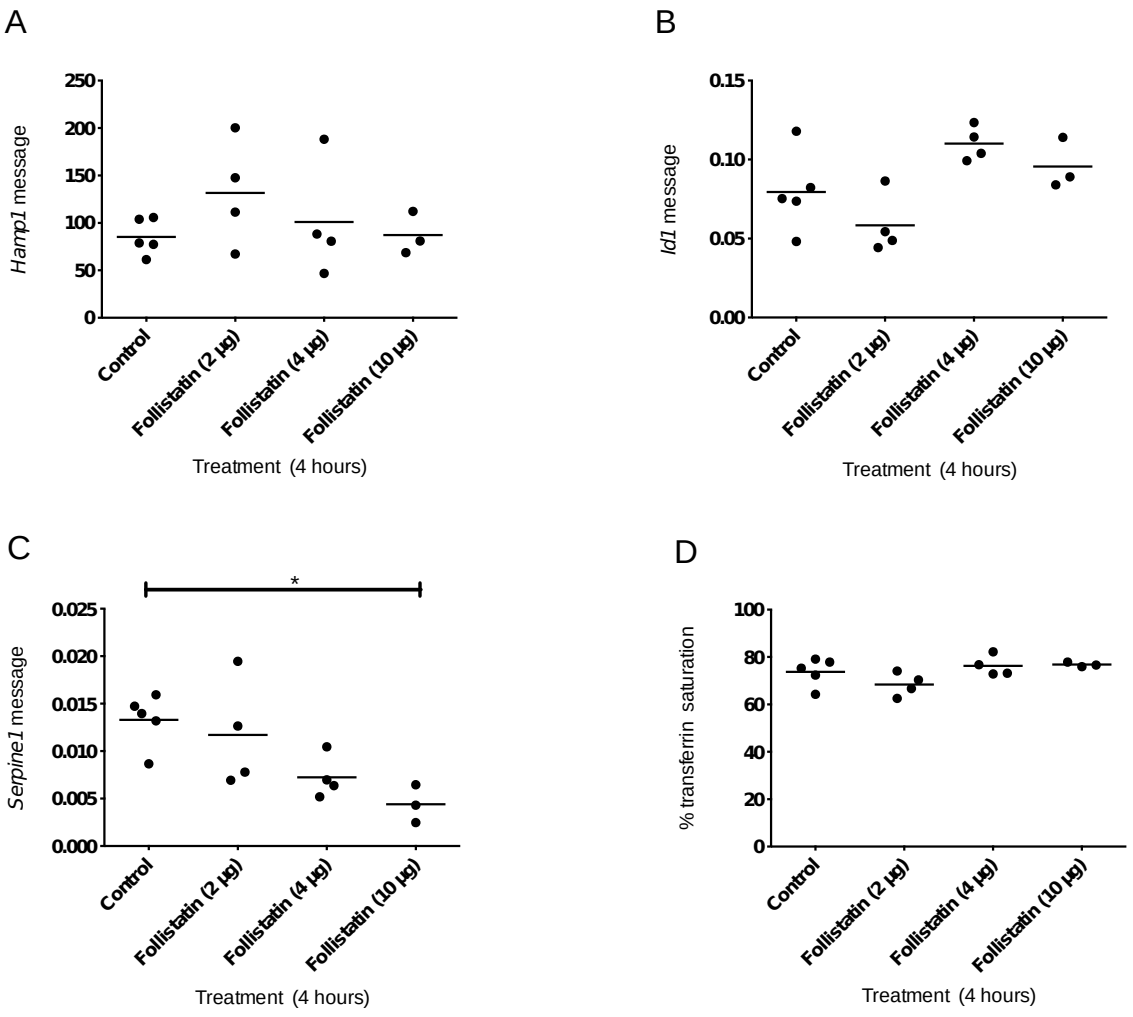

Figure 3.5. Increasing doses of follistatin in mice repress *Serpine1* expression but do not impact *Hamp1* or *Id1* expression, or % transferrin saturation. Mice were injected i.p. with 2, 4, or 10 µg follistatin-315 or PBS only and harvested four h post-injection. Liver *Hamp1* (A) and *Id1* expression (B) were unchanged. *Serpine1* expression (C) was significantly decreased in the 10 µg follistatin-315 group. No change was noted in % transferrin saturation (D). Statistical analyses are Dunn's multiple comparisons tests after Kruskal-Wallis test. *p<0.05.

A time-course experiment was also performed to see how long the effects of a follistatin injection on *Serpine1* persisted in mice. Mice were injected with the highest follistatin-315 dose tested in the titration experiment and harvested 4, 12, or 24 h post-injection. The number of mice used in this experiment was necessarily

small due to limited reagent availability. *Hamp1* was not significantly decreased at any time-point (Figure 3.6A, Mann-Whitney unpaired t tests, all $p>0.05$) in agreement with the findings of the titration experiment. *Id1* showed a trend towards decreased expression in follistatin-treated mice at 24 h, but this was not significant (Figure 3.6B, Mann-Whitney unpaired t tests, all $p>0.05$). *Serpine1* showed a non-significant decrease in follistatin-treated mice at 24 h (Figure 3.6C, Mann-Whitney unpaired t tests, all $p>0.05$). % Transferrin saturation showed a trend towards a decrease at 24 h post-injection (Figure 3.6D, Mann-Whitney unpaired t tests, all $p>0.05$). No changes were significant, although detection of changes was challenging due to the very limited number of mice. Notably, *Hamp1, Id1,* and *Serpine1* all appeared to have altered expression in both control and follistatin-treated groups at different time-points, but expression of all three genes returned to close to baseline at 24 h, possibly indicating the presence of a diurnal rhythm in gene expression.

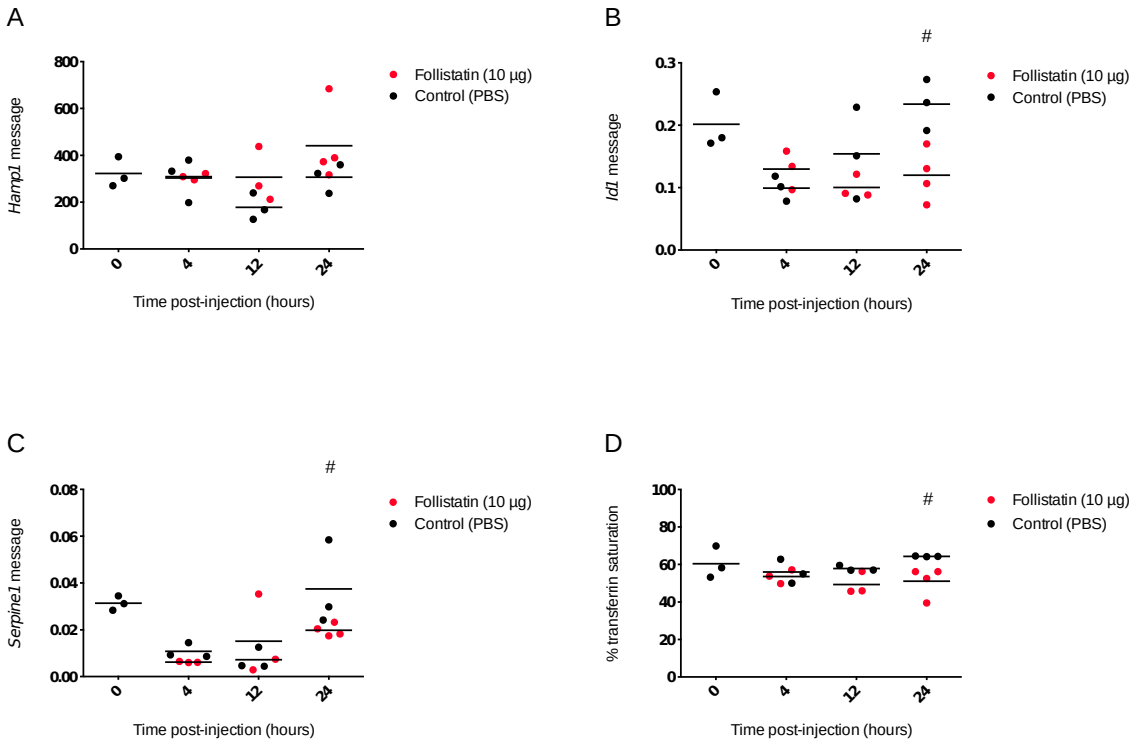

Figure 3.6. Characterization of the effects of a follistatin injection over time in mice. Mice were injected i.p. with 10 µg follistatin or PBS only and sacrificed at 0, 4, 12, or 24 h post-injection. Liver *Hamp1* (A) was not significantly different between treated mice and controls at any measured timepoint. (B) *Id1* showed a trend towards decreasing in follistatin-treated mice at 24 h post-injection., as did *Serpine1* expression (C). % Transferrin saturation (D) was slightly but not significantly decreased in follistatin-treated mice at 24 h. All statistical comparisons are unpaired Mann-Whitney tests comparing controls with treated mice at the same timepoint. # p=.06

To test the hypothesis that activins might be responsible for hepcidin upregulation in malaria-infected mice, follistatin-315 was serially injected into infected mice to attempt to block activin signaling and downstream hepcidin upregulation. Based on the equivocal data from the time-course and titration pilot experiments, we decided to maximize the follistatin dose per injection. Mice were therefore injected with the highest dose of follistatin that was feasible (25 µg in 200 µL PBS) four times, with injections at 12-h intervals, beginning on the morning of

day 6 post-infection and concluding in the evening of day 7 post-infection. As shown in Chapter 2 (Figure 2.1), hepcidin and *Id1* in infected mice increase on day 8 post-infection in this model. In this experiment, all mice were harvested at the same day 8 time-point, 12 h after the last dose of follistatin or control PBS.

There was no significant difference in parasitemia between follistatin-treated and control mice, although there was a trend towards increased parasitemia in follistatin-treated mice (Figure 3.7A). Hepatic expression of *Hamp1*, *Id1*, *Serpine1*, and *Inhbb* were all unchanged between follistatin-treated and control infected mice (Figure 3.7B-E, all Mann-Whitney unpaired t tests, all $p>0.05$). The lack of change of *Hamp1* and *Id1* indicate that any effect of activins on Smad1/5/8 signaling are not inhibited by follistatin. Furthermore, as *Serpine1* (representative of pSmad2/3 signaling) also showed no significant difference between follistatin-treated and untreated infected mice, it was difficult to discern whether these data indicated that follistatin-315 did not prevent hepcidin upregulation in this model of malaria infection, or that follistatin-315 was not biologically active *in vivo* in this experiment at all. In light of this uncertainty, myostatin (*Mstn)* expression was measured in calf muscle as an extra control. Myostatin is increased by activin action and decreased by follistatin [172], and was also unchanged between follistatin-treated and control mice (Figure 3.7E, Mann-Whitney unpaired t test, all $p>0.05$). Furthermore, because of the preliminary nature of this experiment, infected mice were not harvested at any time except at the conclusion of the experiment; hence, it cannot be formally shown that hepcidin, *Id1*, and *Inhbb* were upregulated in this particular experiment. In conclusion, although this study was intended to block activin signaling *in vivo*

during a malaria infection, the results were challenging to interpret. Further studies should attempt to block activins' actions in malaria infection using alternative methods to serial follistatin injection.

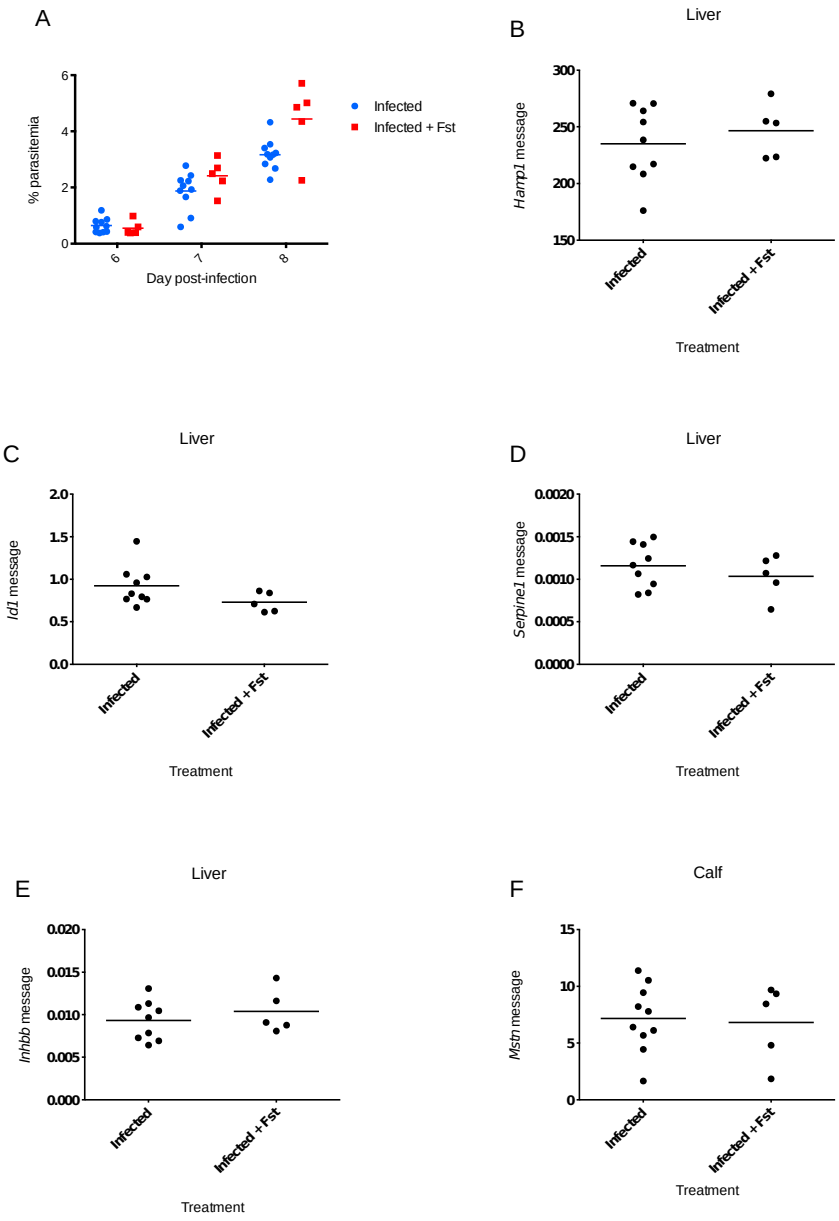

Figure 3.7. Follistatin treatment does not alter hepcidin expression during malaria mouse infection. Mice were injected with 25 µg follistatin (or an equivalent volume of PBS) every 12 h from day 6 of malaria infection to harvest for a total of 4 injections. (A) % Parasitemia does not differ significantly between follistatin-treated and control infected mice. Liver expression of *Hamp1* (B), *Id1* (C), *Serpine1* (D), and *Inhbb* (E) were unchanged. (F) Myostatin (*Mstn)* from calf was also tested as a response to activin signaling, and was also unchanged. Statistical comparisons in dot plots are unpaired Mann-Whitney tests.

3.4. Discussion

This work characterizes the effects of activin A and B proteins and their binding protein, follistatin, on hepcidin expression in cell culture and in both malaria-infected and non-malaria infected mice. Both activin proteins induce increases in *HAMP* message in human hepatoma cells. Prevention of this effect by pre-incubation with LDN indicates that activin proteins are likely acting through the canonical Bmp receptors Alk2 or Alk3, in agreement with Besson-Fournier et al (2012), who also showed that Alk3-Fc, but not Alk2-Fc, could also block hepcidin upregulation by activin B [93]. That activin A and activin B can signal through Alk3 (and, possibly, Alk2) to increase hepcidin and *Id1* message, presumably through pSmad1/5/8, is a new and potentially important finding. Canonically, activins are though to signal through Alk4/5/7 to induce pSmad2/3 signaling, and Bmp proteins to signal though Alk2/3/6 to induce pSmad1/5/8 signaling. However, this prevailing view has been challenged by several recent studies that show that Bmp proteins can act through Alk4/5/7 to stimulate pSmad2/3 under certain conditions [178, 179]. It seems likely that the effects of different ligands on Smad2/3 and Smad1/5/8 signaling pathways are more complex and overlapping than previously thought. More work is needed to explore the effects of Bmp and activin proteins in different cell types, and to elucidate what combination of factors determine which signaling pathways are activated by different ligands during different states of development, physiology, and pathophysiology.

Activins' effects on hepcidin were extended to an *in vivo* model by the injection of activin proteins into iron-deprived mice. Both activin A and activin B

protein produced a significant upregulation of murine hepatic hepcidin expression. To our knowledge, this is the first study to show an effect of exogenously administered activin proteins on hepcidin expression *in vivo*.

No significant changes were observed in either *Id1* expression or % serum transferrin in response to activin treatment in mice. However, due to the prohibitively high cost of commercial reagents, time-course and titration experiments were not feasible, which might have permitted optimization of this system and perhaps observation of greater effects on both gene expression and % transferrin saturation.

The second part of the work presented in this chapter explores the consequences of manipulating levels of the activin-binding protein follistatin both *in vitro* and *in vivo*. Follistatin haploinsufficient mice had no obvious basal differences in hepcidin or *Id1* expression, although the fact that the activin-responsive Smad2/3 indicator gene *Serpine1* was also unchanged in these mice left some doubt as to whether decreased hepatic activin signaling truly occurs in this mouse model.

We therefore began experiments with exogenous follistatin, and demonstrated that both the commercially available follistatin-288 isoform and collaborator-produced follistatin-315 efficiently block the upregulation of *HAMP, ID1,* and *SERPINE1* by activins A and B *in vitro* at similar molecular ratios. Follistatin alone had no effect on gene expression, and showed very little effect on BMP6-stimulated *HAMP* and *ID1* increases, showing that in this *in vitro* system, follistatin's activities are specific to blocking activins.

We then sought to characterize the effects of exogenous follistatin on hepcidin *in vivo.* As follistatin showed no effect on hepcidin expression *in vitro* in the absence of activin treatment, it was a challenge to ascertain whether the follistatin injected into mice was biologically active if no effects on hepcidin or *Id1* was observed. *Serpine1* was measured in murine livers as an indicator for Smad2/3 signaling, and was slightly but significantly decreased in the highest-dose group in a follistatin titration experiment, perhaps indicating that baseline activin signaling through Smad2/3 were inhibited by follistatin. However, in subsequent studies, no clear decreases in hepcidin, *Id1,* or *Serpine1* were noted, although necessarily small group numbers due to restricted follistatin availability may have obscured any differences.

Interestingly, changes were noted in *Hamp1*, *Id1*, and *Serpine1* expression during a time-course experiment that appeared to be related to the time of day at which mice were sacrificed. A search of the literature suggests that this effect is a consequence of naturally occurring diurnal rhythms, not technical problems. Diurnal rhythms are known to affect hepcidin protein levels in humans [180], while serum levels of the protein product of *Serpine1*, plasminogen activator inhibitor-1, exhibit a similar pattern [181]. Limited data is available on the possibility of a diurnal rhythm in *Id1,* but it is known to show diurnal variation in the pineal gland of the rat at the message level [182], which provides weak evidence to support the plausibility of our observation.

We attempted to use follistatin to block the upregulation of hepcidin in a mouse model of malaria infection. Mindful of the marginal results observed in

previous follistatin experiments, we sought to maximize the follistatin administered per mouse throughout malaria infection: resulting in the generation of a protocol involving four injections of 25 µg follistatin-315 to each malaria-infected mouse, as opposed to the previous 10 µg dose. Despite the increased dose of follistatin, however, no clear changes were observed in *Hamp1*, *Id1*, *Inhbb,* or *Serpine1* expression in infected and follistatin-treated mice versus infected mice only. Expression of *Mstn* in the calf muscle was also tested as an alternative Smad2/3-responsive gene, and no change was noted. The lack of effect in either Smad1/5/8 or Smad2/3-responsive genes in this experiment made the results difficult to interpret: it was hard to know if follistatin-315 was exerting effective biological activity.

One interesting finding from these studies is the clear difference between the type I BMP receptor inhibitor LDN and the activin-binding protein follistatin on basal hepcidin expression in cells or mice. LDN causes a reduction in baseline hepcidin expression in cell culture, as shown in Figure 3.1 and previously reported in the literature [95]; a similar effect on hepcidin baseline has been noted in rodent models [95, 183]. In sharp contrast, although follistatin clearly inhibited the activins' effects on gene expression, follistatin administration alone had no effect on hepcidin message in either cell culture or mouse models, even in the single mouse experiment in which a clear effect was observed on the Smad2/3 response gene *Serpine1*. Additionally, no difference in baseline hepcidin values was observed in follistatin-haploinsufficient mice as opposed to their wild-type littermates. These results indicate to us that basal signaling through BMP Type 1 receptors plays a vital

role in homeostatic control of hepcidin expression, while activin proteins likely do not affect hepcidin expression out of the context of inflammation or infection.

In summary, both activin A and activin B significantly upregulate hepcidin and *ID1* in cell culture, and both upregulate hepcidin gene expression in mice. These data support the hypothesis that activins may act *in vivo* to alter hepcidin expression during certain circumstances, including malaria infection. We attempted to use the activin-binding protein follistatin to interfere with activin-modulated hepcidin expression in both uninfected and malaria-infected mice, but were unable to show clearly that the available follistatin protein consistently affected gene expression in mice.

Future experiments should take alternative approaches to the manipulation of activin levels in mouse models of malaria infection. Options include infection (with appropriate littermate controls) of activin A conditional knockout mice (likely to be available soon, H. Drakesmith, personal communication) or a mouse model that constitutively overexpresses follistatin [174]. Follistatin could also be increased via a different method than serial injection, such as overexpression by viral vector. Alternatively, a recently described activin-binding fusion protein, sotatercept (ACE-011) represents a promising alternative to follistatin in this context.

Sotatercept was first described in the context of bone mineralization studies [184]; the sotatercept murine homolog RAP-011 was shown to be efficacious in improving bone mineral density in mice. When these studies were extended to healthy adult women, it was found that as well as improving markers of bone formation, volunteers treated with sotatercept also experienced a transient increase

in hemoglobin, red cell numbers, and hematocrit [185], leading to the supposition that sotatercept might also show erythropoiesis-stimulating agent (ESA) activity through blocking activins' actions.

In mice, RAP-011 was then shown definitely to act as an ESA, an effect that may be partially modulated by the upregulation of erythropoietin (EPO) [146]. RAP-011 was also shown to improve hematological parameters in a mouse model of β-thalassemia, possibly by inhibition of the TGFβ family member growth differentiation factor 11 (GDF11), which is overproduced in β-thalassemia mouse models and patients, and causes ineffective erythropoiesis [186]. The direct effects of sotatercept/RAP-011 on hepcidin, presumably through the inhibition of activins, have not yet been examined, to our knowledge.

In short, the safety of sotatercept has been demonstrated in human clinical trials, and evidence from human studies and from mouse models suggests that sotatercept may improve erythropoiesis. RAP-011, in place of follistatin, should be tested in the context of murine malaria infection to delineate any effects on activin-mediated hepcidin production. Hepcidin inhibition is known to improve responses to ESA [72]. Sotatercept's dual actions as both an ESA and as a hypothesized repressor of hepcidin in the context of malaria infection could make this compound a doubly active agent in improving hematological recovery from malarial anemia.

CHAPTER 4: HEPCIDIN AND ACTIVIN A PROTEINS CO-INCREASE IN THE SERUM OF VOLUNTEERS UNDERGOING CHMI

4.1. Introduction

The first deliberate infections of humans with malaria occurred in the early 1900s, when *Plasmodium vivax* infection was used as a treatment for patients with neurosyphilis [187]. If the patients survived acute illness with the protozoan parasite, the "malaria therapy" could result in the elimination of the – without modern antibiotics - fatal bacterial infection. For this discovery, the controversial physician Julius Wagner-Jauregg was awarded a Nobel Prize in 1927. The therapeutic mechanisms involved remain less than fully understood, but the generation of high fevers was likely involved [188].

In more recent years, CHMI clinical trials with *Plasmodium falciparum* sporozoites have been well established as a safe and effective method to test the efficacy of new vaccine candidates in Phase IIa clinical trials [189]. Data presented in Chapters 2-3 indicate a possible role of activins in hepcidin upregulation in malaria infection. This chapter extends these studies into human volunteers by studying the changes in hepcidin, activin A, and markers of iron status and inflammation in unvaccinated CHMI volunteers used as parasite infectivity controls.

There are several advantages to studying hepcidin changes in this system rather than in natural infections. Hepcidin integrates signaling from iron stores and erythropoietic drive as well as inflammation and infection [73]. In populations with

a high burden of anemia and/or coinfections, these conflicting signals may complicate efforts to study the proximate effects of malaria infection. By utilizing data from volunteers, much of this variation can be eliminated. Additionally, parameters from pre- and post-infection from the same individual can be directly compared to measurements during parasite infection. With this detailed approach, we were able to directly compare changes in hepcidin with changes in activins during human malaria infection.

Only one previous study of hepcidin in CHMI has been carried out: de Mast et al (2009) measured hepcidin in five individuals undergoing CHMI, also as controls for a vaccine trial. The authors found that serum hepcidin increased two days after volunteers were formally diagnosed with malaria infection and started on courses of antimalarial drugs. At the same point, volunteers showed a pronounced decrease in % transferrin saturation and increases in ferritin and IL-6 [66]. As most changes were noted after antimalarial treatment was started, these results left some uncertainty as to whether the hepcidin increase resulted from the *P. falciparum* infection itself, or from the inflammatory stimulus of debris from dead and dying parasites following antimalarial treatment.

Comparison between our study and previous work is slightly complicated by the use of different hepcidin measurement methods. Several commonly-used methods exist for the measurement of serum or plasma hepcidin protein levels. This study utilizes a commercially available enzyme immunoassay (EIA) produced by Bachem, which has been previously reported as a robust tool for hepcidin measurement [70, 73, 190]. In contrast, de Mast et al (2009) measured hepcidin

levels in human serum by a combination of weak cation exchange chromatography and surface-enhanced laser desorption/ionization time of flight mass spectrometry [66], a technique also described elsewhere [191]. In an attempt to standardize the multiple hepcidin detection methods currently in use by different laboratories, two 'round robin' studies have been performed to directly compare available assays, by using them to measure the same standard samples [192, 193]. These comparative studies have shown that although the many methods used to detect hepcidin protein tend to show the same *relative* patterns (identifying the same samples as high or low-hepcidin), the absolute values of hepcidin each method determines for identical samples can vary [192, 193]. Hence, although we hypothesized we might see similar patterns of hepcidin protein upregulation as reported in de Mast et al (2009), it could not be assumed that absolute hepcidin protein concentrations would be directly comparable [66].

To the best of our knowledge, no studies have been performed examining activin protein serum levels in the context of malaria infection. A report has been published describing a human enzyme-linked immunosorbent assay (ELISA) for activin B protein [162], but at the time of writing, this ELISA had not been made commercially available. However, activin A is known to increase in the serum of patients suffering from septicemia [150, 151], hepatitis C virus infection with or without concurrent schistosomiasis [152], and patients undergoing treatment for acute respiratory failure [153]. In this study, a commercially available ELISA for activin A (previously demonstrated in [151, 152]) was utilized to measure serum activin A protein levels during malaria infection.

This chapter examines, through correlative analysis, the hypothesis that increased levels of circulating activin proteins may upregulate hepcidin in malaria infection. The data details changes in iron- and inflammation-related parameters, hepcidin, and activin A protein in the sera of experimentally infected volunteers throughout CHMI trials. Hepcidin and activin A proteins show correlated increases during infection.

4.2. Materials and Methods

Trial information

Samples analyzed in this study were from 18 malaria-naïve unvaccinated volunteers from three separate UK CHMI clinical trials conducted to assess the efficacy of novel vaccines. Trials were named NCT01623557, NCT00890760, and NCT01142765, (also termed VAC045, MAL034B, and VAC039, respectively), all were registered with ClinicalTrials.gov [194-196]. Briefly, volunteers were healthy male and female UK adults aged 18-50. All volunteers gave written informed consent to participate in the CHMI studies and for their samples to be stored and used for further investigations to assess host responses and immunity to malaria. All trials were conducted in accordance with Good Clinical Practice (GCP), and the principles of the Declaration of Helsinki. Trials were approved by the Oxfordshire Research Ethics Committee. The results of each trial and details of all necessary ethical and regulatory approvals are reported elsewhere [194-196].

Experimental human malaria infection and sample collection

As detailed elsewhere [194], five *Anopheles stephensi* mosquitoes infected with *P. falciparum* 3D7 clone sporozoites were allowed to bite each volunteer. The day of infection is termed the day of challenge and abbreviated C. From day C+6.5 until C+21, volunteers were assessed once or twice daily by Giemsa-stained thick smear for the presence of blood parasites. Samples were collected at the same time-points for later analysis of *P. falciparum* parasitemia by quantitative PCR (qPCR).

Upon the detection of a parasite by thick smear (day of diagnosis, abbreviated DoD, typically also the point of maximal parasitemia), a course of treatment with Riamet or Malarone was initiated [194-196]. Larger volumes of blood were also collected at set time-points during the studies for other analyses (depicted in Figure 4.1). In all three trials, samples were collected at the day before C (C-1), at DoD, and after resolution of infection (C+35). Additionally, in NCT01623557, samples were also collected at DoD+2, two days following the diagnosis of malaria and the initiation of antimalarial treatment. In NCT01142765 and NCT00890760, the DoD+2 timepoint was not examined, but samples were taken at C+11 unless DoD had occurred prior to 11 days post-challenge.

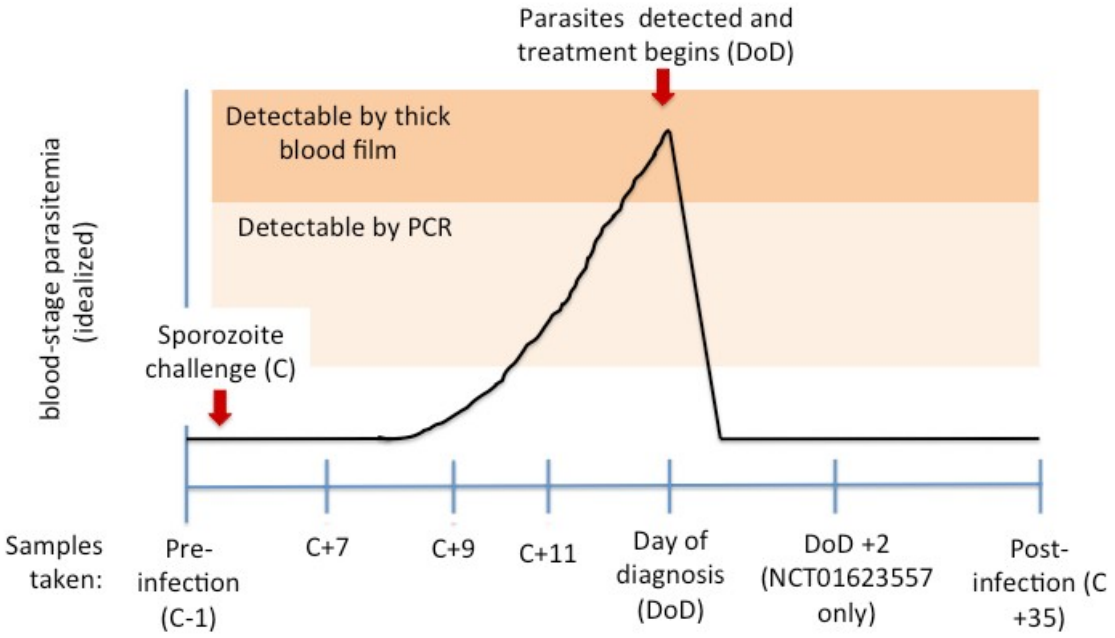

Figure 4.1. CHMI trial schematic. Volunteers are infected at day of challenge (C, red arrow). Parasites develop in the liver and emerge approximately 1 week later. Thick smears and serum samples for future parasitemia analysis by PCR are taken once to twice daily from C+6.5 until a parasite is detected by thick smear (DoD), at which point antimalarial treatment is initiated (red arrow). DoD is also the timepoint at which most volunteers experience maximum parasitemia. Retrospective analysis of parasitemia using PCR allows analysis with greater resolution: volunteers typically become PCR-positive prior to detection of parasites by thick blood smear. Further serum samples for analysis are taken at set trial timepoints.

Blood-stage parasite PCR

Full details of the qPCR are detailed elsewhere [194, 195] and were performed by members of the Draper group. Briefly, DNA was extracted from filtered whole blood using a modification of the Qiagen QIAamp Blood mini kit [194]. Taqman PCR primers targeted the *P. falciparum* 18S ribosomal gene (full primer sequences are available online [194]). qPCR was run on an Applied

Biosystems Step 1 Plus PCR system, incorporating a standard curve on each plate that allowed results to be reported as parasites/mL blood. The lower limit of detection (LOD) of the assay is 5 parasites/mL, and the lower limit of quantification of the assay is 20 parasites/mL.

% Transferrin, CRP, and ferritin measurements

Serum iron, UIBC, CRP, and ferritin in human samples were measured using an Abbott Architect cSystem Analyser by C. Webster and P. Sturges (Department of Biochemistry, Birmingham Heartlands Hospital, UK). TIBC was calculated by serum iron + UIBC. Transferrin saturation (%) was calculated by: (serum iron/TIBC) x 100%.

Hepcidin EIA

The 25-amino acid bioactive isoform of hepcidin was detected in volunteers' sera using the hepcidin-25 (human) EIA kit (Bachem). Samples were run in duplicate. A dilution series of standard hepcidin-25 was run on each plate with values ranging from 25 ng/mL - 0.05 ng/mL. Sample values were interpolated using logistic 4-parameter nonlinear curve fitting in GraphPad Prism software. Freeze-thaws of standards, samples, and reagents were kept to a minimum, and where possible all reagents for these studies were pooled, aliquoted, and frozen before use.

Samples were diluted in peptide-free human serum diluent (Bachem) at initial dilutions of 1:8. If hepcidin readings initially fell outside the linear portion of

the standard curve, samples were rerun at appropriate dilution and the new value substituted. If samples exhibited a high coefficient of variance (>15%) between duplicates, they were rerun and the new value substituted. Samples which did not produce detectable hepcidin at the lowest possible dilution (typically 1:1), were assigned a value of 50% LOD (LOD = 0.118 ng/mL), multiplied by the dilution at which they were run.

Human activin A ELISA

Activin A levels were measured using a R&D solid-phase sandwich ELISA (kit #DAC00B). The R&D protocol was followed with the following adjustment: 350 µL of wash buffer was used instead of 400 µL for the wash steps. Samples and standards were run in duplicate; samples with high coefficient of variance (>15%) were rerun on a new plate. No samples exhibited values below the LOD.

Analysis

All qRT-PCR and iron measurement data processing were performed in Excel. All figures, graphs, and statistical analysis were generated using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA).

4.3. Results

Comparing parasitemia from three trials

Data from three separate CHMI trials were included in this study to allow analysis of an adequate number of samples. Parasitemia data between trials was compared to identify any key inter-trial differences (Figure 4.2, full parasitemia data in A-C). The time that elapsed from C to DoD did not differ significantly between trials and ranged from 8 to 14 days (Figure 4.2D, Kruskal-Wallis test, $p>0.05$). Similarly, trials did not differ in time from infection to time to blood-stage patency, defined as a positive PCR measurement (≥ 20 parasites/mL, Figure 4.1E, Kruskal-Wallis test, $p>0.05$).

Blood-stage parasitemia varied between volunteers at DoD because volunteers were diagnosed using qualitative thick-smear techniques, and then parasitemia was retrospectively analyzed with the more sensitive technique of qPCR. Parasitemia at DoD varied more than two orders of magnitude between different volunteers, from a low of 90 parasites/mL in volunteer 10 (Trial NCT00890760) to a high of 47,965 parasites/mL in volunteer 6 (Trial NCT01623557). However, no significant differences were observed between parasitemia at DoD between trials (Figure 4.2F, Kruskal-Wallis test, $p>0.05$). Because intra-trial differences were larger than inter-trial differences, we were able to combine the three trials for future analyses.

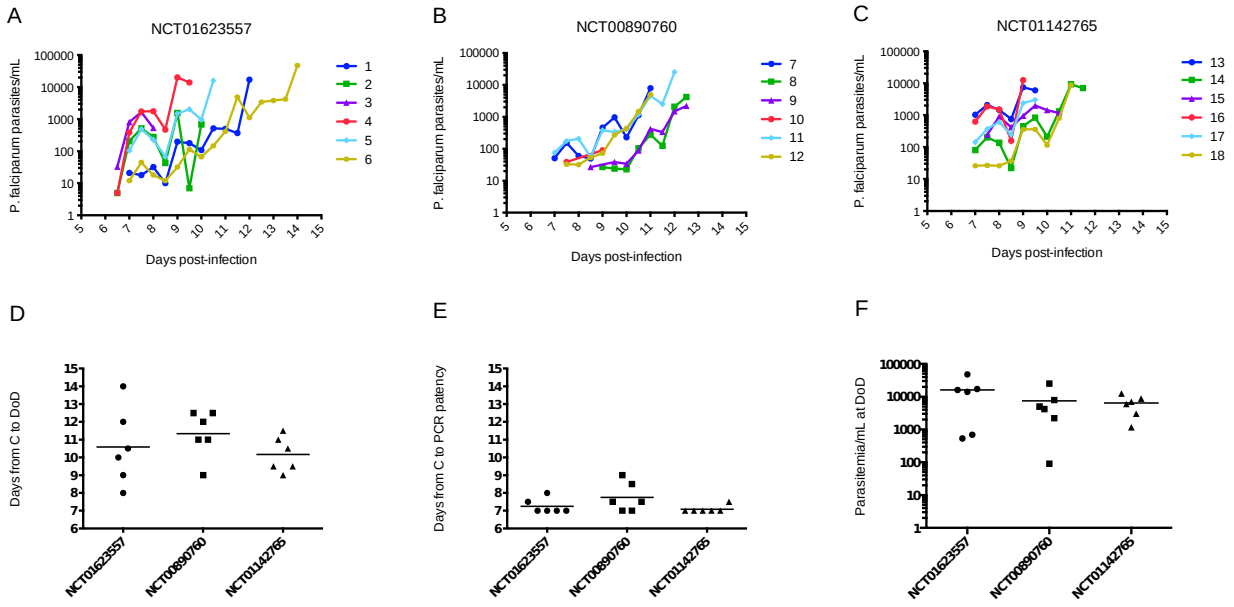

Figure 4.2. Parasitemia does not differ between three CHMI trials. A, B, and C show parasitemia as measured by PCR in trials NCT01623557, NCT00890760, and NCT01142765, respectively. Each individual volunteer was issued with a study code number and is depicted using a different color and symbol throughout all graphs. The three trials did not differ significantly in days elapsing from C to DoD by microscopy (D), days to parasitemia patency by qPCR (E), or parasitemia at DoD (F). Comparisons are Kruskal-Wallis tests.

Markers of iron status and inflammation

Iron status marker % transferrin saturation was variable between volunteers at C-1, but overall showed a decrease towards DoD (this decrease persisted to DoD+2 in NCT01623557, Figure 4.3A), and a return towards baseline values at the C+35 timepoint. The finding that % transferrin saturation decreases in experimental malaria infection of healthy volunteers is consistent with previous studies [66]. One volunteer (volunteer 17 in NCT01142765, shown in light blue in Figure 4.3C) showed very high % transferrin saturation at baseline.

Across all volunteers, % transferrin saturation was on average decreased on DoD as compared to either uninfected timepoint C-1 or C+35 (Figure 4.3D, Dunn's multiple comparisons test, $p<0.05$ for both comparisons). As sex is frequently associated with differences in iron-related parameters, we examined data to see if any baseline differences existed between male and female volunteers. Sex was not associated with significant differences in % transferrin saturation at C-1, DoD, or C+35 timepoints (Figure 4.3E, Mann-Whitney tests, all $p>0.05$).

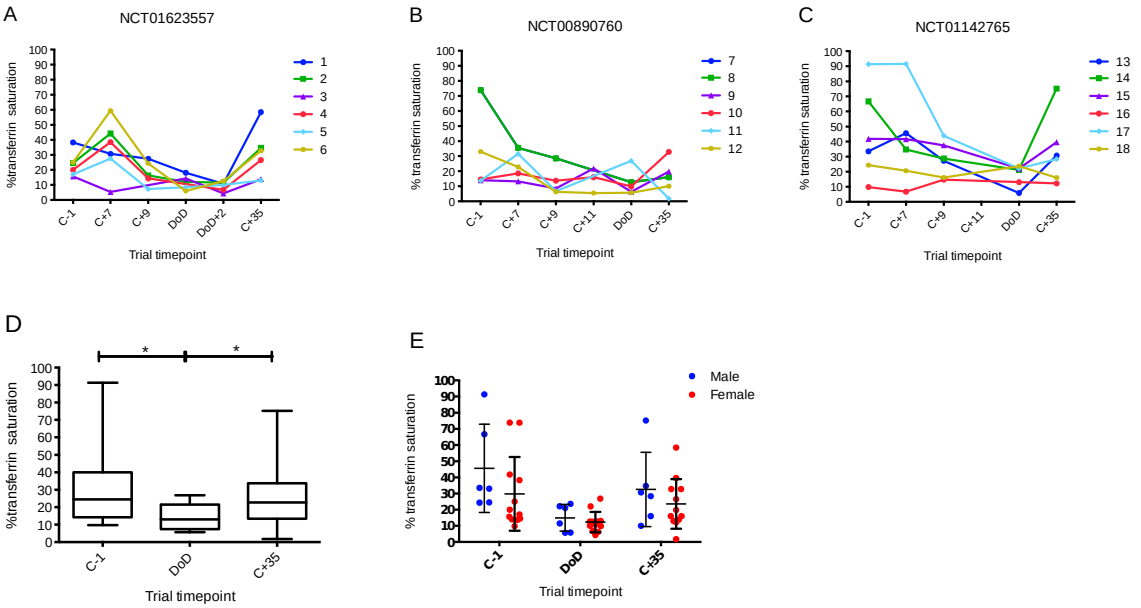

Figure 4.3. % Transferrin saturation decreases at DoD and at DoD+2. % Transferrin saturation at all available timepoints are shown for each trial (A-C). Volunteers are depicted as in Figure 4.2. (D) Overall, volunteers exhibited a significant drop in % transferrin saturation at DoD as compared either to pre- or post-infection levels. (E) % Transferrin saturation did not differ significantly between male (n=6), and female (n=12) volunteers before or after infection or at day of diagnosis. Statistical comparisons in box and whiskers plot are Dunn's multiple comparisons tests after Friedman test. Statistical tests in male-female comparison are unpaired Mann-Whitney tests for males and females at each timepoint.* $p<0.05$.

Finally, we compared the change (hereby abbreviated Δ) in % transferrin saturation at DoD with the parasitemia level experienced by each volunteer at DoD (Δ = [value at DoD] – average of [values at C-1 and C+35]). No correlation was noted between Δ % transferrin saturation during infection and parasitemia (Spearman's correlation, $p>0.05$, $r=0.22$, not shown).

Acute phase markers were examined throughout the trials. De Mast et al (2009) showed that ferritin, which increases during inflammation, showed a large but transient increase two days after treatment initiation (equivalent to DoD+2 timepoint in NCT01623557) [66]. Similarly, in a majority of NCT01623557 volunteers, the maximum levels of acute phase markers CRP and ferritin were observed on DoD+2, following treatment, rather than at DoD (Figure 4.4A and 4.4D, Volunteer 4 had insufficient serum available for analysis at DoD). The increases in CRP and ferritin observed in NCT00890760 and NCT01142765 (Figures 4.4B-C, 4.4E-F), which did not include samples taken at the DoD+2 timepoint, are less than those observed in NCT01623557.

Across all three trials, however, CRP was increased on DoD as compared to either C-1 or C+35 levels (Figure 4.4G, Dunn's multiple comparisons test, $p<0.05$ for both comparisons). Ferritin was increased on DoD as compared to C+35 but not C-1 (Figure 4.4H Dunn's multiple comparisons test, $p<0.001$ for DoD vs. C+35 only). These results suggest that although some increase in acute phase parameters is associated with parasitemia, a greater impact on both CRP and ferritin is observed post-treatment at DoD+2. This may be due to the inflammatory stimulus of dying parasites and debris produced after treatment. Ferritin was considerably more

variable at C-1 and C+35 than was CRP, likely reflecting variation in volunteers' baseline iron status. Indeed, ferritin level varied by sex: it was clearly higher in male volunteers at the C-1, DoD, and C+35 timepoints (Figure 4.4I, Mann-Whitney tests, all $p<0.05$). CRP levels did not vary by sex (not shown, Mann-Whitney tests, all $p>0.05$).

Neither Δ CRP nor Δ ferritin were associated with parasitemia or Δ % transferrin saturation (all Spearman's correlation, all $p>0.05$, not shown). Δ Ferritin and Δ CRP showed a trend towards correlation (Spearman's correlation, $p>0.05$, $r=0.36$, not shown), but this did not achieve significance.

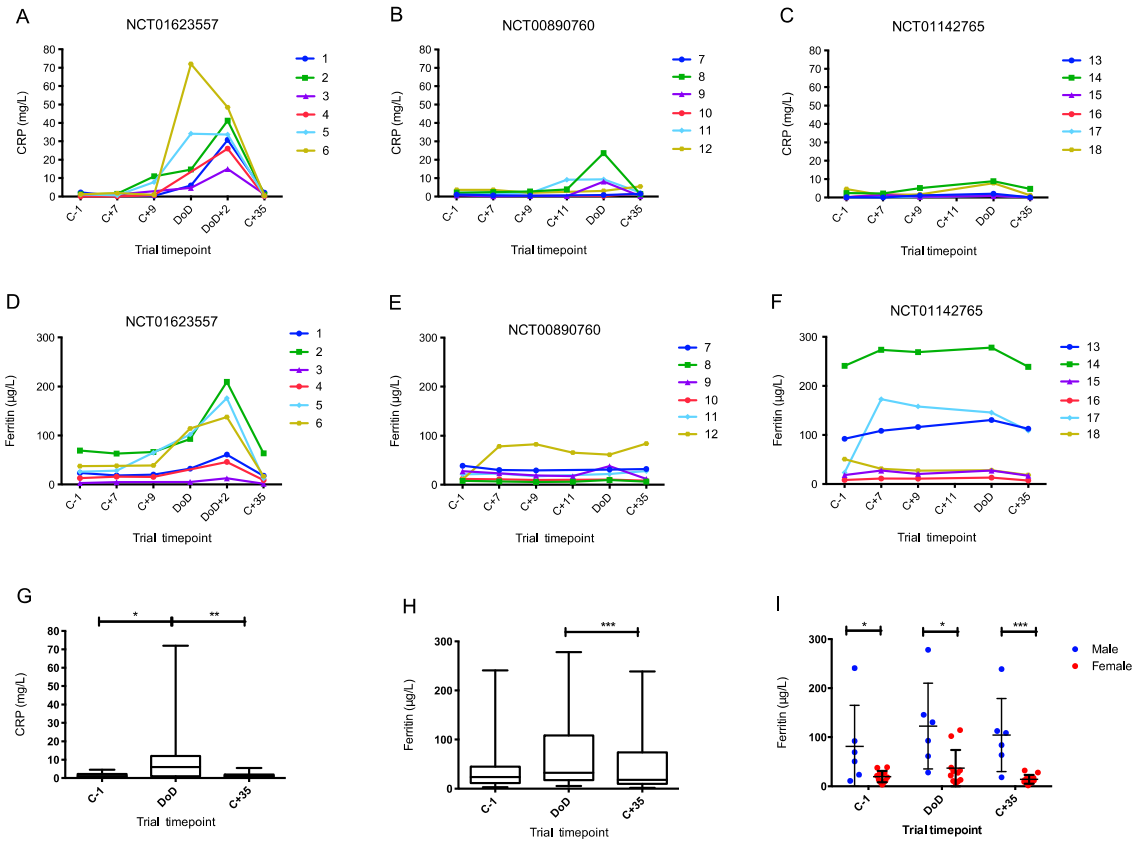

Figure 4.4. Acute phase markers CRP and ferritin increase on DoD and DoD+2. CRP (A-C) and ferritin (D-F) at all available timepoints are shown for each trial. Both CRP and ferritin increase markedly on DoD+2 in NCT01623557, less so on DoD across all three trials. Both CRP (G) and ferritin (H) are significantly upregulated on DoD overall, although ferritin is considerably more variable at baseline. One source of variability may be sex; ferritin was significantly higher in men throughout the experiment (I). Statistical comparisons in box and whiskers plots are Dunn's multiple comparisons tests after Friedman test. Statistical tests in male-female comparison are unpaired Mann-Whitney tests for males and females at each timepoint. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Hepcidin and activin A protein

Hepcidin was measured in all available serum samples using the commercially available EIA kit from Bachem. Hepcidin is variable at baseline but increases at DoD in all three trials (Figure 4.5A-C) and at DoD+2 in NCT01623557

(Figure 4.5A). Hepcidin increases overall on DoD as opposed to either C-1 or C+35 (Figure 4.5D, Dunn's multiple comparisons test, $p<0.05$ for both comparisons). Hepcidin at C-1 and C+35 showed a sex difference, with the male volunteers exhibiting significantly higher hepcidin levels than the females, but this effect lost significance during infection (Figure 4.5E, Mann-Whitney tests, all $p<0.05$ except at DoD). Δ Hepcidin was not significantly correlated with parasitemia (Spearman's correlation, $p>0.05$, $r=0.23$, not shown) or Δ % transferrin saturation (Spearman's correlation, $p>0.05$, $r= -0.18$, not shown), but was positively correlated with Δ CRP (Figure 4.5F, Spearman's test, $p>0.05$, $r=0.6$) and with Δ ferritin (Figure 4.5G, Spearman's correlation , $p>0.05$, $r=0.55$).

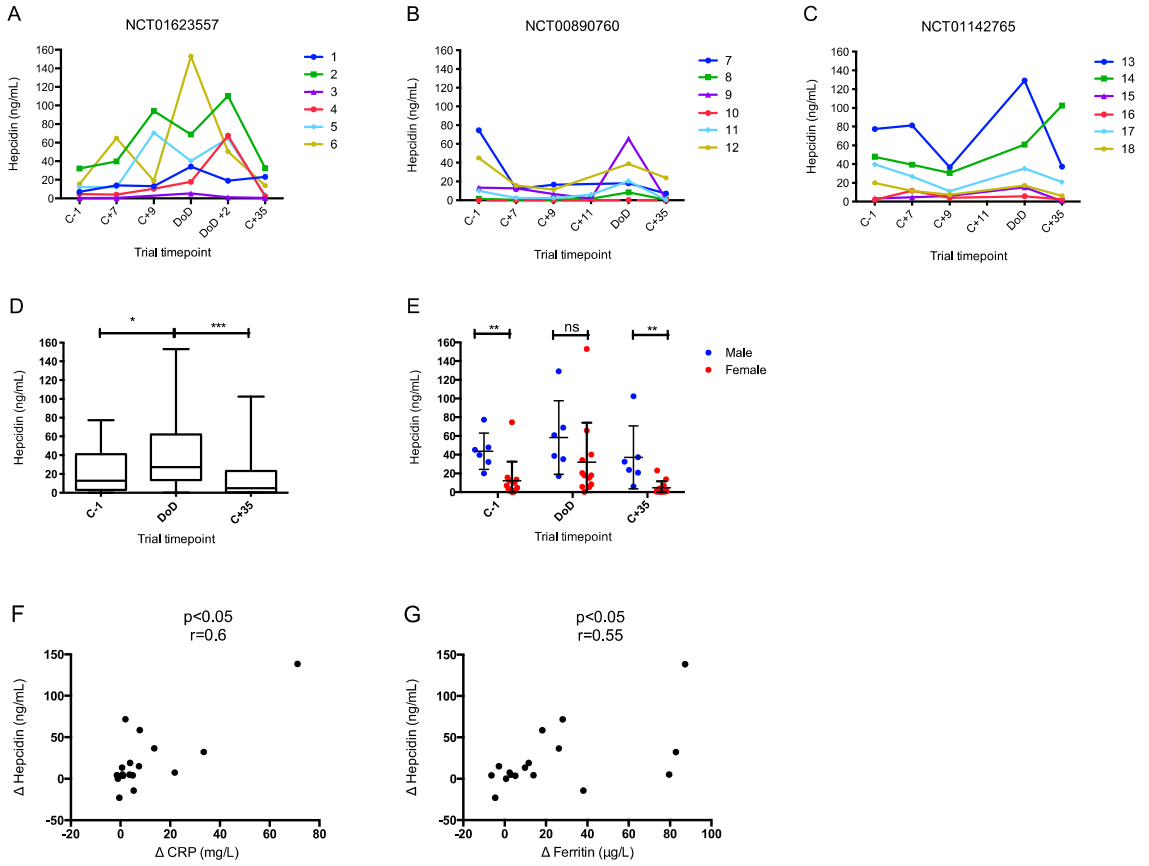

Figure 4.5. Serum hepcidin protein increases during malaria infection in volunteers and correlates with CRP and ferritin. Individual hepcidin protein measurements at all available timepoints shown for each trial (A-C). (D) Hepcidin is significantly increased on DoD over both C-1 and C+35. (E) Hepcidin in male volunteers is significantly higher than in female volunteers at C-1 and C+35 but not DoD. Δ Hepcidin is significantly correlated with acute phase markers CRP (F) and ferritin (G). Statistical comparison in box and whiskers plot is Dunn's multiple comparisons tests after Friedman test. Statistical tests in male-female comparison are unpaired Mann-Whitney tests for males and females at each timepoint. Correlation analyses are Spearman's tests. * p<0.05, **p<0.01, *** p<0.001.

In the murine model of malaria infection presented in Chapter 2, hepatic upregulation of activin A message was not noted, but hepatic message levels of activin A have specifically been shown to not associate with protein serum levels

[139, 140]. Therefore, activin A was measured in the serum of the volunteers undergoing CHMI.

Due to insufficient sera available for multiple timepoints, activin A measurements were restricted to samples from the 3 timepoints C-1, DoD, and C+35 in trials NCT00890760 and NCT01142765. Although activin A levels were variable at baseline throughout all three trials (Figure 4.6A-C), overall, activin A was increased on DoD as compared to the C+35 timepoint, and showed a non-significant trend towards an increase at DoD compared to C-1 (Figure 4.6D, Dunn's multiple comparisons test, $p<0.05$ for DoD vs. C+35 only). To our knowledge, this is the first report of any changes in activin A associated with malaria infection. Activin A showed slightly higher baseline values in male volunteers at C-1, but showed no difference on DoD (Figure 4.6E). A strong correlation was evident between Δ hepcidin and Δ activin A protein: those volunteers who demonstrated the most pronounced hepcidin increases at DoD also showed the greatest activin A increases (Figure 4.6F, Spearman's correlation, $p<0.001$, $r=0.80$). Δ Activin A was not correlated with parasitemia at DoD (Spearman's correlation, $p>0.05$, $r=0.4$, not shown) or with Δ % transferrin saturation (Spearman's correlation, $p>0.05$, $r=0.09$, not shown), showed a slight correlation with CRP (Figure 4.6G, Spearman's correlation, $p<0.05$, $r=0.54$), and was not correlated with ferritin (Spearman's correlation, $p>0.05$, $r=0.09$, not shown).

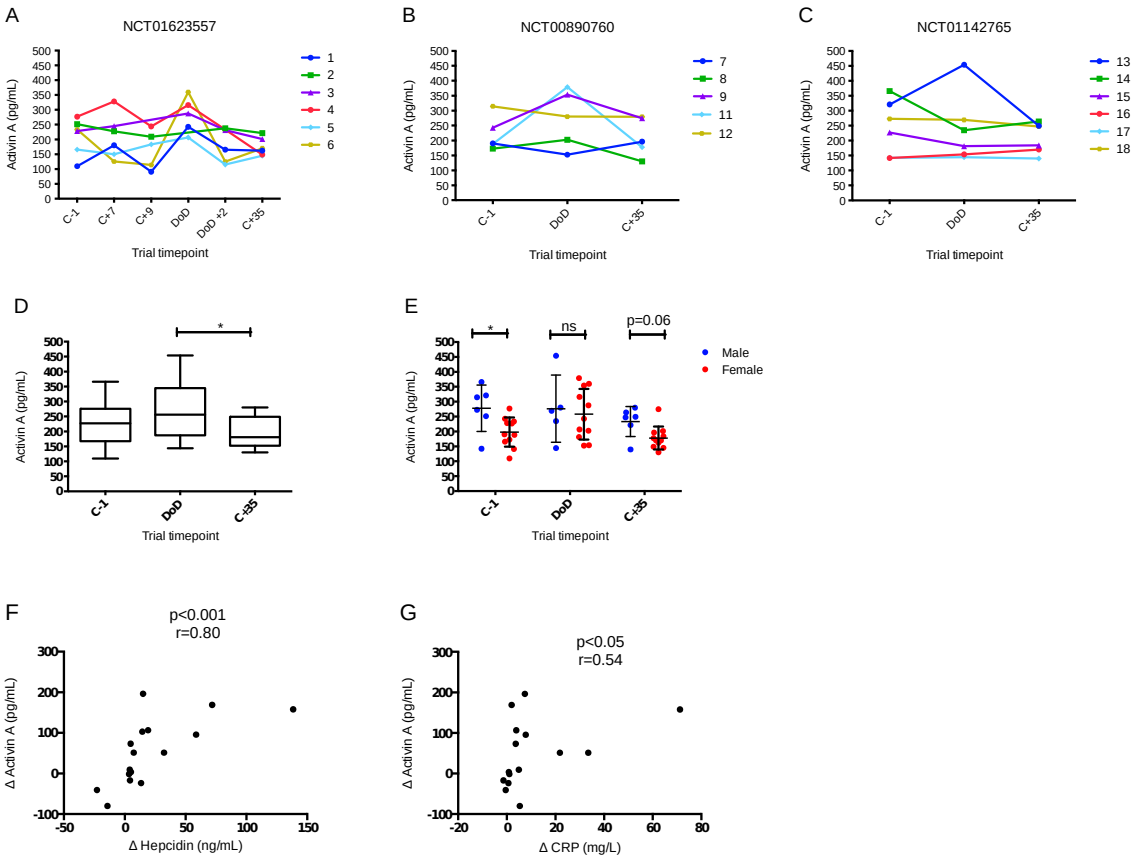

Figure 4.6. Activin A increases during malaria infection and changes in activin A and hepcidin are significantly correlated. All timepoints were measured for NCT01623557 (A) but due to shortages of serum at many intermediate timepoints, activin A was only measured at timepoints C-1, DoD, and C+35 for NCT00890760 (B) and NCT01142765 (C). One volunteer (10, NCT00890760) was omitted due to insufficient serum availability at DoD. (D) Activin A is significantly increased on DoD over C+35 and shows a trend towards increase over C-1. (E) Activin A in male volunteers is significantly higher than in female volunteers at C-1 but not other timepoints. Δ hepcidin and Δ activin A were significantly correlated (F). Δ Activin showed a correlation with Δ CRP (I), but did not correlate significantly with Δ ferritin, Δ % transferrin, or parasitemia (not shown). Statistical comparison in box and whiskers plot is Dunn's multiple comparisons tests after Friedman test. Statistical tests in male-female comparison are unpaired Mann-Whitney tests for males and females at each timepoint. Correlation analyses are Spearman's tests. * $p<0.05$.

Two male volunteers (14 and 17, both NCT01142765) exhibited very high ferritin levels across most timepoints, with levels above 100 µg/L. The same volunteers showed high % transferrin saturation at several timepoints (60-70% for

14 and over 80% for 17 pre-infection, shown in Figure 4.3C). These unusually high levels of ferritin and transferrin saturation in these two volunteers could either indicate laboratory test problems, possibly stemming from sample hemolysis (although none was observed), or be ascribed to a physiological cause such as undiagnosed hemochromatosis. Hemochromatosis is among the more prevalent of heritable genetic conditions, especially in northern European populations, and for reasons that are not fully understood, the penetrance of hemochromatosis mutations is more severe in males [197]. These two volunteers with high ferritin and transferrin saturation were both males (as mentioned above, female volunteer 8 in NCT00890760 had high transferrin saturation at baseline but no elevation of ferritin), but information was not available regarding their ethnic background. However, in a pool of 18 volunteers, including only 6 males, the presence of even one volunteer with hemochromatosis is extremely unlikely. Hepcidin between these two volunteers was markedly different: 14 exhibited one of the highest baseline hepcidin measures across all three trials, perhaps indicative of high iron stores overall, but 17 showed hepcidin levels that were unremarkable as compared to other volunteers. Although the possibility cannot be formally excluded that one or both volunteers may have had undiagnosed hereditary hemochromatosis, it is a more likely explanation that laboratory error may have occurred with regard to the ferritin and % transferrin saturation measurements; regrettably, limited sample volumes precluded the retesting of serum.

4.4. Discussion

This chapter demonstrates that in volunteers undergoing CHMI, serum concentrations of hepcidin and activin A proteins increase together. % Transferrin saturation decreased during malaria infection, as acute phase markers ferritin and CRP increase. Hepcidin was increased at both DoD and two days later, after the initiation of antimalarial drug administration. Activin A was variable at baseline but was significantly increased at DoD.

The upregulation of hepcidin at DoD somewhat contrasted with the only previous study of hepcidin in CHMI, which showed hepcidin upregulation only after administration of antimalarial drugs (equivalent to DoD+2) and hence left some formal uncertainty as to whether malaria infection itself or the treatment of malaria infection and corresponding parasite death were responsible for hepcidin upregulation [66]. Our findings conclusively show that in CHMI, upregulation of hepcidin can occur prior to the initiation of antimalarial treatment.

The change in each parameter during infection was calculated. Interestingly, the Δ changes in ferritin and CRP were not correlated, likely reflecting considerable variation in ferritin baseline due to different volunteer iron status (associated with sex, among other factors); and that this variation does not exist with regards to CRP levels. Hepcidin at DoD was correlated with Δ ferritin and Δ CRP, and most strongly correlated with Δ activin A. Previous findings (detailed fully in Chapters 2-3) suggested a role for activin proteins in hepcidin upregulation in malaria infection, and the co-increase of hepcidin and activin A in CHMI supports this hypothesis. Indeed, the moderate response we observe in volunteers infected in CHMI trials

may be more pronounced in naturally infected individuals in the field, in whom parasitemia levels can greatly exceed the low parasitemia that is achieved in CHMI.

No studies have yet been published that use the hepcidin Bachem EIA to analyze serum hepcidin values in adults, hence, there was no clear formal reference range with which to compare these findings. However, the adult female volunteers, 11/12 of whom had basal hepcidin levels ≤16 ng/mL, showed comparable hepcidin levels to those reported in a study of hepcidin in children in a malaria-endemic area prior to the malaria season (also measured by Bachem EIA, geometric mean of 11.2 ng/mL or 3.8 ng/mL, depending on study site) [73]. The 6 male volunteers exhibited notably higher hepcidin levels, ranging from 20-80 ng/mL, likely reflecting the greater iron stores of adult men. The sex difference noticed in this chapter is in keeping with previous work that describes higher ferritin and hepcidin levels in healthy men than in healthy women of menstruating age, an effect possibly due to the monthly iron cost of menstrual blood loss and the subsequent homeostatic repression of hepcidin [198].

Activin A has only been measured in healthy volunteers in a limited number of studies. Basal activin levels in healthy adults have been reported as 70 pg/mL [199], or 110 pg/mL [153], or, in an outlying study, 5,700 pg/mL [152]. This variation in normal values may reflect the use of different assays. In this study, participants had average activin A baseline values of 226 pg/mL.

A limitation of this study was our inability to assay activin B in malaria-infected human volunteers. A report has been published describing a human ELISA for activin B protein [162], but at the time of writing, this ELISA had not been made

generally available. However, in two studies using this ELISA, some correlation between activin A and activin B serum levels was noted in pathophysiologic conditions. One found that patients with acute respiratory failure showed higher levels of both activin B and activin A proteins [153], the other noted that activin A and B were correlated in patients with type 2 diabetes but not healthy controls [199]. Taken together, these findings provide weak evidence that serum activin B protein levels may increase with activin A in certain disease states. Measurement of activin B in CHMI volunteers and naturally infected volunteers is a vital next step in the analysis of the role of activins in iron control in malaria infection.

In summary, this work demonstrates that changes in hepcidin and activin A serum protein levels are correlated in volunteers undergoing CHMI infection. This is the first report of an increase in serum activin A protein in the context of malaria infection, and the first study to directly compare serum hepcidin and activin A.

CHAPTER 5. *PLASMODIUM FALCIPARUM*-INFECTED RED BLOOD CELL-DERIVED MICROPARTICLES ELICIT CYTOKINE RESPONSES FROM HUMAN WHITE BLOOD CELLS

5.1. Introduction

Hepcidin is upregulated at the message level in livers of mice infected with blood-stage malaria infection (Chapter 2 and [67, 68]) and is increased in the serum of malaria-infected humans (Chapter 4 and [63-66]). However, previous pilot experiments by this lab have shown that direct co-culture of human hepatoma cells with *P. falciparum*-infected red blood cells (iRBC) does not result in hepcidin upregulation (A. Armitage and H. Drakesmith, personal communication). Therefore, the mechanism of hepatic hepcidin upregulation in blood-stage malaria infection is likely to be dependent on increased cytokine production by one or more non-parenchymal cell types, not the direct detection of blood-stage parasites by hepatocytes. This study aims to identify the pathogen-associated molecular pattern (PAMP) that initially stimulates production of circulating cytokines that subsequently induce increased hepcidin production from the liver.

The mechanism by which white blood cells detect the presence of malaria infection is controversial. Early studies focused on correlating parasite life-stage with production of cytokines. Parasites inhabiting human red blood cells grow from ring-stages into trophozoites, and then into multinucleated schizonts, before lysing to release infectious merozoites that then infect a new wave of red blood cells.

Schizont rupture, which occurs approximately every 48 h [200], has been linked to maximal cytokine release, and has been hypothesized to cause the periodic fevers that characterize malaria infection. In one early study, authors showed that in PBMC co-cultured with infected red blood cells, peak production of TNFα protein occurred after schizont rupture [201]. More recently, schizont rupture has been implicated by studies that show that laboratory parasite strains, which often have life-cycle times that are consistently greater than or less than 48 h, produce different temporal patterns of innate immune activation that are consistent with schizont rupture driving cytokine increases [202].

If schizont rupture is linked to innate immune activation, then what PAMP is actually sensed, and by which receptor? Different groups working with murine or human parasites have implicated parasite glycosylphosphatidylinositol (GPI), an anchor of membrane proteins that is exposed subsequent to schizont rupture [203-207], parasite DNA sequence motifs [208], DNA bound to hemozoin, the product of parasite detoxification of heme [209], or DNA complexed to proteins [210, 211]. GPI is thought to be detected primarily by through membrane-associated TLR2, with a lesser role for TLR4 [204]. Conversely, parasite DNA is thought to be detected via endosomal TLR, especially TLR9 [209-211]. However, a study that treated human PBMC with parasite DNA motifs indicated that cell response was contingent on a cytosolic DNA detection pathway incorporating stimulator of interferon genes (STING), tank-binding kinase 1 (TBK1), and interferon (IFN) response factors 3 and 7 [208]. In summary, the exact parasite PAMP and host receptor combination that results in recognition of malaria infection remains under discussion. Initially, the

aim for this part of the project was to test a variety of parasite PAMP, including parasite DNA, GPI, and hemozoin, and examine which were capable of provoking hepcidin-stimulatory cytokine production from PBMC. Following numerous technical setbacks in pilot experiments, however, we chose to focus in on a particular class of PAMP that were relatively straightforward to isolate and study: microparticles.

Microparticles are circulating membrane-bound entities (≤1 μm in diameter) that are formed by vesiculation of plasma membranes (reviewed in [212]). The characteristics of microparticles are determined by their cellular source; microparticles can come from a variety of cells, including red blood cells and platelets (reviewed in [213]). Conversely, exosomes are smaller (30-150 nm in diameter) and are derived from late endosomes [214, 215].

Serum microparticle levels are known to increase in patients infected with *P falciparum* [216] or *P. vivax* [217], although it is not entirely clear if the increase in circulating microparticles is primarily due to high microparticle production by iRBC [216] [218], or increased production by platelets [217]. Certainly, microparticles derived from iRBC are hypothesized to act as carriers of PAMP: Combes et al (2005) noted that microparticles isolated from the plasma of *P. berghei*-infected mice stimulated TNFα protein production from murine bone marrow-derived macrophages [219]. Later studies confirmed that microparticles collected from the serum of mice infected with the murine parasite *P. berghei,* or from *ex vivo* parasite culture, could stimulate TNFα protein production from murine macrophages [218]. This effect was found to be abrogated in macrophages obtained from TLR4- and

myeloid differentiation primary response 88 (MyD88)-knockout mice, with reduced production of TNFα also noted in TLR2- and TLR9-knockouts [218]. The immunostimulatory nature of microparticles has been confirmed in one *ex vivo* human study, in which microparticles collected from *P. falciparum* culture were found to stimulate cytokine production from human PBMC, macrophages, and neutrophils [220].

New studies have also implicated microparticles as carriers not only of PAMP, but also of information to other parasites. Microparticles derived from iRBC were found to stimulate gametocytogenesis in *P. falciparum* cultures [220]. This new finding implicates microparticles as a part of a population density sensing mechanism that enables parasites to integrate information about parasite population densities before committing to irreversible gametocyte differentiation. Additionally, an independent study found that parasite-derived exosome-like vesicles could carry genetic material between parasites, providing a means for genes, such as those conferring drug resistance, to spread rapidly between parasites within the same host. Like microparticles, these exosome-like vesicles also were found to increase differentiation into gametocytes [214]. Hence, microparticles and exosomes may be both useful to the parasite and recognizable to the host.

Only a single study, however, has thus far examined the effects of microparticles derived from the human parasite *P. falciparum* on cytokine production by human cells [220], and this study did not address the question of which TLR are required to sense parasite PAMP. The data presented in this chapter describes the derivation and characterization of *P. falciparum* microparticles, and

the effects of these microparticles on cytokine upregulation from PBMC. The microparticle-stimulated upregulation of cytokines is partially blocked by the endosomal acidification inhibitor bafilomycin A. In addition, previous work (Chapters 2-4) has suggested a role for activin proteins in hepcidin upregulation in malaria infection. Current evidence suggests that stored activin A protein may be produced and released into the circulation by bone-marrow derived cells, and that activin A can also be produced *de novo* from circulating white blood cells [155, 156]. PBMC co-cultured with either whole schizonts or schizont-derived microparticles are shown to increase production of activin A message, further supporting the hypothesis that activin A may contribute to the modulation of hepcidin in malaria infections.

5.2. Materials and Methods

Human PBMC preparation and culture

PBMC were isolated using a Ficoll gradient (GE Healthcare) from the heparinized blood of consenting healthy adult donors according to the Weatherall Institute of Molecular Medicine local procedures, as described elsewhere [62, 132]. Cells were plated in RPMI-1640 media (10% FCS, also supplemented with 2mM glutamine, 100U/mL penicillin, and 0.1mg/mL streptomycin, all from Sigma) to a final concentration of 5 x 10^6 cells/mL, with 1 mL/well in a 12-well plate. The Ficoll gradient procedure does not include the collection of the more dense neutrophil fraction; hence, PBMC as referred to in this chapter does not include neutrophils.

P. falciparum culture and concentration

All parasites used were of the laboratory strain A4. Parasites were cultured as previously described [221, 222]. Late-stage trophozoites and schizonts were synchronized by a Plasmagel density gradient as previously described [223] on the day of experimentation, with parasitemia confirmed by thin smear. Mycoplasma is a frequent commensal of laboratory *Plasmodium* cultures: mycoplasma testing by PCR was performed within two days of each experiment, usually on the day of experimentation itself. New cultures of A4 were also periodically thawed to prevent infection. Experiments in which parasites were found to be mycoplasma positive were discarded and are not included in analysis. Parasite culture, synchronization, and mycoplasma testing were performed by R. Pinches.

Generation and characterization of microparticles

Microparticles were isolated following the erythrocyte-derived microparticle protocol described in Couper et al (2010) [218]. Briefly, the required quantity of concentrated iRBC or uninfected red blood cells (uRBC) was collected, washed 3x times in PBS, and subjected to three cycles of alternating freeze-thaws and sonication. The resulting mixture was then spun at 17,000*g* for 5 min. The supernatant was harvested, diluted 1:4 in citrated heparinized PBS (chPBS), and spun at 18,000*g* for 90 minutes. The supernatant from the second spin was discarded. The resulting pellet was resuspended in chPBS at the starting volume of schizonts (i.e. microparticles derived from a starting volume of 100 µL of packed infected red cells were diluted in 100 µL chPBS). uRBC were subjected to the same procedure and the microparticles obtained used as controls.

For microparticle size characterization, microparticles were diluted in chPBS with 1µm polystyrene beads (Polysciences, Inc.), and analyzed by fluorescence-activated cell sorting (FACS) analysis. To release their protein and DNA content, microparticle membranes were disrupted by suspending microparticles in a solution of 0.1% Triton X-100 SigmaUltra (Sigma Aldrich) for 30 min. Protein analysis of microparticles was accomplished using the Pierce BCA Protein Assay Kit (Thermo Scientific) kit according to manufacturers' instructions. *Plasmodium* genomic DNA (gDNA) levels in microparticles was analyzed using a 40S ribosomal gene probe, S5, for SYBR green quantitative real-time PCR, with the assistance of S. Kyes.

Microparticle or whole uRBC/iRBC treatment

For microparticle experiments, unless otherwise stated, PBMC were co-cultured with 50 µL microparticles for 24 h. Whole uRBC and iRBC experiments were previously performed by A. Armitage and R. Pinches; as stated, PBMC were co-cultured with 10^7 *P. falciparum* iRBC or an equivalent amount of control uRBC for 3 h as previously reported [132]. After appropriate incubation time, RNA was extracted from both adherent and nonadherent cells using Qiashredder homogenization (Qiagen) followed by RNeasy Mini Kit extraction (Qiagen).

TLR inhibitors

Cells were pre-treated with endosomal acidification inhibitors bafilomycin A (100 nM, Sigma), Chloroquine (30 µM), NH_4Cl (20 mM), cathepsin B inhibitor Z-FA-fmk (10 µM, Sigma), or TLR4 antagonist LPS-RS (25 µg/mL, Invivogen). Inhibitors were all added to PBMC 1 h prior to microparticle treatment. All reagents except chloroquine and LPS-RS were gifts from M. Hipp, chloroquine was a gift from the Newbold lab.

Previously described methods

RNA quantification, cDNA synthesis, and qRT-PCR were all performed as described in Chapter 2.

5.3. Results

Characterization of microparticles

Isolated microparticles were initially characterized by appearance and protein and DNA content. Microparticles were examined by FACS for size (forward scatter, Figure 5.1, y-axis) and granularity (sideways scatter, Figure 5.1, x-axis). 1 µm-diameter polystyrene beads were included as a size control in each experiment to ensure that the size of microparticles was constant between experiments (Figure 5.1A). Figure 5.1B and 5.1D depict representative FACS plots of uRBC and iRBC-derived microparticles. Microparticles were typically slightly smaller than the beads, from 0.1-1 µm diameter. Treatment with Triton X-100, a mild detergent, completely ablated detection of both uRBC and iRBC microparticles (Figures 1C and 1E). From these results, it can be concluded that these microparticles are lipid-bound.

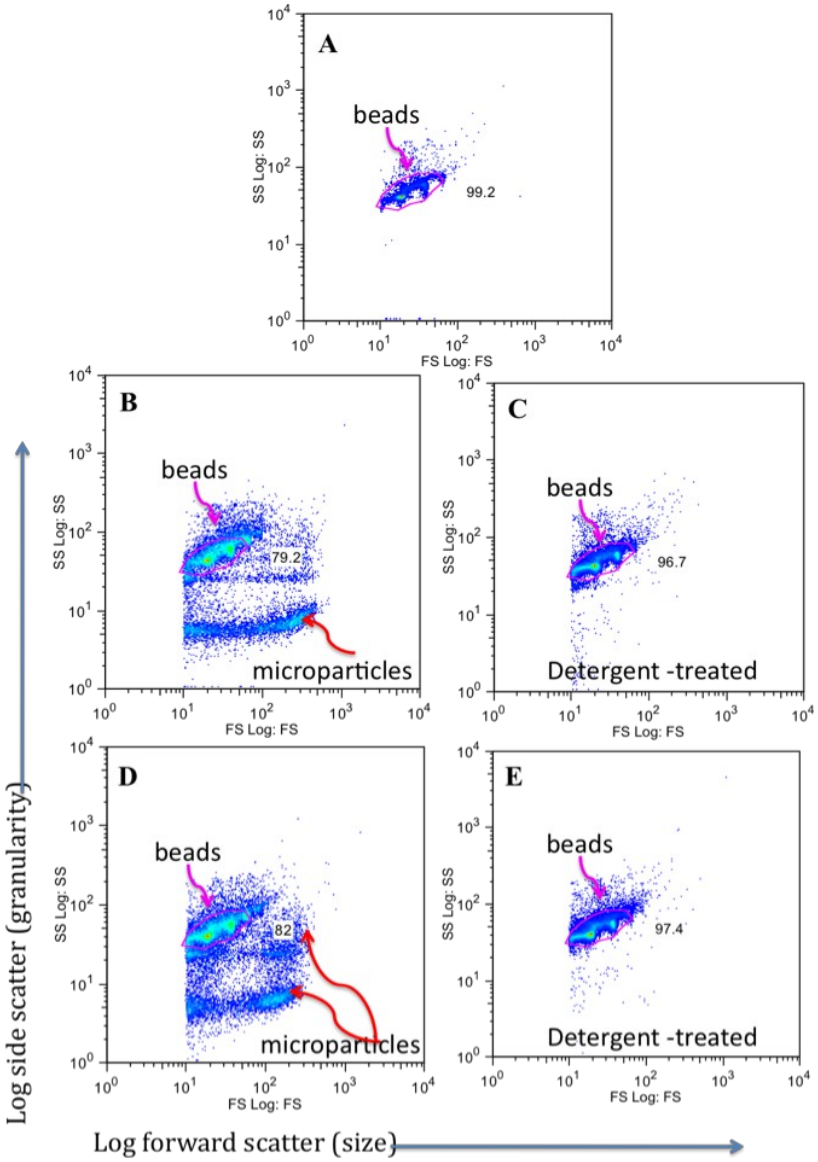

Figure 5.1. Microparticles are membrane-bound entities that are disrupted by detergent treatment. (A) shows size control beads only (circled). (B) shows microparticles derived from uRBC. (C) uRBC microparticles are no longer present when solution has been treated for 30 min with Triton X-100, a mild detergent. (D) iRBC-derived microparticles appear more variable in side scatter than uRBC microparticles, and are also destroyed by Triton X-100 treatment (E).

The protein and DNA content of microparticles were analyzed. Protein content of microparticles was measured by Bradford assay against a standard curve.

Figure 5.2A shows the protein content detected: uRBC-derived microparticles had an average protein concentration of 6 µg/µL, and iRBC-derived microparticles had a slightly lower protein concentration of 3.5 µg/µL. To measure the *P. falciparum* gDNA content of microparticles, qRT-PCR was performed for the *P. falciparum* 40S ribosomal protein S5 gene. Figure 5.2B shows the qRT-PCR findings from a typical microparticle experiment. All uRBC-derived microparticles samples were negative for *P. falciparum* gDNA, while all isolates of iRBC-derived microparticles were positive. Extrapolating from a control gDNA sample of known concentration, the concentration of gDNA in iRBC-derived microparticles was estimated to be approximately 0.25 ng/µL. A standard treatment of 50 µL iRBC-derived microparticles would therefore contain 12.5 ng of gDNA and 175 µg protein, while 50 µL uRBC-derived microparticles would contain 300 µg protein and no detectable parasite gDNA.

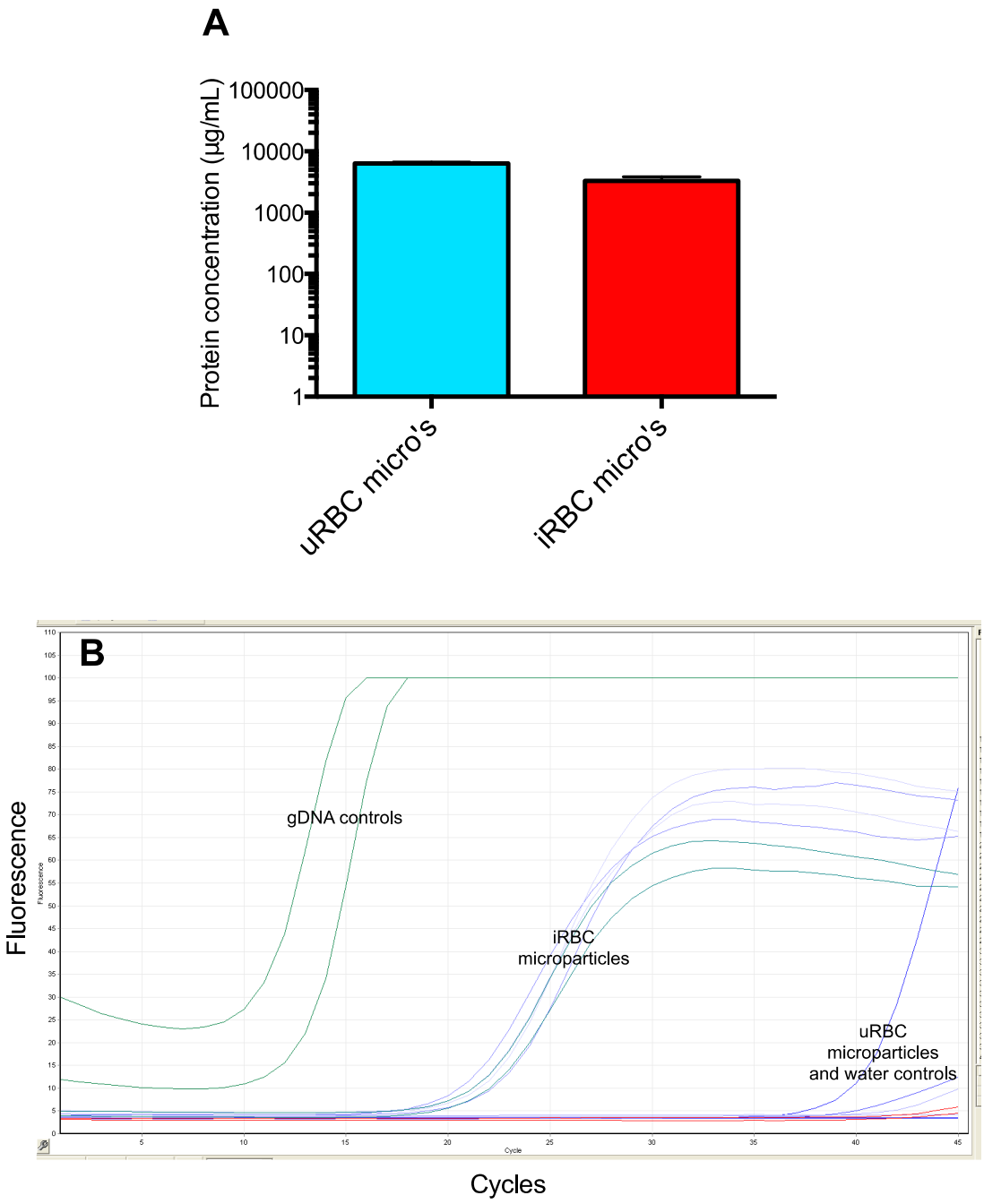

Figure 5.2. Microparticles contain both proteins and parasite DNA. (A) uRBC microparticles and iRBC microparticles (*n*=3 in each group) were tested for protein content using a Bradford assay. uRBC microparticles contained approximately 6.4 µg/mL solution, while iRBC microparticles contained 3.3 µg/mL. (B) qRT-PCR of microparticles for *Plasmodium falciparum* 40S ribosomal gene S5. Green lines at the left show *P. falciparum* DNA included as a positive control (255 ng/µL). Central lines are iRBC microparticles (*n*=2). At right are uRBC microparticles and water controls, none of which demonstrated detectable amplification. iRBC microparticles were calculated to contain 0.25 ng DNA/µL.

Effects of iRBC-derived microparticles on cytokine production

Having produced *P. falciparum* iRBC-derived microparticles and defined them as lipid-bound entities containing parasite gDNA and protein, the next step was to characterize the effects of microparticles on cytokine production by circulating white blood cells. PBMC were co-cultured with uRBC- and iRBC-derived microparticles. Initial measurement of cells' responses was assessed by examining expression of *TNFα,* which frequently has been used as a measure of white blood cell activation by PAMP, and *IL-6*, another major inflammatory cytokine that is known to have a role in stimulating hepcidin upregulation.

To optimize our treatment of PBMC, time-course and titration experiment were performed. PBMC from a single donor were co-cultured with 50 µL uRBC or iRBC-derived microparticles, PBS as a negative control, or LPS as a positive control, all for 1, 4, 12, 24 or 36 h. Untreated cells were also harvested at time 0h to provide reference values. Both *IL-6* and *TNFα* expression in response to iRBC-derived microparticles peaked at 12-24 h, while uRBC-derived microparticles did not elicit cytokine increases at any timepoint (Figure 5.3A-B). All future experiments use a 24-h incubation. The kinetics of cytokine upregulation in response to LPS differed from those in response to iRBC-derived microparticles, with LPS responses peaking at earlier timepoints.

A titration experiment revealed that iRBC-derived microparticles elicited increasing cytokine production reaching a maximum at treatment with microparticles derived from 50 µL packed iRBC (Figure 5.3C-D). At no dose did

uRBC-derived microparticles appreciably alter gene expression. All future experiments use a dose of microparticles derived from 50 µL packed iRBC at a 24 h timepoint.

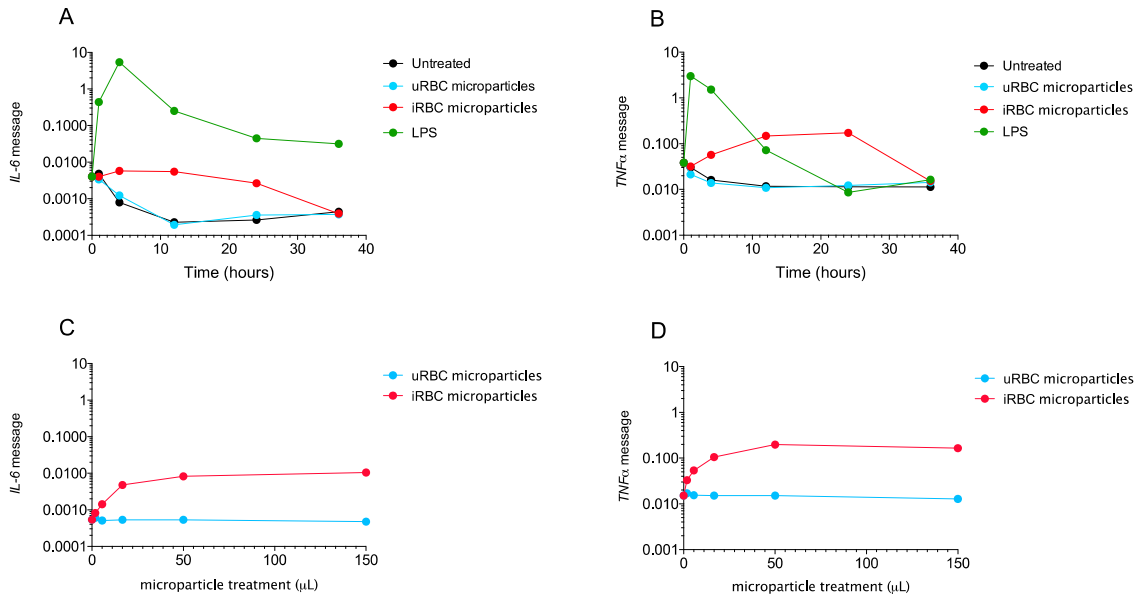

Figure 5.3. Timecourse and titration of microparticles. PBMC were co-incubated with uRBC microparticles, iRBC microparticles, or LPS as a positive control. Both *IL-6* (A) and *TNFα* (B) upregulation in PBMC treated with iRBC microparticles peak at 12-24 h. uRBC microparticles do not upregulate *IL-6* or *TNFα*. Both *IL-6* and *TNFα* (C and D) show peak upregulation with 50-150 µL iRBC-derived microparticles/mL. All further experiments were performed with 50 µL microparticles and at 24 h co-culture.

Treatment with iRBC-derived microparticles reliably and consistently elicits induction of both *IL-6* (Figure 5.4A, Dunn's multiple comparisons test, $p<0.01$) and *TNFα* (Figure 5.4B, Dunn's multiple comparisons test, $p<0.001$) from PBMC obtained from multiple donors. Microparticles derived from *P. falciparum* therefore represent a plausible candidate for the parasite factor(s) that initiate cytokine upregulation and, eventually lead to hepcidin upregulation.

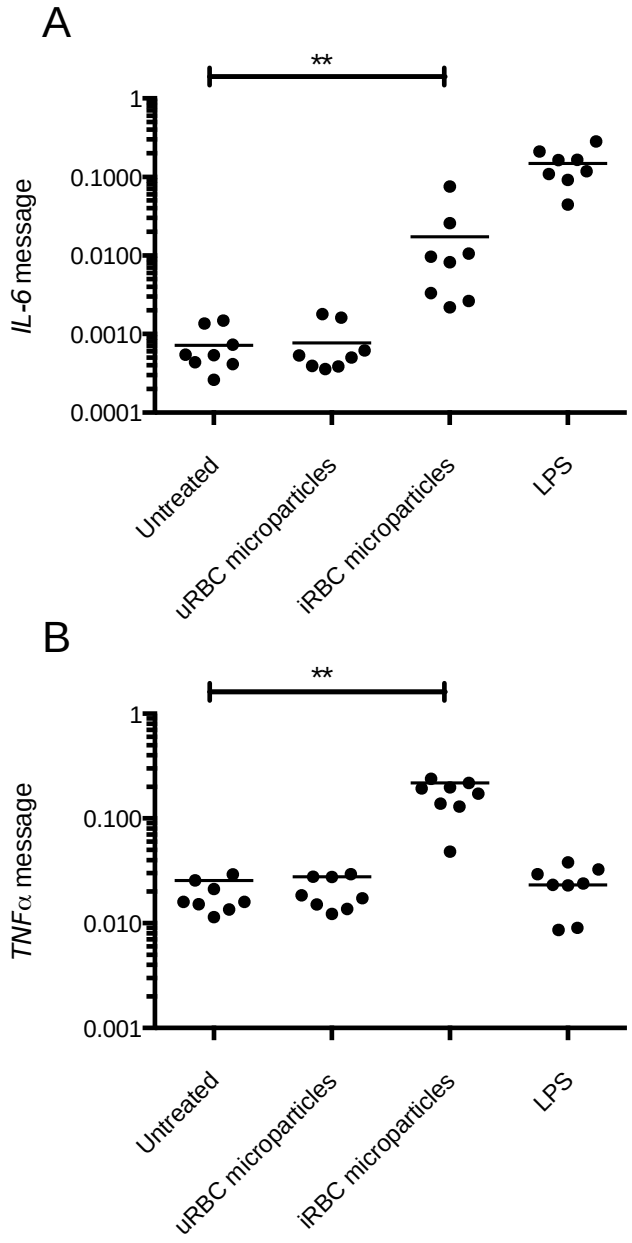

Figure 5.4. iRBC-derived microparticles consistently upregulate *IL-6* and *TNFα* in PBMC from multiple healthy donors. PBMC (n= 8) were co-cultured with 50 µL uRBC microparticles, iRBC-derived microparticles, or LPS for 24 h. iRBC microparticles, but not uRBC microparticles, caused significant upregulation of both *IL-6* (A) and *TNFα* (B) mRNA. Each donor is represented by a single dot, experiments were performed in biological duplicate. Statistical comparisons are Dunn's multiple comparisons' test after Friedman's test. ** p<0.01.

In order to further investigate the mechanism of microparticle recognition by PBMC, an assortment of TLR antagonists were used to attempt to block cytokine upregulation in PBMC. Multiple pilot experiments were performed (not shown) to examine the effects of cathepsin inhibitors (Z-FA-fmk) [224], endosomal acidification inhibitors (bafilomycin A, chloroquine, NH_4Cl), and TLR4 antagonists (LPS-RS). None of these compounds produced an appreciable decrease in *TNFα* expression from iRBC-treated PBMC (not shown). LPS-RS and NH_4Cl appeared to cause a minor decrease in *IL-6* expression, but both compounds also produced a considerable *IL-6* upregulation when used to treat PBMC alone, as did chloroquine, making these results challenging to interpret.

The most promising inhibitor noted was the endosomal acidification inhibitor bafilomycin A. This experiment was repeated with different PBMC donors (n=5), and found that overall, bafilomycin A inhibited *IL-6* with marginal significance in iRBC-derived microparticles-treated PBMC without appreciably affecting controls (Figure 5.5A, Wilcoxon matched-pairs signed rank test, p=0.06), but had no clear effect on *TNFα* (Figure 5.5B, Wilcoxon matched-pairs signed rank test, p>0.05). Bafilomycin A, by preventing endosomal acidification and potentially blocking the action of endosomal TLR receptors, may prevent the activation of some intracellular signaling pathways following the treatment of PBMC with iRBC-derived microparticles.

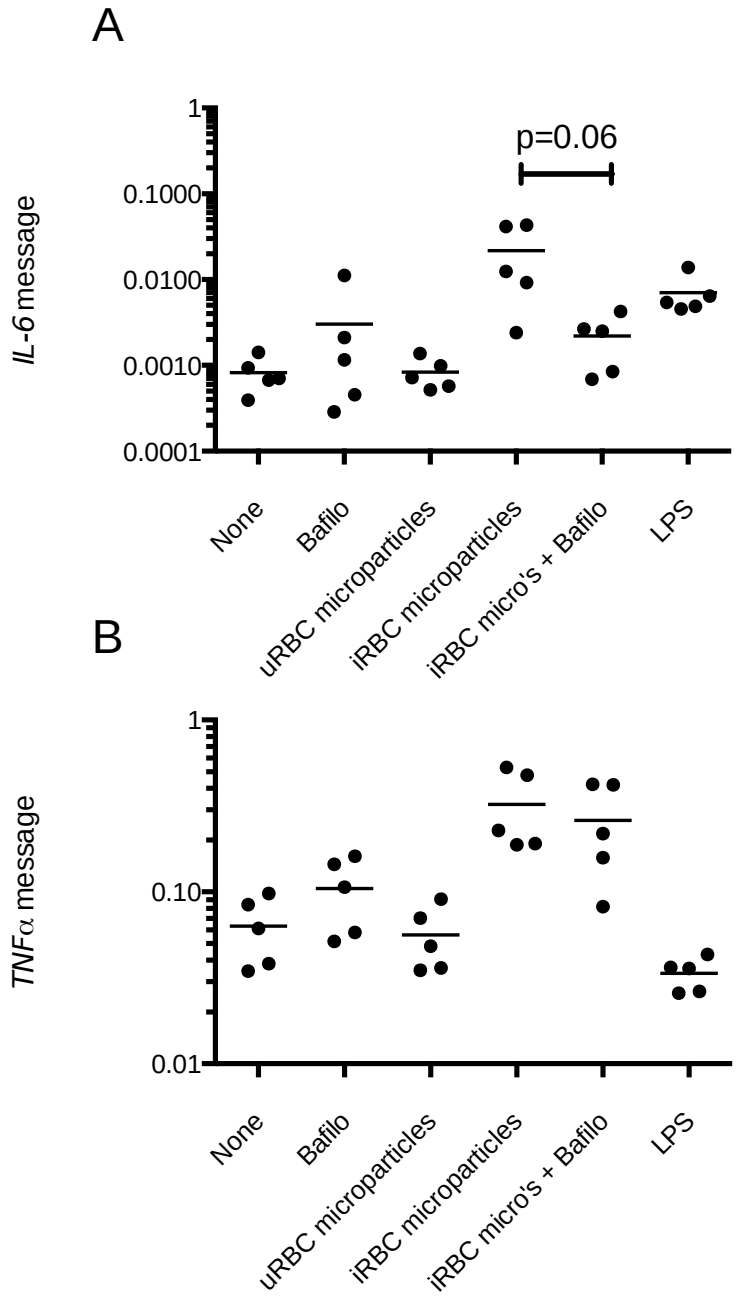

Figure 5.5. Bafilomycin A, an endosomal acidification inhibitor, partially blocks the cytokine upregulation elicited by iRBC-derived microparticles. (A) *IL-6* upregulation is partially inhibited by bafilomycin A (abb. as "Bafilo") with marginal significance. (B) *TNFα* is not inhibited by addition of bafilomycin A. Donors (n=5) are depicted as in Figure 5.4. Statistical tests are Wilcoxon matched-pairs signed-rank test on iRBC- treated vs. iRBC+bafilomycin A-treated cells.

Finally, we explored whether whole iRBC or microparticles might stimulate production of activin A from white blood cells. Previous studies (see Chapter 2-4) had implicated activin A and B proteins as playing a role in hepcidin control in malaria infection. Armitage et al (2009) had demonstrated that hepcidin message was upregulated in PBMC co-cultured with whole *P. falciparum* iRBC [132]. This experiment was reanalyzed and *INHBA* message measured from PBMC samples co-cultured with uRBC or iRBC for 3 h. Both hepcidin (Figure 5.6A, Dunn's multiple comparisons test, $p<0.01$) and activin A (Figure 5.6B, Dunn's multiple comparisons test, $p<0.05$) were upregulated in PBMC co-cultured with whole iRBC, but not in PBMC co-cultured with uRBC.

INHBA expression was also shown to be upregulated in a representative standard microparticle (PBMC +/- iRBC microparticles or uRBC microparticles for 24 h, Figure 5.6C). Although further studies are needed, these data suggest that PAMP carried by iRBC-derived microparticles or whole iRBC may cause PBMC to increase production of activin A, and therefore lead eventually to an upregulation of hepcidin, during malaria infection. Activin B mRNA (*INHBB)* was difficult to detect in PBMC and did not appear to show an appreciable change in response to either schizonts or to microparticles (not shown).

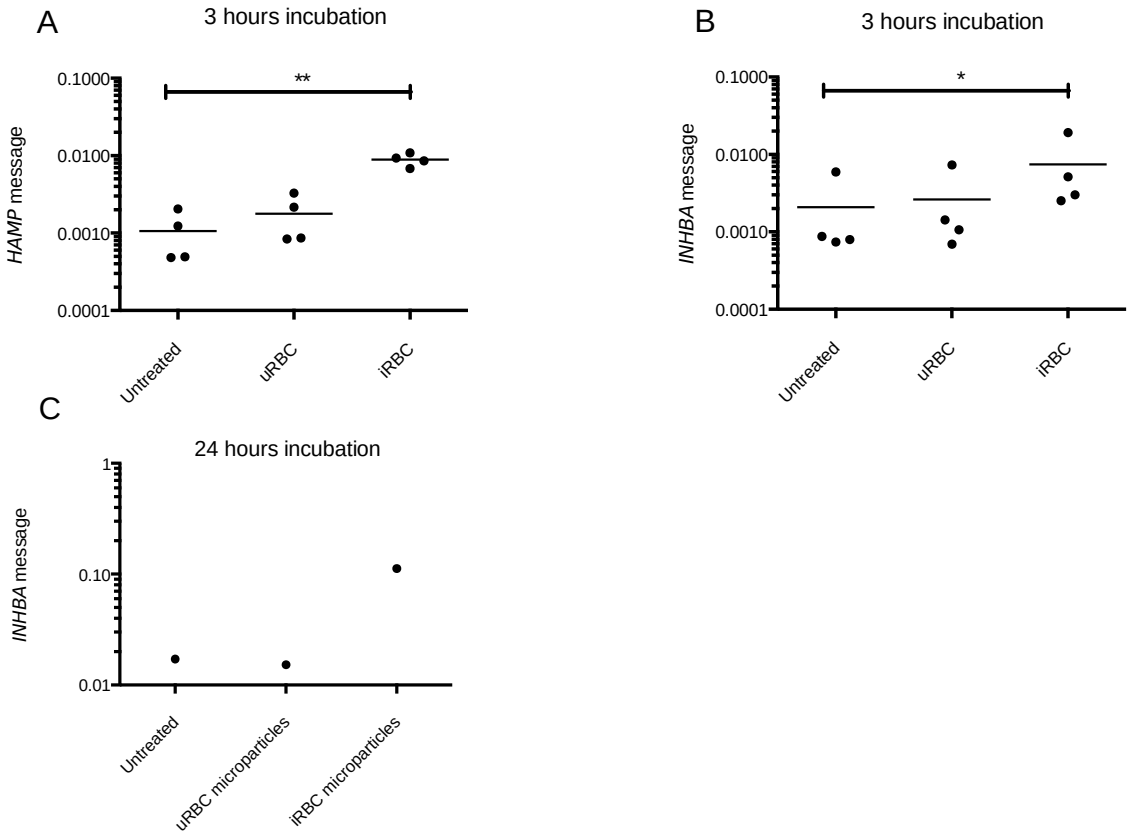

Figure 5.6. PBMC upregulate activin A message in response to both whole iRBC and iRBC-derived microparticles. An experiment was re-analyzed in which PBMC (*n* donors =4) were co-cultured with iRBC or uRBC for 3 h [132]. Both *HAMP* (A) and *INHBA* (B) were elevated at the message level in PBMC co-cultured with iRBC, but not those co-cultured with uRBC. (C) Microparticles also stimulate increased production of *INHBA*. One donor's PBMC were incubated with 50 µL microparticles for 24 h as in previous figures: microparticles from uRBC but not iRBC stimulated an increase in *INHBA* message. Comparisons are Dunn's multiple comparisons tests after Friedman test. * $p<0.05$, ** $p<0.01$.

5.4. Discussion

This study presents the derivation of microparticles from cultures of the malaria parasite *P. falciparum.* These microparticles are membrane-bound entities that contain proteins and parasite gDNA. Microparticles derived from iRBC, but not uRBC, reproducibly elicit a potent cytokine response from human PBMC at the transcriptional level. This effect is partially abrogated by the pre-administration of the endosomal acidification inhibitor bafilomycin A. Finally, activin A mRNA is induced in PBMC by either iRBC-derived microparticles or whole iRBC, providing a potential mechanism for the increases in activin A peptide in the serum of malaria-infected volunteers described in Chapter 3.

P. falciparum-derived microparticles consistently induced increased cytokine expression. Since microparticles may carry different *P. falciparum*-derived PAMP, such as GPI or parasite-derived nucleic acids, a range of experiments were performed with different TLR inhibitors. Bafilomycin A, a highly selective inhibitor of H^+-ATPase, the proton pump that is required for the acidification of endosomes [225], inhibited *IL-6* mRNA production across donors, but had less of a clear effect on *TNFα*. Endosomal TLR, which are likely to be affected by bafilomycin A treatment, comprise TLR3, TLR7, TLR8, and TLR9. All four recognize different nucleic acid PAMP: TLR3 recognizes double-stranded RNA [226], TLR9 recognizes unmethylated single-stranded DNA [227], and TLR7 and 8 recognize single-stranded RNA, as well as small-molecule antiviral compounds [228, 229]. Bafilomycin A therefore likely blocks PBMC detection of parasite nucleic acids; these

data are in keeping with observations that TLR9-knockout murine macrophages are less responsive to *P. berghei* iRBC-derived microparticles [218].

There is corroborative evidence for an effect of endosomal inhibitor compounds on cytokine responses in malaria infection. One study found that chloroquine, also an endosomal acidification inhibitor, could greatly reduce TNFα serum levels in a mouse model of malaria infection [230]. A novel synthetic antagonist of endosomal TLR restricted TNFα production in splenic-derived dendritic cells from malaria-infected mice [231].

The discrepancy between bafilomycin A's effects on *IL-6* and *TNFα* inhibition is intriguing. Bafilomycin A administration noticeably inhibits *IL-6* mRNA production, but not *TNFα*. Transcriptional effects of TLR stimulation are mediated through multiple mechanisms that can be MyD88- dependent or -independent (reviewed in [232]), but it is not immediately obvious why *TNFα* and *IL-6* should be differently affected at the message level.

One limitation of this study is that we did not formally show that the membrane-bound bodies studied were microparticles or exosomes. The distinction between microparticles and exosomes is based on both their size and their origin. Microparticles (100-400 nm diameter) are thought to be released by vesiculation, while exosomes (30-150 nm) are derived from endosomes [214, 215]. The particles isolated in this work ranged from 10 nm in diameter to 1,000 nm, and no specific steps were taken to eliminate the presence of smaller particles beyond the disposal of supernatant that may have contained particles too small to be pelleted out during centrifugation. It is therefore possible that these microparticle preparations

contained exosomes, and/or the iRBC-derived exosome-like bodies described by Regev-Rudzki et al (2013) [214], as well as microparticles. Further work should fractionate the particles by size to determine which size category appears to exhibit the most immunostimulatory properties. Another major limitation of this study is that we were unable, in numerous pilot experiments not presented here, to investigate the immunostimulatory effects of other malaria-derived PAMP, such as parasite gDNA or membrane GPI. Therefore, this study is limited only to microparticles.

Previous work (Chapters 2-4) demonstrated that activin A and B may be involved in hepcidin upregulation in malaria infection. Both proteins upregulate hepcidin *in vitro* and *in vivo*; activin B message is upregulated in malaria-infected mouse liver tissue, and activin A protein increases with hepcidin in the serum of malaria-infected volunteers. This work shows that PBMC, when co-incubated either with iRBC derived-microparticles or with whole iRBC, upregulate activin A at the message level. The response of PBMC to *P. falciparum*-infected red blood cells and the microparticles that are produced during infection represents a plausible mechanism for increased circulating activin A levels in malaria infection.

Future work should continue to elucidate the effects of parasitized red blood cells and parasite products, including microparticles, on activin A production by white blood cells. Both message and whole activin A protein should be measured by ELISA in different cell types following co-culture with iRBC or microparticles. Neutrophils in particular are known to release preformed activin A in response to treatment with different cytokines [154]. Carefully designed *in vitro* experiments

could enable a better understanding of what cell types might be responsible for the systemic activin A protein increases noted in Chapter 4.

Moreover, an important unanswered question is the relative contributions of increased activin A and activin B to hepcidin upregulation in malaria infection. As stated in Chapter 4, we were unable to obtain a reliable ELISA for activin B protein, but as soon as this is possible, relative levels and changes in both activins' serum protein levels should be measured and compared during natural human infections and/or CHMI. Might the short-term release of preformed activin A protein (as noted in multiple animal models following inflammatory stimuli, for example [139, 140, 149]), stimulate the initial hepcidin increase, then the *de novo* synthesis of both activins continue to maintain raised hepcidin levels? Studies that examine the timing of the increases of both activin proteins, to compare and contrast to the elevation of hepcidin, may help to answer this question.

Finally, are both activins A and B required for hepcidin upregulation in malaria infection? Knockout or knockdown mouse models may prove particularly important for assessing the differing requirements for activins A and B in hepcidin upregulation. If activin B or activin A can be knocked out (the latter requiring a conditional knockout or a tissue-specific knockout, as *Inhba*$^{-/-}$ mice exhibit neonatal lethality [159]) what are the effects on hepcidin expression at baseline and during infection? Can higher upregulation of activin compensate for loss of the other? These issues present fruitful areas for further study. Given the relative technical difficulty of malaria infections, as well as the long time (~8 days) that elapses between sporozoite infection and hepcidin upregulation, it is possible that some of

these studies should be carried out using a more tractable model of hepcidin upregulation, such as LPS injection.

CHAPTER 6: MEASUREMENT OF HEPCIDIN IN CORD SAMPLES FROM A LONGITUDINAL COHORT AND AN ASSESSMENT OF EPIGENETIC ASSOCIATIONS WITH HEPCIDIN AND CYTOKINE CONCTRATIONS AT BIRTH

6.1. Introduction

Susceptibility to malaria is controlled by multiple host and parasite factors. Host genotype has been estimated to control only approximately 25% of variation in malaria risk [233], and the remainder is still partially unexplained. A previous study in a longitudinal cohort in Muheza, Tanzania demonstrated that TNFα and IL-1β levels in cord blood predict parasite densities and risk of severe malaria in infancy and early childhood [120]. The data presented herein expands the findings from this previous work, firstly by analyzing cord blood hepcidin in this cohort, and in a second part of this study, by exploring the possible contributions of epigenetic changes in DNA methylation to individual differences in levels of cord hepcidin and cytokines.

Hepcidin levels and function during the neonatal period have only recently begun to be explored. Evidence linking cord hepcidin values with maternal hepcidin or maternal iron stores is mixed. In a rodent model of pregnancy, iron stores in the fetus appear to be maintained at the expense of maternal iron stores [234]. Meanwhile, hepcidin expression in the fetal liver is altered in response to maternal iron stores, with hepcidin message decreasing in the fetuses of rat dams fed an iron

deficient diet [234, 235]. At term, maternal serum hepcidin levels are low, likely reflecting the intense iron requirements of pregnancy, especially in the third trimester. Cord blood hepcidin values in healthy neonates have been noted to be higher than mothers' serum hepcidin values; no correlation was observed between the two [121, 122].

The studies that have examined hepcidin in cord blood have observed various correlations between hepcidin values and other iron status markers. All studies reporting both cord blood hepcidin and ferritin values noted a positive correlation between the two [121, 122, 124, 236], with one exception where hepcidin and ferritin were not correlated [123]. sTfR was reported as inversely correlated with hepcidin in one study [121], in another, an index based on the sTfR/ ferritin ratio was inversely associated with hepcidin [122]. One study noted an inverse association between hepcidin and EPO [121], two others found no association between hepcidin and EPO [123, 237].

Several studies suggest that hepcidin in neonates and infants may increase in response to inflammatory or infectious stimuli. Hepcidin in low birth-weight infants associates with neonatal sepsis [236, 238]. However, in the only study to date that has examined cord serum hepcidin in a cohort where malaria was endemic, cord hepcidin levels were not altered due to placental malaria, but this study was relatively limited in in sample size [125]. Only one study, to our knowledge, has compared cord blood CRP and IL-6 with cord blood hepcidin in a healthy population of premature and term neonates; the authors found no association in either case [124].

The question of whether hepcidin is functionally active as an iron-regulating hormone in neonates is currently under investigation. A study focusing on iron absorption from milk at 6 and 9 months of life showed that iron absorption may not be regulated at all at 6 months of age, but is regulated at 9 months [239]. However, the increase in hepcidin associated with sepsis in neonates [236, 238] and the correlations detailed above between iron indices and hepcidin indicate a probable functional role in both iron homeostasis and response to infection, as in later life.

The first part of this chapter presents an analysis of cord hepcidin from participants in a longitudinal cohort study in a malaria endemic-area, and compares hepcidin with iron markers and other cytokines already assayed in these samples. Cord hepcidin is also analyzed to assess any correlation with the future risk of iron deficiency (ID), severe malaria (SM), or severe malarial anemia (SMA).

The second part of this study characterizes the epigenetic variation that may underlie differing levels of hepcidin or cytokines in cord blood. Genetic polymorphisms in cytokine-encoding loci have been frequently noted in populations in geographical areas in which malaria is endemic (e.g. [240, 241]). However, to our knowledge, the role of epigenetic variation has not been studied in this context. Epigenetic variation has also been shown to be associated with susceptibility to a variety of diseases, including systemic lupus erythematosus [242], asthma [243], and diabetes [244]. Epigenetic variation can indicate any non-genetic heritable alteration that can affect gene expression: including histone methylation, histone acetylation, or DNA methylation at CpG (cytosine-phosphate-guanine) genome sites. The work presented here focuses on DNA methylation, which is currently the best

studied epigenetic change, as one factor that may help to explain the differing cytokine and hepcidin levels noted in this cohort at birth.

DNA methylation was initially explored in the context of CpG methylation within gene promoters, which frequently have an unusually high proportion of unmethylated CpG sites (CpG islands, reviewed in [245]). Increased methylation at these promoter sites has been viewed as a long-term means of decreasing gene transcription, particularly in the cases of X-chromosome inactivation and parent-of-origin-specific imprinting of genes (reviewed in [246] and [247]). However, further work has showed that methylation at other genomic sites can affect gene expression in more subtle ways. Primarily, studies have linked within-gene methylation with increased transcriptional activity [248, 249], although other studies have shown that increased methylation within a gene may block transcriptional elongation [250]. Furthermore, these relationships may vary by cell type [251]. In general, variations in DNA methylation in different gene regions appear to be important for gene expression control, but the precise transcriptional consequences of changing methylation at a particular CpG site cannot be predicted with confidence.

As large-scale analyses of DNA methylation have only recently become available, previous studies comparing DNA methylation with protein levels in an epidemiological context are limited. One study has demonstrated that changes in DNA methylation at a promoter CpG site in IL-2, as measured in cord blood, was associated with asthma exacerbation and hospital admissions in a cohort of children followed from birth to 8 years of age [128]. Promoter methylation at a CpG site in the estrogen receptor α gene was associated both with lower transcription and

protein levels of estrogen receptor α in the context of systemic lupus erythematosus in adult women [130]. Another study has shown that *TNFα* promoter methylation in PBMC of women was negatively associated with TNFα protein levels in serum [129]. In this study, the methylation of each CpG site within the genes encoding cytokines and hepcidin are compared independently with the cord concentration of the protein product of the appropriate gene.

Materials and Methods

Cohort and clinical monitoring procedures

Cohort samples in this study were from the Mother-Offspring Malaria Studies (MOMS) study at Muheza Designated Hospital, Tanzania. Descriptions of this study cohort, and findings from it, have been previously described elsewhere [24, 30, 120, 252]. This study was ethically approved by the Institutional Review Boards of Seattle BioMed, the National Medical Research Coordinating Committee in Tanzania, and the International Clinical Studies Review Committee of the Division of Microbiology and Infectious Diseases at the US National Institutes of Health. Mothers were recruited at the time of parturition and provided informed consent for themselves and their children.

From birth to 1 year of age, well children were monitored by clinical visits every two weeks; clinical visits were performed monthly in children above the age of 1 year. Visits included a thin smear for malaria analysis. Routine sample collection for further analysis, including assessment of iron status, was performed at 3, 6, and 12 months of age, and then every 6 months. Children who presented at the mobile clinics or central hospital were treated appropriately, and samples were also taken at sick visits.

Clinical definitions of ID, SM, and SMA

ID was defined as published in this cohort [24] using a combination of ferritin and CRP measurements. Ferritin values <30 ng/mL were defined as ID in conjunction with low CRP values (<8.2 µg/mL); when CRP was high (>8.2 µg/mL), ferritin values <70 ng/mL were defined as ID.

Because ID status can change throughout life and there were differences between the number of samples for iron measurement that were obtained per child, a continuous variable was calculated that was representative of the iron status of each child throughout the study. The proportion of ID visits was defined as for each child as follows: (visits categorized as ID between 24-100 weeks of age)/(total visits between 24-100 weeks of age at which samples were collected)

SM was defined by World Health Organization (WHO) criteria (also reported in the context of this cohort [120, 253]) as any of the following symptoms with a malaria-positive blood smear: prostration, Hb <50 g/L, respiratory distress, hypoglycemia, or ≥2 convulsions in 24 hours. SMA was also defined in accordance with WHO criteria, as Hb <50 g/L in association with a malaria-positive blood smear.

Processing of cord blood and serum samples

Cord samples were obtained as previously described [120]. Samples of whole blood were frozen for future epigenetic analysis. Serum samples were spun at 3000 *g* for 3 min to pellet out cells, serum was then frozen. All samples were kept at -80°C until thawed for testing, and freeze-thaws were kept to a minimum.

Statistical analyses of cord hepcidin data

All analyses except comparison between cord hepcidin and ID visits and longitudinal assessment of hepcidin were performed by E. Brickley using *Stata* (StataCorp LP version 12). Tabular data is presented in Word. ID visit comparison was performed in R with support from R. Morrison. Cord hepcidin comparison to peripheral serum concentrations of hepcidin at 76-100 weeks of age was performed in GraphPad Prism (GraphPad Software, Inc., La Jolla, CA). P values are for two-sided tests, and the level of statistical significance was set at $P \leq 0.05$. All cytokine measurements that were below the LOD were replaced by 50% of the LOD.

Epigenetic sample selection, gDNA extraction, and analysis

Samples for epigenetic analysis were selected to represent groups within the cohort of significant biological interest, such as participants who experienced severe malaria and participants born to mothers of different gravidities and placental malaria status. Participants were excluded from analysis if they were human immunodeficiency virus (HIV) infected, twins, stillbirths, or if they were lost to follow-up or died in the neonatal period.

Cord blood samples were extracted using one of two methods depending on the amount of sample available. If >300 µL blood was available, the Qiagen QIAamp DNA Midi Kit (Vacuum Protocol) was used according to manufacturer's protocol. If

<300 µL was available, the Qiagen QIAamp DNA Mini Kit (Vacuum Protocol) was used. DNA concentration and purity were assessed using a Nanodrop ND-1000 Spectrophotometer (Wilmington, DE, USA). A subset of these samples were extracted by E. Kabyemela. No difference was observed between DNA yields and purity from the two protocols. DNA methylation was measured by the 450k Illumina HumanMethylation microarray as previously described [131], by the Johns Hopkins University genetic resources core facility in collaboration with J. Bressler.

Beta values, data normalization and processing

Raw 450k Illumina HumanMethylation data were processed using the lumi software pipeline [254-256] by K. Shen and R. Morrison. Quality control, color balance adjustment, background correction, and quantile normalization were performed using lumi in R. Probes located in the X or Y chromosomes were excluded from analysis. Data are presented as beta values. Beta value = [methylated probe intensity]/[methylated probe intensity + unmethylated probe intensity]. Therefore, more methylated CpG sites will have a higher beta value. Beta values were logit transformed before analyses for normality.

Statistical analyses of epigenetic data: gene regions, false discovery rate, and sex

This study focuses on probes that were classified as associated with genes that encoded proteins for which we had cord serum concentration data. Probe binding regions were classified as transcription start site (TSS) (defined as TSS1500,

1500 base pairs (bp) upstream to start of promoter), promoter (1000 bp upstream of 5'UTR), 5' UTR (start of gene to first exon), exons, introns, or 3'UTR (end of last exon to end of gene).

False discovery rate (FDR) was defined in this study using a novel method. For probes in a given gene *X,* FDR was determined by randomly choosing *K* probes from the total ~450,000 probe database at random, where *K* is the number of probes in gene *X.* This process was repeated 25,000 times to calculate the likelihood that methylation at a randomly selected CpG site from within the genome shows a correlation with protein X as significant as does a given CpG site in *X.* Spearman's correlations were used to compare methylation beta-values at CpG sites with log-transformed protein levels.

Site methylation-protein correlations were designated as significant if and only if both FDR<0.05, and Spearman's p<0.05. Correlations that were significant are highlighted with bold lines in graphs.

In previous epigenetic studies, sex has been established as an effect modifier in humans and murine models [257-259]. Each gene was analyzed for any modifying effect of sex. For a given gene *X,* associations were calculated between each CpG site within *X* and protein X with males and females separately analyzed. Correlations were ranked by significance. The Mann-Whitney test was used to test the resulting distribution against the null hypothesis that sex had no modifying effect and that significant probe-protein relationships should be equally distributed between sexes. If p<0.05, relationships between methylation in *X* and X protein levels were significantly different between sexes, and further analyses for X were stratified by

sex. All analyses of epigenetic data were performed in R with assistance from R. Morrison.

White blood composition in cord blood samples

White blood cell composition was measured using an impedance-based analyzer (Abbott Cell Dyne 1200) as reported in [24]. White blood cells were divided into lymphocytes, granulocyte, and mid-cells (eosinophils, basophils, monocytes, and precursors [260]). % of each cell type was calculated as follows: (cell type of interest/(granulocyes+lymphocytes+mid-cells))*100%. Analysis was performed in GraphPad Prism (GraphPad Software, Inc., La Jolla, CA). In this chapter, PBMC is used to refer to white blood cells inclusive of neutrophils, as cells were not fractionated prior to DNA extraction.

Identifying SNPs in probe target sites

The National Center for Biotechnology Information (NCBI) maintains a database of all known human SNPs (dbSNP) [261]. SNPs were extracted that fall within the ~60 bp target region of each probe of interest in dbSNP and are reported in Table 6.

Previously described methods

Human hepcidin EIA was performed as in Chapter 4.

6.3. Results

Hepcidin levels in cord blood vary by placental malaria status and marginally by sex, but not by season of birth, genotype, birth weight, or maternal age or gravidity

Untransformed hepcidin cord values were compared by different biographical, biological, and maternal characteristics and examined for any differences (Table 6.1, all statistical tests are Kruskal-Wallis tests). Notably, children born to mothers with placental malaria had significantly higher cord hepcidin (Table 6.1, $p<0.05$). The sex of the participant had a trend towards an association with hepcidin values, with female children slightly more likely to exhibit high hepcidin values ($p=0.06$). No difference was noted between participants born during different seasons (malaria season defined as high between May and October, low between November and April, as previously reported [120]), thalassemia or sickle cell genotype, or maternal gravidity (divided into primi-, secondi- and multigravidae) (all $p>0.05$). Neither birth weight nor maternal age, both of which were divided into tertiles for the purposes of analysis, associated with hepcidin level (both $p>0.05$).

	Characteristic	Hepcidin Median (ng/mL) (IQR)	***P value***
Questionnaire Data	Sex		0.0631
	Female, n=341	31.6 (13.6, 55.9)	
	Male, n=370	27.1 (14.6, 46.1)	
	Transmission season at delivery		0.1885
	Low, n=341	29.3 (15.9, 49.7)	
	High, n=370	27.2 (13.0, 50.5)	
Biological Measurements	Thalassemia		0.4563
	α2/ α2, n=321	29.6 (15.2, 50.2)	
	α2/ α3.7, n=294	26.1 (13.7, 50.2)	
	α3.7/ α3.7, n=85	27.9 (12.6, 46.0)	
	Sickle cell trait		0.9681
	AA, n=593	27.9 (14.0, 50.1)	
	AS,n=116	28.9 (14.8, 47.3)	
	Birth weight		0.5207
	Low (2.00-3.00 kg), n=255	29.4 (14.2, 51.3)	
	Middle (3.03-3.40 kg), n=268	28.5 (15.5, 50.1)	
	High (3.41-4.50 kg), n=187	26.8 (13.1, 48.2)	
Maternal Characteristics	Maternal gravidity		0.4894
	Primigravid, n=203	31.3 (14.2, 50.8)	
	Secundigravid, n=167	28.4 (15.5, 50.1)	
	Multigravid, n=341	27.7 (12.7, 48.2)	
	Maternal age		0.6621
	Low (18-22 y), n=260	27.4 (15.7, 50.2)	
	Middle (23-28 y), n=231	29.4 (14.7, 49.7)	
	High (29-45 y), n=220	26.9 (11.6, 50.7)	
	Placental malaria		***0.0150***
	No, n=613	27.6 (13.3, 48.2)	
	Yes, n=98	34.2 (18.8, 63.6)	

Table 6.1. Placental malaria associates with higher cord hepcidin levels. Untransformed hepcidin levels are compared between neonate groups subdivided by different characteristics. Hepcidin median values for each group are shown, with interquartile-range (IQR) in parentheses. P values are Kruskal-Wallis tests.

Hepcidin levels in cord blood samples correlate with iron status measurements, acute phase indices, and cytokines

Hepcidin concentrations in cord blood were compared with measures of iron status and cytokines previously obtained. Hepcidin was inversely correlated

with sTfR (Table 6.2, all tests are Spearman's rank correlation with Bonferroni correction, $p<0.05$, $r= -0.14$), positively correlated with acute phase marker C-reactive protein (CRP, Table 6.2, $p<0.05$, $r=0.18$), and positively correlated with ferritin, which is both an acute phase protein and a marker of iron status (Table 6.2, $p<0.05$, $r=0.29$). Hepcidin was not associated with EPO (Table 6.2, $p>0.05$, $r= -0.05$).

Table 6.2. Cord hepcidin correlates with sTfR, CRP, and ferritin, but not EPO.

	Hepcidin	**EPO**	**sTfR**	**CRP**
EPO	-0.0547			
sTfR	-0.1351*	0.1168*		
CRP	0.1821*	0.0101	0.3648*	
Ferritin	0.2851*	0.1341*	-0.1073	0.0501

Table 6.2. Cord hepcidin correlates with sTfR, CRP, and ferritin, but not EPO. Correlations are Spearman's rank correlation with Bonferroni correction performed using untransformed measurements (n= 591). Numbers are Spearman's rank correlation coefficients, *$p<0.05$.

The correlations between hepcidin and other cytokines were examined. Hepcidin was significantly correlated with TNFα (Table 6.3, all correlations are Spearman's rank correlation with Bonferroni correction, $p<0.05$, $r=0.27$), IL-6 ($p<0.05$, $r=0.14$), and IL-10 ($p<0.05$, $r=0.16$). Hepcidin was not correlated with TNF-receptor 1 (TNF-RI), TNF-receptor 2 (TNF-RII), IL-1β, IL-4, IL-5, or IFN-γ (all in Table 6.3, all $p>0.05$). The other correlations noted between cytokines have been previously reported [120]; as previously noted, significant correlations are evident between both pro- and anti-inflammatory cytokines in these cord samples.

	Hepcidin	TNFα	TNF-RI	TNF-RII	IL-1β	IL-4	IL-5	IL-6	IL-10
TNFα	0.2710*								
TNF-RI	0.1157	0.4107*							
TNF-RII	-0.0478	-0.1077	0.1310*						
IL-1β	0.0690	0.6522	0.4552*	-0.0646					
IL-4	-0.0325	0.2332*	0.1288*	-0.0227	0.2406*				
IL-5	0.0396	0.3988*	0.2222*	-0.0808	0.3470*	0.1727*			
IL-6	0.1351*	0.1545*	0.2047*	0.1858*	0.3794*	0.1310*	0.1409*		
IL-10	0.1610*	0.3219*	0.2169*	0.2178*	0.3375*	0.2244*	0.2654*	0.4569*	
IFN-γ	0.0720	0.3583*	0.1659*	0.0130	0.3231*	0.3221*	0.2520*	0.1746*	0.3454*

Table 6.3. Cord hepcidin correlates with TNFα, IL-6, and IL-10. Correlations are Spearman's rank correlation with Bonferroni correction performed using untransformed cytokine measurements (*n*= 711). Numbers are Spearman's rank correlation coefficients, *p<0.05.

Hepcidin levels in cord blood do not correlate with hepcidin in peripheral blood at 76-100 weeks of age

In a previous analysis of data derived from this cohort, TNFα protein concentration in neonatal cord blood was shown to be predictive of peripheral TNFα levels during childhood (up to 3 years) [120]. Other cytokines previously examined, however, did not show a similar correlation (IL-6, IL-5, and IL-10) or were noted to correlate with cord levels only up to 1 year of life (IL-1β).

An initial subset of samples were examined for any correlation between cord hepcidin and hepcidin from peripheral blood samples taken during scheduled well-child visits. Peripheral samples were taken from well-child visits at 76 weeks of age when available (*n*=68); when samples from 76-week visits were not available, samples from week 100 were substituted (*n*=20). No difference was noted between

the distribution of hepcidin values at week 76- and week 100 timepoints (Mann-Whitney test, $p>0.05$, not shown). The subset of children examined was not random, but was selected to include representative children of both sexes from mothers of every gravidity status.

Hepcidin concentration in cord blood was not correlated with peripheral blood samples taken from 76-100 weeks (Figure 6.1, Spearman correlation, $p>0.05$, $r=0.15$). Interestingly, hepcidin was markedly lower in peripheral blood at 76-100 weeks than it was in cord samples (Figure 6.1, Wilcoxon paired t-test, $p<0.0001$, geometric mean 34.11 ng/µL in cord blood vs. 8.00 ng/µL in peripheral samples). This decrease likely reflects the suppression of hepcidin due to the intense need for iron during a period of rapid growth.

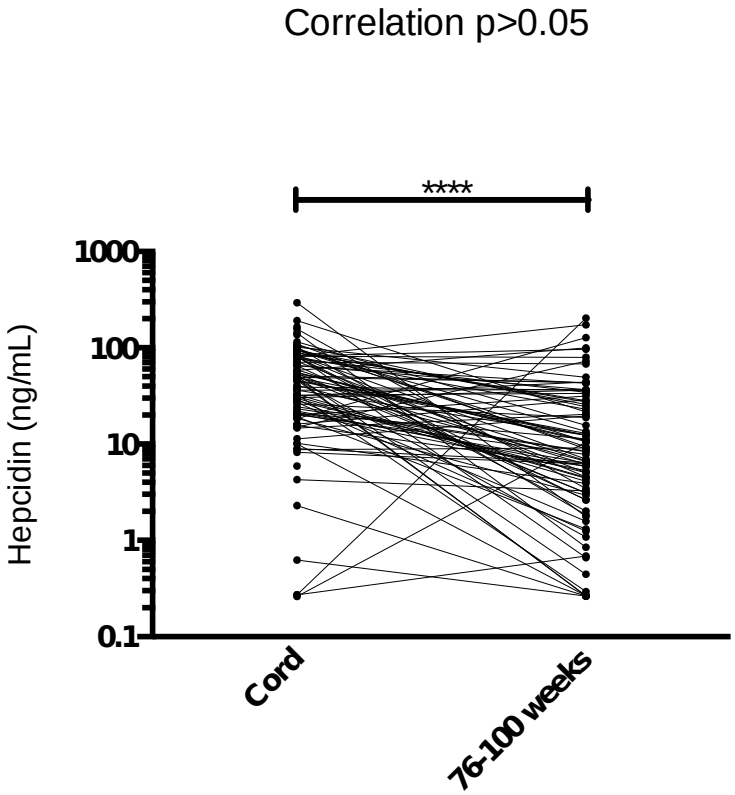

Figure 6.1. **Hepcidin at 76-100 weeks is not correlated with cord values.** Hepcidin was measured in a pilot subset of peripheral blood samples from well child visits at 76 weeks (*n*=68) or 100 weeks (*n*=20) of age, and compared with cord blood samples from the same children. No correlation was noted (Spearman's correlation test, p>0.05). Values at 76-100 weeks were significantly lower than those in cord blood (Wilcoxon matched-pairs signed rank test, p<0.0001).

Hepcidin levels in cord blood are not associated with visits with iron deficiency

Hepcidin cord values were examined for any association with future risk of iron deficiency. The percentage of visits for each child that were categorized as ID (detailed in Materials and Methods) was compared with hepcidin. As sex is known to modulate iron levels at different age groups, male and female children were considered both as separate groups and combined. No correlation was observed between the proportion of ID (termed "IronDef" in figure) visits and with cord hepcidin in either or both sexes (Figure 6.2, Spearman's correlation, all p>0.05).

Figure 6.2. Hepcidin in cord samples does not predict proportion of visits with ID. Percent of visits called as ID ("IronDef") are compared with cord hepcidin for male and female participants separately. Each dot represents a single child; color of the dot indicates sex. No correlation was significant for males, females, or both combined (all Spearman's correlations, all $p>0.05$).

Hepcidin levels in cord blood are not associated with increased risk of SM or SMA

Previously, IL-1β cord levels have been shown to predict the future risk of SM [120]. SM is defined as fulfillment of one of multiple pathophysiological criteria, including prostration, hypoglycemia, convulsions, respiratory distress, or severe anemia [120]. Because distinct mechanisms may be involved in the development of these different clinical signs, and because it was our judgment that any effects of

hepcidin would be most likely to alter the risk of SMA, these data were also analyzed for any relationship between cord hepcidin and SMA only.

Biomarker	SD	WHO SM (*n*=95) HR (95% CI)	*P value*
Hepcidin (Univariate)	2.72	1.09 (0.89, 1.34)	*0.399*
Hepcidin (Multivariate)	2.72	1.09 (0.89, 1.33)	*0.414*

Table 6.4. Hepcidin values are not associated with occurrence of SM. Hepcidin (*n*= 696 with full clinical data, *n*=95 cases) is considered as a continuous variable and square-root transformed for normality. Univariate model considers hepcidin against SM risk as a sole risk factor, multivariate model adjusts for shared frailties: village, sex, delivery transmission season, thalassemia, sickle cell trait, birth weight, maternal gravidity, and placental malaria status. SM is defined as in [253] according to WHO criteria. P values are calculated using Wald tests; hazard ratios (HR) are from Cox Proportional Hazards Model.

The link between cord hepcidin and risk of severe malaria in this cohort (*n*=696 children with hepcidin measurements and complete clinical data, *n*=95 SM cases, results shown in Table 6.4) was analyzed using univariate analysis or a multivariate model that accounted for shared frailties (fully listed in Table 6.4 legend). Hepcidin was not a significant predictor for SM risk using either of the methods explored (both Wald tests, both p>0.05). No significant effect of hepcidin on risk by univariate or multivariate analysis was present when analysis was restricted to SMA cases (*n*=696 children with hepcidin measurements and complete clinical data, *n*=36 SMA cases, Table 6.5).

Biomarker	SD	WHO SMA (Hb<50 g/L, *n*= 36) HR (95% CI)	*P value*
Hepcidin (Univariate)	2.72	1.02 (0.73, 1.42)	*0.927*
Hepcidin (Multivariate)	2.72	0.97 (0.69, 1.36)	*0.851*

Table 6.5. Hepcidin values are

not associated with SMA occurrence. Hepcidin (*n*= 696 children with full clinical data, *n*=36 SMA cases) is considered as in Table 6.4. SMA is defined according to WHO criteria of Hb <50 g/L. P values are calculated using Wald tests; HR are from Cox Proportional Hazards Model.

DNA methylation in hepcidin and cytokine genes correlates with concentrations in cord blood

Environmental influences, gestational age, genetic variation, or epigenetic differences may all play a role in determining the variable levels of cytokines and hepcidin observed in cord samples. Epigenetic contributions were assessed by performing a full-genome DNA methylation assay on gDNA from a subset of cohort participants. gDNA was obtained from cord blood samples; as red blood cells are non-nucleated, gDNA is presumed to be primarily from PBMC including neutrophils. Analyses were restricted to CpG sites in the TSS regions, promoters, 5' UTR, exons, introns, and 3'UTR of genes encoding measured cytokines and hepcidin. Methylation levels at CpG sites were examined for any correlations with the cord concentrations of the appropriate protein.

As a first step, methylation-protein correlations for each gene were analyzed for any modifying effect of sex. Correlations between CpG methylation and TNFα protein levels were significantly different between male and female neonates ($p<0.0001$, Mann-Whitney test). There was also a significant difference in the relationship between CpG methylation and hepcidin protein levels by sex ($p<0.05$, Mann-Whitney test). Therefore, CpG sites occurring in these two genes were analyzed separately by sex in all future analyses. All other cytokine analyses are

shown with male and female data combined. Correlations (as in Methods) were defined as significant if both FDR and Spearman's P values were <0.05.

No significant correlations were noted between CpG methylation and protein levels for ferritin, IFN-γ, IL-1β, or IL-6 (not shown). TNFα concentrations were significantly correlated with *TNFα* methylation status at a number of CpG sites (Figure 6.3A-B); however, most of these correlations were only observed when analysis was restricted to male neonates. In male neonates, methylation at two CpG sites in the promoter region of the gene were positively associated with TNFα protein values, methylation at one promoter site was negatively associated with protein levels. One exonic site was positively associated with protein levels; negative associations were observed at four CpG sites within exons and a single site in the 3'UTR (Figure 6.3A). Notably, correlations in females were almost completely absent, with methylation at only a single CpG site within an exon showing an inverse relationship with protein levels (Figure 6.3B).

A

Gene	Probe ID	Probe Location	Gender	Spearman R	FDR	Spearman P
TNFα	cg23384708	Exon	Male	-0.3629936	0.01324	0.006453859
TNFα	cg24452282	Promoter	Male	-0.3501145	0.02096	0.008782976
TNFα	cg06825478	3'UTR	Male	-0.3478057	0.04968	0.009269863
TNFα	cg20477259	Exon	Male	-0.3396887	0.00812	0.011171919
TNFα	cg03037030	Promoter	Male	0.3384982	0.02744	0.011477422
TNFα	cg04425624	Exon	Male	0.3362615	0.0448	0.012070905
TNFα	cg09637172	Exon	Male	-0.3204243	0.0378	0.017078832
TNFα	cg05952498	Exon	Male	-0.300763	0.03968	0.025666264
TNFα	cg01569083	Promoter	Male	0.2825448	0.03108	0.036611155

B

Gene	Probe ID	Probe Location	Gender	Spearman R	FDR	Spearman P
TNFα	cg21467614	Exon	Female	-0.3121804	0.03736	0.010993219

Figure 6.3. Methylation values in different CpG sites associated with the *TNFα* gene are associated with TNFα protein levels in male neonates. (A-B) Graphs show logit-transformed beta values compared to log-transformed TNFα concentration. Each dot represents a single individual's measurement , color-coded by region of gene in which probe was located. For each site, line shows relationship (linear model). Significant correlations are highlighted by bold lines and the probe number that corresponds with that site is included both in table and graph. Tables are ordered according to Spearman P value. (A) Nine significant correlations exist between CpG site methylation and TNFα in male neonates (*n*=64). (B) In female neonates (*n*=71), conversely, only one CpG site within an exon is significantly negatively associated with cytokine levels.

Hepcidin had one site within an intron at which methylation was negatively correlated with hepcidin cord protein levels in female neonates but not in male neonates (Figure 6.4A-B).

A

B

Gene	Probe ID	Probe Location	Gender	Spearman R	FDR	Spearman P
HAMP	cg27273033	Intron	Female	-0.254158	0.01664	0.041051529

Figure 6.4. Methylation values in one CpG site in an intron of the *HAMP* gene is associated with hepcidin protein levels in female neonates. Data is presented as in Figure 6.3. No significant correlations are noted in male neonates (A) but one CpG site in an intron is significantly negatively associated with log-transformed hepcidin values in female neonates (B).

All other cytokines were analyzed without first subdividing for sex. Correlations between methylation at two *IL-10* intronic sites were significantly negatively correlated with cytokine levels of IL-10 (Figure 6.5A). Methylation of *TNF-RI* at three exon probe sites were positively associated with TNF-RI levels (Figure 6.5B). Finally, the methylation status at one site in the promoter region of *TNF-RII,* and one site in the 5' UTR region, were negatively associated with TNF-RII protein levels.

A

Gene	Probe(ID	Probe(Location	Spearman(R	FDR	Spearman(P
IL5L0	cg15096505	Intron	-0.3504588	0.00572	0.00020053
IL5L0	cg17067005	Intron	-0.2093169	0.04984	0.02969632

B

Gene	Probe(ID	Probe(Location	Spearman(R	FDR	Spearman(P
TNF7R1	cg26391891	Exon	0.29793244	0.00504	0.00085844
TNF7R1	cg11268190	Exon	0.26137932	0.02632	0.00359041
TNF7R1	cg16429476	Exon	0.19467697	0.01936	0.03109711

C

Gene	Probe(ID	Probe(Location	Spearman(R	FDR	Spearman(P
TNF7R11	cg13836770	Promoter	/0.2715279	0.03456	0.00306309
TNF7R11	cg24682307	5'UTR	/0.2430282	0.04388	0.00828485

Figure 6.5. Methylation values in *IL-10*. *TNF-RI*, and *TNF-RII* show some correlations with respective cytokine levels. Data is presented as in Figure 6.3-4, but male and female neonates are combined for the purposes of analysis (*n*=135). (A) IL-10 log protein levels are negatively associated with methylation at two sites in an intron. (B) TNF-RI is positively associated with methylation at three sites within exons, and (C) TNF-RII is negatively associated with methylation at two sites in the gene's promoter.

Correlations between gene methylation and protein levels are unlikely to result from variation in PBMC composition, except in the case of TNF-RI

PMBC are comprised of a heterogeneous mixture of cells that may exhibit different DNA methylation patterns as a result of differentiation [262]. Hence, inter-individual differences in PBMC composition may be misinterpreted as differences in methylation. In the individuals for whom both cord blood cell type composition and epigenetic data had been measured, cell type composition was compared with methylation levels at the 18 probe sites that showed significant correlations with their protein products (sites of interest). Analyses for methylation in *TNFα* and *HAMP* genes were subdivided by sex (n=50 males, n=61 females, the remaining individuals did not have available cell composition data). For other cytokines, males and female data were analyzed together (n=111). Logit(beta) values for each site of interest were compared with % lymphocytes, % granulocytes, and % mid-range white cells.

No significant correlations was observed between the percentage of any of the three cell types to methylation levels at 15/18 probe sites examined (all Spearman's correlation tests, all $p>0.05$, not shown). The only significant correlations were noted for probes cg26391891, cg11268190, and cg16429476. Notably, all were sites of interest associated with exons of the *TNF-R1* gene. All three had logit(beta) values that were significantly positively correlated with the percentage of lymphocytes, not associated with % mid-range cells, and negatively

correlated with the % of granulocytes (Figure 6.6A-C, respectively, all Spearman's correlation tests, p and r values shown on graphs).

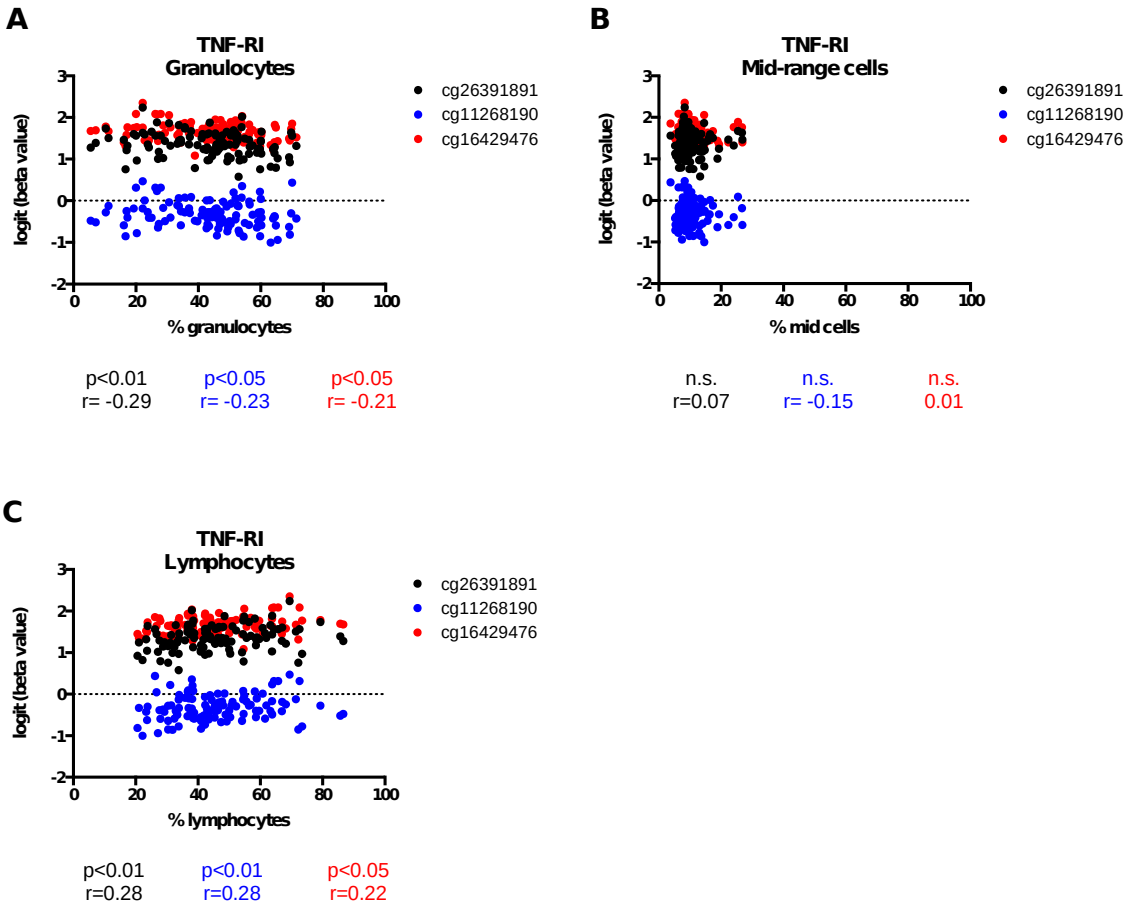

Figure 6.6. Methylation values at probe sites in *TNF-RI* are significantly associated with cell type. % Granulocytes (A), % mid-range cells (B), and % leukocytes (C) were compared to logit (beta) values for the three probes in *TNF-R1* that showed significant correlations with the protein product. Each symbol in dot plots represents a single individual's measurement (n=111); each probe is depicted in a different color. All correlations are Spearman's correlation tests.

Epigenetic analysis may be confounded by SNPs at several probe sites

SNPs at the site of a CpG probe can, by altering the sequence to which the probe binds, cause what appear to be inter-individual differences in methylation levels [263]. Studies performed specifically to examine the effects of SNPs on the

HumanMethylation450k Illumina assay have estimated that 4.3% of Illumina probes may have a documented SNP at the CpG probe-binding site [264]. dbSNP was examined for the presence of known human SNPs in the 18 sites of interest; SNPs were located within the probe-binding area of 9 of them, including the probe-binding area within *HAMP* (Table 6).

Gene	**Probe ID**	**Probe Location**	**SNP (dbSNP)**
HAMP	cg27273033	Intron	rs74887182:35773676
TNFα	cg24452282	Promoter	rs4248160:31542693
TNFα	cg01569083	Promoter	rs41297589:31543262
TNFα	cg03037030	Promoter	
TNFα	cg04425624	Exon	
TNFα	cg21467614	Exon	
TNFα	cg23384708	Exon	
TNFα	cg20477259	Exon	
TNFα	cg09637172	Exon	
TNFα	cg05952498	Exon	
TNFα	cg06825478	3'UTR	rs191617007:31546014
IL-10	cg15096505	Intron	rs190113461:206943569
IL-10	cg17067005	Intron	rs140487354:206945304 rs3024490:206945311
TNF-RI	cg11268190	Intron	
TNF-RI	cg26391891	Exon	rs200900510:6439066
TNF-RI	cg16429476	Exon	rs200029309:6439013
TNF-RII	cg13836770	Promoter	
TNF-RII	cg24635257	Promoter	rs36205948:12226905

Table 6.6 SNPs are present at 9/18 probe sites of interest. NCBI database was used to identify SNPs at the sites of probes from the analyzed genes. Analysis was limited to SNPs at which significant association between methylation level and protein levels had been detected.

6.4. Discussion

Cord levels of some cytokines have been shown to be predictive of the risk of both malaria parasitemia and of severe malaria syndromes in early childhood [120]. Hepcidin fulfills a unique role: controlling iron metabolism both during normal homeostasis and in the context of infection. This work presents an analysis of hepcidin levels in cord blood samples from a cohort in Muheza, Tanzania, and a first investigation into the epigenetic mechanisms that may underlie the individual differences in hepcidin and cytokine levels at birth.

The only biographical variables that were significantly associated with increased cord hepcidin values were placental malaria, and with marginal significance, female sex. The association of increased cord blood hepcidin with placental malaria is novel: the only previous report to examine cord hepcidin in a malaria-endemic area found no effect of placental malaria on cord hepcidin [125]. A different hepcidin quantification assay is used herein, but round-robin comparison of different hepcidin assays showed that although absolute numbers tend to vary by assay, trends are generally conserved, as mentioned previously in Chapter 4 [192, 193]. Hence, the different findings reported in van Santen et al (2011) are more likely to result from differences in cohort size and composition rather than technical variability. This previous study was smaller than ours in number of participants (n=69 neonates in [125] as opposed to n=711 in the study reported here), and subtle effects would therefore have been more likely to be detected in the analysis reported herein. However, no trend towards higher hepcidin values in cord blood from neonates whose mothers had experienced placental malaria was evident in the

previous study. Other explanations for these different results may be found in the differences between study participants: the previous study restricted its analysis to primigravidae, while the data reported here considers all gravidity categories. Furthermore, van Santen et al (2011) conducted their study in a cohort localized in Gabon, in an area of "stable meso- to hyperendemic malaria transmission" [125], whereas the Tanzanian cohort reported here typically experiences intense seasonal transmission. It is possible that differences in participant demographics, ethnicity, or malaria transmission patterns may account for the distinct findings.

The presence of raised hepcidin in the cord blood of neonates whose mothers have placental malaria is of potential clinical importance. It is plausible that upregulated hepcidin by the fetus, in response to placental malaria, serves to restrict maternal-fetal iron transfer, thereby resulting in lower iron stores at birth (although some studies suggest that placental malaria is not associated with lowered neonatal iron stores [265]) or, if hepcidin remains elevated, to reduce post-birth iron acquisition. Upregulation of hepcidin at the message level has already been noted in placental tissue from pregnant women with placental malaria [266], and it is possible that an unknown proportion of the hepcidin we have measured is partially sourced from the placenta rather than the fetal liver.

The fact that cord blood hepcidin correlates with CRP may indicate that hepcidin's upregulation in the fetus in placental malaria is modulated partially by inflammatory signaling. Chapters 2-5 describe the possible involvement of activin A and activin B proteins in hepcidin upregulation in malaria infection in rodent models and in adults infected in CHMI trials. Activin A has previously been

measured in cord blood in several studies [267], and shown to be upregulated in neonates from pregnancies in which pre-eclampsia occurred [268], in the cord blood of neonates whose mothers who had been treated with selective serotonin re-uptake inhibitors during pregnancy [269], and in hypoxic neonates [270]. As methods of measuring activin B are still novel, to our knowledge, activin B has not yet been measured in cord blood. The measurement of activin A and activin B in cord blood, and comparison with hepcidin and with placental malaria status are logical next steps for these studies.

In addition, the finding of marginally higher hepcidin cord blood values in females is worthy of further investigation. A single study found that cord serum hepcidin did not vary significantly by sex [121], but as the difference we noted was only marginally statistically significant with a sample size of 711 participants, it is possible that this relatively subtle effect may not have been detected in the smaller cohort previously reported (n=191 neonates in [121]). A sex-related difference in hepcidin at birth is an important contribution to our evolving understanding of differences in iron metabolism throughout life. Adult men and post-menopausal women tend to have higher iron-related indices and higher baseline hepcidin levels than women of childbearing age [198]. This has largely been thought to be due to the monthly iron loss of menstruation in women of child-bearing age and the subsequent need for more efficient iron intake than men. In infants, evidence exists to suggest that hepcidin may be lower in males than females, following the immediate post-birth period. A study of healthy Zimbabwean non-anemic infants at 3, 6, and 9 months of age revealed that hepcidin was non-significantly lower in male

infants [271]. A separate study of Kenyan infants at 6 months found that hepcidin was lower in males and correlated with lower iron-related indices [272]; lower iron indices in male infants have additionally been noted elsewhere [273, 274]. The data presented here adds to our total knowledge of sex-related hepcidin differences throughout life. Summarizing the available information, it appears that hepcidin may be marginally higher in female neonates, that higher female hepcidin values persist throughout infancy and possibly childhood, but that this trend reverses during puberty, likely coinciding with menarche.

The data presented in this chapter confirm a positive association of cord blood hepcidin with ferritin and the negative association of hepcidin with sTfR in these neonatal samples, in agreement with reports from the literature. As stated in the Introduction (Chapter 6), the majority of studies that have examined cord blood hepcidin and ferritin find a positive correlation between the two, and an inverse correlation with sTfR. In this study, hepcidin was not found to correlate with EPO, in agreement with some studies [123, 237], but not all [121]. These findings may indicate that hepcidin in the perinatal period, in this population, is affected by inflammation and iron status, but not by hypoxia.

Several of the iron indices' correlations were unexpected. EPO and sTfR were correlated, as previously reported in adults [275] and in cord blood [123]. The observed correlation between CRP and sTfR is harder to explain. It is possible that the neonates who are more inflamed (high CRP) also exhibit iron deficiency (higher sTfR). If inflammation is also associated with iron deficiency in these neonates, then the lack of correlation between ferritin and CRP might also be explained, if ferritin is

both downregulated by iron deficiency and upregulated by the inflammatory stimuli that increase CRP. Finally, that ferritin should exhibit a *positive* correlation with EPO is surprising and not immediately explicable.

Serum cord levels of pro- and anti-inflammatory cytokines in this cohort have been previously found to be significantly positively associated with each other [120]. In the data presented in this chapter, hepcidin was found to be significantly correlated with TNFα, IL-6, and IL-10, but not other cytokines. Hepcidin and IL-10, and hepcidin and IL-6, have previously been shown to be correlated in multiple studies examining circulating cytokines in malaria-infected children [103, 104], and both IL-10 and IL-6 have been proposed as cytokines responsible for the proximate control of hepcidin in malaria infection [276]. The correlation of these factors with hepcidin in cord blood suggests that the co-regulation of interleukins and hepcidin begins in the prenatal period. Fewer prior data are available to support the strongest correlation, between TNFα and hepcidin, reported in this chapter, but one of the above-cited studies looking at circulating cytokines in malaria-infected children found an association between TNFα and hepcidin [104].

In a limited pilot study, no association was noted between cord hepcidin measurements and serum levels in early life, at a timepoint (76-100 weeks) when TNFα, but not any other cytokine, retains a correlation with cord values [120]. Hepcidin in the serum is known to increase after oral intake of iron [277, 278], and displays a diurnal rhythm independent of oral intake [180]. Most samples were taken in the morning, but there was variation both in the time of sample collection and in the proximity of this time to oral intake. Both of these factors are potential

sources of variation in hepcidin levels after birth. Given these limitations, it is perhaps not surprising that no evidence was observed to support a genetic or epigenetic effect continuing into infancy. Similarly, the data were analyzed to identify any ongoing effect of hepcidin levels at birth on iron levels or malaria susceptibility throughout life. Cord hepcidin was not found to be a predictor for iron deficiency, or for clinical presentation with severe malaria or severe malarial anemia. However, again, clinical outcomes are likely to be affected by multiple other host or parasite factors, making any but the clearest effect challenging to observe.

As evidenced by the association of cord levels of TNFα and IL-1β with future cytokine levels and incidence of severe malaria [120], levels of some cytokines in cord blood may be indicative of genetic or epigenetic differences that persist through childhood and thereby have important implications for disease susceptibility. Epigenetic contributions to neonatal cytokine levels have not been well explored. In this work, differences in DNA methylation in cytokine- and hepcidin-encoding genes are found to associate with levels of their protein products in cord samples. Several significant correlations, both positive and negative, were noted between the cord protein concentration of TNFα, hepcidin, IL-10, TNF-RI, and TNF-RII, and the levels of methylation at different CpG sites within their respective genes. Interestingly, methylation at different sites within the introns, exons, and UTR portions of several genes showed significant correlations with protein levels, as well as sites within the promoter regions. Moreover, of the promoter sites that showed a correlation with protein levels, several showed a *positive* correlation. These data bolster claims that that the general model of transcriptional suppression

by promoter methylation may be incomplete [246], and the actual relationships between gene transcription and methylation more complex.

Epigenetic studies frequently find that sex has a modifying effect in both human studies and rodent models [257-259, 279], although the reasons for this are not yet fully understood. TNFα protein-methylation correlations were prominently skewed by sex: strong correlations existed between many CpG sites' methylation and TNFα cord values, but almost entirely in male neonates. Hepcidin also showed a modifying effect of sex: a significant correlation between methylation at one site was significantly associated with protein levels in females but not males. The proximal reason for an epigenetic sex bias in these genes is unclear: *TNFα* and *HAMP* are on autosomal chromosomes.

PBMC are frequently used in epigenetic studies due to the relative ease of sample collection; however, multiple authors have suggested that methylation analyses should be performed on sorted cell populations due to differences between DNA methylation in different cell types [262], or that computational analyses should be used to determine PBMC compositions from epigenetic data [280]. We attempted to address this issue by analyzing cell composition (in terms of %lymphocytes %granulocytes, and %mid-range cells) and whether cell composition was associated with methylation at key CpG sites. Cell composition did not correlate with methylation at the majority of sites examined, with the notable exception of three sites in the *TNF-RI* gene. PBMC samples with a greater % of lymphocytes showed an increase in methylation at all three sites in the exons of the *TNF-RI* gene, suggesting that *TNF-RI* gene body CpG sites may be more methylated in lymphocyte cells than

in granulocytes, although we were not able to find previous reports of this in the literature. Notably, the lack of any association observed between methylation status in other genes' probe sites and cell composition indicates that the other 15 correlations observed between cytokines' methylation status and protein levels are not likely due to changes in cellular composition. However, other published studies have been able to subdivide cells into different and more precise categories by using a variety of cellular markers [262], and the possibility cannot be excluded that a more subtle shift in composition of PBMC is responsible for the apparent differences in methylation.

Finally, multiple studies in populations affected by malaria, similar to this particular cohort, have documented polymorphisms in many of the cytokine-encoding genes described above (some examples: [281-283]). SNPs at the site of a CpG probe can affect methylation levels [263], even if the SNP is not precisely at the site of probe binding [284-286]. The dbSNP database has records of SNPs at a significant subset of this study's probe sites of interest.

Some authors [264] have opined that the most appropriate action to take with Illumina 450k probes that may harbor CpG site SNPs is to exclude them from analysis altogether. However, we would posit that, in this particular case, exclusion of all the CpG probes that may harbor SNPs in their target sequence is too general a prohibition for both technical and biological reasons. Technically, the dbSNP database is an evolving resource, our knowledge of human genetic diversity is incomplete, and the SNPs currently recorded in the dbSNP database may not always apply to our specific Tanzanian population. Secondly, SNPs occur in genes under

selective pressure; variation in methylation may occur in similar locations for the same reason. To exclude sites that may harbor SNPs risks excluding the methylation changes that may of be most important biological significance independent of genotype. Therefore, instead of excluding probes that may be associated with SNPs, at the time of writing, work is underway to genotype our population at pertinent sites in the cytokine and hepcidin genes, to permit direct comparison of genotype and epigenotype at these important loci. These studies should enable us to ascertain whether epigenotype-protein correlations may be truly attributable to genetic differences, or whether the effects observed represent truly novel associations of epigenetic changes with protein levels.

In summary, these data present the first large-scale study of hepcidin levels in cord blood samples from a cohort study in which malaria is endemic. Cord levels of hepcidin were increased in neonates whose mothers had experienced placental malaria. Cord hepcidin correlated with neonatal levels of some iron markers and cytokines, but did not predict future hepcidin levels or clinical outcomes. Epigenetic difference in cytokine and hepcidin-encoding genes and their correlation with their protein products are also presented. These preliminary results still must be validated by genetic sequencing of crucial loci, but this work is a first step towards the establishment of an epigenetic contribution to neonatal cytokine levels and subsequent clinical outcomes.

CHAPTER 7: DISCUSSION

While iron supplementation or iron replete status are linked with malaria susceptibility, malaria infection can precipitate potentially life-threatening anemia. Hepcidin, the master controller of iron metabolism, has been shown to increase in uncomplicated malaria infection, and may contribute to the dyserythropoiesis and anemia of malaria, modulate susceptibility to superinfection, and inhibit the utilization of oral iron.

On the global level, malaria remains a major cause of anemia, and pharmacologic means of alleviating anemia during or following malaria infection are badly needed, for use in conjunction with the appropriate antimalarial treatments. A first step towards the development of these agents is an improved understanding of the mechanisms behind the hepcidin increase during malaria infection, and the bulk of the data presented in this thesis are aimed at addressing that goal.

Hepcidin increases in the context of non-malarial infections have been primarily found to be associated with an increase in the activity of the Stat3 signaling pathway; however, the evidence from a murine model of malaria infection presented in Chapter 2 challenges this prevailing view. No correlation was observed between multiple indicators of Stat3 pathway activity and hepcidin expression in this model. Conversely, the Smad signaling pathway indicator gene *Id1* was co-upregulated with hepcidin during increased parasitemia. These data may contrast with previous studies of hepcidin upregulation in the context of other murine mouse infections [62], but they agree indirectly with a study that suggests that hepcidin

increases post-LPS administration may be modulated through the Smad signaling pathway [93], and confirm previous reports of *Id1* upregulation in malarial infection [67].

Further studies in the same model revealed that despite the apparent involvement of the Smad signaling pathway in hepcidin increase in infection, *Bmp* genes themselves were either unchanged or downregulated in multiple tissues at the message level. However, a recent report was published during this work that detailed the involvement of TGFβ superfamily member activin B on hepcidin upregulation via the Smad signaling pathway, in an inflammatory context [93]. In our model of malaria infection, hepatic expression of activin B was increased and correlated with *Id1*.

The upregulation of hepcidin by activin B and activin A have only been described in a single report each, and the two studies were performed in different human hepatoma cell lines [84, 93]. The experiments presented in chapter 3 confirm these reports and show that both activin A and B upregulate hepcidin in HepG2 hepatoma cells and in healthy iron-deprived mice, adding to the plausibility of an effect of activins on hepcidin during malaria infection.

To test the applicability of these *in vitro* and animal model findings to human infection, samples were obtained from human CHMI experiments. Hepcidin protein and activin A were found to co-increase in the serum of volunteers during *P. falciparum* infection. The changes in hepcidin and activin A during infection were significantly correlated between volunteers; indeed, this correlation was more pronounced than were the correlations between hepcidin and changes in acute

phase markers ferritin or CRP. Taken together, the data presented in the first part of this thesis indicate a likely role for activin proteins in hepcidin upregulation in the context of malaria infection, via Smad signaling.

A secondary aim in this thesis was to attempt to clarify what malarial PAMP is recognized by circulating PBMC and initiates the systemic cytokine production that eventually leads to hepcidin upregulation. iRBC-derived microparticles from rodent parasite *P. berghei* have previously been described as cytokine-stimulatory [218, 219]. A single study has shown a cytokine-stimulatory role for *P. falciparum* iRBC-derived microparticles; this study also demonstrated that microparticles may have a role in promoting gametocytogenesis in *P. falciparum* culture. The data presented in Chapter 5 demonstrate that microparticles derived from *P. falciparum* iRBC elicit *IL-6* and *TNFα* upregulation from PBMC. This upregulation is partially inhibited by the endosomal acidification inhibitor bafilomycin A, adding further evidence to previous studies that have shown a role for endosomal TLR in recognition of malaria infection [209-211]. Pilot studies indicate that iRBC-derived microparticles elicit message-level increases of *INHBA* from PBMC. Additionally, reanalysis of a published experiment [132] showed that PBMC also upregulate *INHBA* when co-cultured with whole iRBC. Together, these data indicate that iRBC-derived microparticles can act as a trigger of cytokine upregulation in malaria infection, possibly via recognition by an endosomal TLR. These findings also suggest a solution to the conundrum set up by data presented in Chapters 2 and 4, in which activin A protein was found to increase in human serum but to decrease at the

message level in the livers of malaria-infected mice. The increased levels of activin A protein noted during human infection may come from circulating PBMC.

What is the relative importance of activin B and activin A upregulation in malaria, and what physiological source does each protein come from? Due to technical limitations, discussed fully in Chapter 5, we have only been able to form an incomplete picture of how activins change in human malaria infection and in murine models. Activin B message increases in the livers of malaria-infected mice, and activin A protein in the serum of human volunteers infected in CHMI trials. However, no data yet exists on the levels of both proteins in murine serum, and of activin B in human serum, during malaria infection. Measurement of these parameters is crucial for advancing our understanding of the precise roles of different activins. Additionally, the use of mice with organ-specific activin gene knockouts may help to shed light on this question. Moreover, the roles of activins C and E remain completely unexplored; neither is upregulated at the message level in the livers of mice injected with LPS [93], but nor is activin A, which increases in the serum presumably as a result of protein release or message-level upregulation in other tissues. Activin proteins C or E may also have an as-yet unexplored role in hepcidin control that can be examined by a combination of *in vitro* and *in vivo* studies.

Why should activins and the Smad signaling pathway be responsible for hepcidin upregulation in malaria infection, while in acute viral and fungal infections[62], the Stat3 signaling pathway appears to control hepcidin increases? Two theoretical possibilities are the need for hepcidin upregulation in a relatively

long term-rather than acute infection, and the need to upregulate hepcidin using the dominant Smad signaling pathway.

As opposed to the fungal and viral infections in which hepcidin upregulation was previously analyzed in murine models [62], malaria infection has a relatively indolent course. The mouse model of malaria infection presented in this manuscript requires eight days to exhibit hepcidin upregulation, and most mice are not outwardly symptomatic at that time. Children who live in highly malaria-endemic areas may be exposed to several hundred infective bites a year, have several episodes of febrile malaria, and frequently carry parasites asymptomatically as well [287]. Upregulation of inflammatory cytokines such as IL-6 occurs mostly during febrile episodes and, while likely critical for parasite control during some infections, also contributes to the development of life-threatening severe malaria. Moreover, a febrile malaria episode reduces the subsequent production of inflammatory cytokines in response to parasite stimuli, suggesting that production of IL-6 and other inflammatory cytokines are limited to protect the health of the host [288]. If hepcidin upregulation was contingent upon increased Stat3 signaling, repeated upregulation of IL-6 might be required, which could be harmful; perhaps Smad signaling by activins has therefore evolved to be a more tolerable primary method of controlling hepcidin in malaria infection. Against the theory that increased activins may be more tolerable than increased IL-6 is a murine study that shows that raised activin A following LPS injection in mice contributes to mortality [139], but this was not replicated in a following mouse study that treated mice with whole bacteria instead of LPS [170], and has not been yet shown in human studies.

Additionally, several studies have shown that Smad signaling is in some ways dominant to Stat3 signaling [94, 95]. Possibly due to the unique red blood cell tropism of the malaria parasite, malaria growth is closely tied to the iron status of the host [289]. Perhaps the need to efficiently control iron levels in a malaria infection requires the use of the most effective hepcidin upregulation pathway. It might be beneficial to discover if hepcidin upregulation is also controlled by this pathway in the context of other red blood cell-tropic parasitic infections, such as babeiosis.

What are the practical implications of activin protein involvement in hepcidin increase? The inhibition of hepcidin in conjunction with antimalarial treatment would likely permit more effective iron absorption and speed recovery from malarial anemia. Multiple methods are being explored to develop and test safe and effective anti-hepcidin treatments (reviewed in [290]), but none so far have achieved clinical use. Moreover, use of hepcidin-inhibiting drugs risks over-inhibition of hepcidin and over-raising iron levels (which itself may contribute to disease susceptibility). Drugs targeted at the factors upstream of hepcidin upregulation should only restore hepcidin to the levels appropriate for the host's iron status and therefore are a compelling alternative option for hepcidin control during or following malaria infection.

The activin-binding protein follistatin has been previously used in mouse models of acute inflammation and found to decrease mortality [139], and follistatin has been discussed as a potential clinical option to block activins in disease conditions [145]. Data presented in Chapter 3 describes the attempted use of

follistatin-315 to block activins' actions on hepcidin during malaria infection in a murine model. This series of experiments was affected by technical problems *in vivo*. Follistatin-315 blocked activin-mediated hepcidin and *Id1* upregulation *in vitro,* but had no effect on gene expression in untreated cells, indicating that activins likely do not control hepcidin outside of inflammation and infection. In subsequent murine experiments, follistatin administration did not show a notable effect on gene expression in uninfected mice. Consequently, when follistatin-315 failed to affect hepcidin expression in malaria-infected mice, it was not clear whether the protein was not biologically active, that the protein was active but cleared too quickly from the circulation to exert any meaningful effect, or whether follistatin-315 truly inhibited activin proteins but did not affect hepcidin expression. A promising alternative is the use of the activin trap sotatercept (in mice, RAP-011), discussed in Chapter 3. As both an ESA and a hypothesized repressor of activin-mediated hepcidin increase, sotatercept represents a promising option to speed recovery from malarial anemia, in conjunction with appropriate malaria treatments.

An additional aspect of hepcidin control in malaria infection requires further investigation. As stated in the Introduction, apparent hepcidin suppression has been noted in children with severe malarial anemia [103, 104]. One possible mechanistic explanation for this clinical observation is that increased erythropoietic drive leads to increased levels of the newly described hormone ERFE [117], which then overrides the activin-mediated upregulation of hepcidin when the anemia is sufficiently severe.

Infections with the murine parasite *Plasmodium chabaudi* have been well-described as a model of severe malarial anemia (reviewed in [291]), and recent preliminary work by our laboratory (Armitage et al, unpublished) has shown that *Erfe* message is increased in the bone marrow of *P. chabaudi*-infected mice, while hepatic hepcidin message is suppressed. At the time of writing, a collaborative experiment is planned to examine if hepcidin is suppressed in ERFE$^{-/-}$ mice during *P. chabaudi* infection. An additional test that should be completed is the measurement of ERFE protein levels in samples from patients with severe malarial anemia as compared to those with uncomplicated malaria infection. These experiments will increase our understanding of hepcidin suppression in severe infection, a counterpoint to the work presented here that delineates the mechanism of hepcidin upregulation. Therapeutic strategies should take the hepcidin suppression described into consideration: children with very severe malarial anemia (and presumably high ERFE) may not require hepcidin-repressing agents until parasitemia is successfully controlled and Hb levels increasing.

In addition to the different hepcidin responses in malaria infection, hepcidin in healthy individuals may partially determine baseline iron status, thereby affecting malaria susceptibility through modulation of the iron available to the parasite. A report has demonstrated that levels of some cytokines at birth may predict future cytokine levels and susceptibility to parasitemia and severe malaria [120]. Hepcidin levels in the cord blood of the same cohort are described in Chapter 6, in an attempt to identify any analogous effect. Hepcidin was found to vary by placental malaria status, with cord blood hepcidin significantly higher in cord blood from neonates

whose mothers had placental malaria. The precise implications of these data remain to be explored. Do neonates with high hepcidin levels absorb subsequent oral iron less effectively? Are activins involved in prenatal hepcidin upregulation in response to placental malaria?

Furthermore, which proximate factors determine the levels of cytokines and hepcidin in cord blood? Epigenetic alterations are a recently recognized contributor to disease risk. The 450k HumanMethylation Illumina assay was used to obtain methylation values for CpG sites throughout the genome, and changes in DNA methylation in the appropriate genes were compared with different cord protein levels of cytokines and hepcidin. These data, presented in Chapter 6, show multiple statistically significant correlations between methylation at different sites and with the protein level of the appropriate product. Interestingly, the direction of the correlations varied, with no obvious association by CpG location with positive or negative correlations. The epigenetic literature paints a complex portrait of how methylation at different gene sites may affect gene expression [246]; the data presented in this thesis suggests that methylation may have an important role in controlling cytokine and hepcidin levels at birth, but does not explain the precise mechanisms involved in transcriptional regulation by methylation at different sites. As the methylation assay can be affected by SNPs at probe sites, these findings must be considered in the light of full sequencing of gene regions of interest, a process that is now underway, but provisionally this represents a significant advance in our understanding of epigenetic control of neonatal cytokine and hepcidin levels.

Iron metabolism changes can be a part of malarial pathogenesis, while at the same time healthy iron levels may contribute to malaria susceptibility. Understanding the mechanisms involved in both phenomena will inform our treatments for malaria and our preventative measures against it. This thesis presents work in multiple systems that implicate recently described hepcidin control proteins, activin A and activin B, in the upregulation of hepcidin during malaria infection. The final data chapter of the thesis examines hepcidin cord levels, in a cohort from a malaria-endemic area, as a prognostic factor for future hepcidin levels and for clinical outcomes, and delves into the epigenetic mechanisms underlying neonatal levels of hepcidin and cytokines. The hepcidin-malaria relationships described herein may help to develop new therapeutics that improve recovery from anemia after malaria infection, and also shed light on innate immune defenses against this ancient human pathogen.

SOURCES CITED

1. Murray CJL RL, Lim SS, Andrews KG, Foreman KJ, Haring D, Fullman N, Naghavi M, Lozano R, Lopez ADL: **Global malaria mortality between 1980 and 2010: a systematic analysis.** *The Lancet* 2012, **379:**413-431.
2. Ojukwu JU, Okebe JU, Yahav D, Paul M: **Oral iron supplementation for preventing or treating anaemia among children in malaria-endemic areas.** *Cochrane Database Syst Rev* 2009**:**CD006589.
3. Okebe JU, Yahav D, Shbita R, Paul M: **Oral iron supplements for children in malaria-endemic areas.** *Cochrane database of systematic reviews* 2011**:**CD006589.
4. Kassebaum NJ, Jasrasaria R, Naghavi M, Wulf SK, Johns N, Lozano R, Regan M, Weatherall D, Chou DP, Eisele TP, et al: **A systematic analysis of global anemia burden from 1990 to 2010.** *Blood* 2013.
5. Meremikwu MM, Donegan S, Sinclair D, Esu E, Oringanje C: **Intermittent preventive treatment for malaria in children living in areas with seasonal transmission.** *Cochrane Database Syst Rev* 2012, **2:**CD003756.
6. Oppenheimer SJ, Gibson FD, Macfarlane SB, Moody JB, Hendrickse RG: **Iron supplementation and malaria.** *Lancet* 1984, **1:**389-390.
7. Oppenheimer SJ, Macfarlane SB, Moody JB, Harrison C: **Total dose iron infusion, malaria and pregnancy in Papua New Guinea.** *Transactions of the Royal Society of Tropical Medicine and Hygiene* 1986, **80:**818-822.
8. Murray MJ, Murray NJ, Murray AB, Murray MB: **Refeeding-malaria and hyperferraemia.** *Lancet* 1975, **1:**653-654.
9. Murray MJ, Murray AB, Murray MB, Murray CJ: **The adverse effect of iron repletion on the course of certain infections.** *British medical journal* 1978, **2:**1113-1115.
10. Murray MJ, Murray AB, Murray CJ: **An ecological interdependence of diet and disease? A study of infection in one tribe consuming two different diets.** *Am J Clin Nutr* 1980, **33:**697-701.
11. Sazawal S, Black RE, Ramsan M, Chwaya HM, Stoltzfus RJ, Dutta A, Dhingra U, Kabole I, Deb S, Othman MK, Kabole FM: **Effects of routine prophylactic supplementation with iron and folic acid on admission to hospital and mortality in preschool children in a high malaria transmission setting: community-based, randomised, placebo-controlled trial.** *Lancet* 2006, **367:**133-143.
12. Desai MR, Mei JV, Kariuki SK, Wannemuehler KA, Phillips-Howard PA, Nahlen BL, Kager PA, Vulule JM, ter Kuile FO: **Randomized, controlled trial of daily iron supplementation and intermittent sulfadoxine-pyrimethamine for the treatment of mild childhood anemia in western Kenya.** *The Journal of infectious diseases* 2003, **187:**658-666.
13. Ouedraogo HZ, Dramaix-Wilmet M, Zeba AN, Hennart P, Donnen P: **Effect of iron or multiple micronutrient supplements on the prevalence of anaemia among anaemic young children of a malaria-endemic area: a**

randomized double-blind trial. *Tropical medicine & international health : TM & IH* 2008, **13:**1257-1266.

14. Roth DE BR, Ojukwu JU, Okebe JU, Yahav D, Paul M. : **Commentary on 'Oral iron supplementation for preventing or treating anaemia amoung children in malaria-endemic areas' with a response from the review authors. .** *Evidence-Based Child Health: A Cochrane Review Journal* 2010, **5:**1186-1188.
15. Spottiswoode N, Fried M, Drakesmith H, Duffy PE: **Implications of malaria on iron deficiency control strategies.** *Adv Nutr* 2012, **3:**570-578.
16. Zlotkin S, Newton S, Aimone AM, Azindow I, Amenga-Etego S, Tchum K, Mahama E, Thorpe KE, Owusu-Agyei S: **Effect of iron fortification on malaria incidence in infants and young children in Ghana: a randomized trial.** *JAMA : the journal of the American Medical Association* 2013, **310:**938-947.
17. Prentice AM, Verhoef H, Cerami C: **Iron fortification and malaria risk in children.** *JAMA : the journal of the American Medical Association* 2013, **310:**914-915.
18. Soofi S, Cousens S, Iqbal SP, Akhund T, Khan J, Ahmed I, Zaidi AK, Bhutta ZA: **Effect of provision of daily zinc and iron with several micronutrients on growth and morbidity among young children in Pakistan: a cluster-randomised trial.** *Lancet* 2013, **382:**29-40.
19. Veenemans J, Mank T, Ottenhof M, Baidjoe A, Mbugi EV, Demir AY, Wielders JP, Savelkoul HF, Verhoef H: **Protection against diarrhea associated with Giardia intestinalis Is lost with multi-nutrient supplementation: a study in Tanzanian children.** *PLoS neglected tropical diseases* 2011, **5:**e1158.
20. Tielsch JM, Khatry SK, Stoltzfus RJ, Katz J, LeClerq SC, Adhikari R, Mullany LC, Shresta S, Black RE: **Effect of routine prophylactic supplementation with iron and folic acid on preschool child mortality in southern Nepal: community-based, cluster-randomised, placebo-controlled trial.** *Lancet* 2006, **367:**144-152.
21. Chen K, Zhang X, Li TY, Chen L, Wei XP, Qu P, Liu YX: **Effect of vitamin A, vitamin A plus iron and multiple micronutrient-fortified seasoning powder on infectious morbidity of preschool children.** *Nutrition* 2011, **27:**428-434.
22. Zimmermann MB, Chassard C, Rohner F, N'Goran E K, Nindjin C, Dostal A, Utzinger J, Ghattas H, Lacroix C, Hurrell RF: **The effects of iron fortification on the gut microbiota in African children: a randomized controlled trial in Cote d'Ivoire.** *Am J Clin Nutr* 2010, **92:**1406-1415.
23. Litton E, Xiao J, Ho KM: **Safety and efficacy of intravenous iron therapy in reducing requirement for allogeneic blood transfusion: systematic review and meta-analysis of randomised clinical trials.** *BMJ* 2013, **347:**f4822.
24. Gwamaka M, Kurtis JD, Sorensen BE, Holte S, Morrison R, Mutabingwa TK, Fried M, Duffy PE: **Iron deficiency protects against severe Plasmodium falciparum malaria and death in young children.** *Clin Infect Dis* 2012, **54:**1137-1144.

25. Rouault TA: **The role of iron regulatory proteins in mammalian iron homeostasis and disease.** *Nat Chem Biol* 2006, **2:**406-414.
26. Torti FM, Torti SV: **Regulation of ferritin genes and protein.** *Blood* 2002, **99:**3505-3516.
27. Nyakeriga AM, Troye-Blomberg M, Dorfman JR, Alexander ND, Back R, Kortok M, Chemtai AK, Marsh K, Williams TN: **Iron deficiency and malaria among children living on the coast of Kenya.** *J Infect Dis* 2004, **190:**439-447.
28. Jonker FA, Calis JC, van Hensbroek MB, Phiri K, Geskus RB, Brabin BJ, Leenstra T: **Iron status predicts malaria risk in Malawian preschool children.** *PLoS One* 2012, **7:**e42670.
29. Senga EL, Harper G, Koshy G, Kazembe PN, Brabin BJ: **Reduced risk for placental malaria in iron deficient women.** *Malar J* 2011, **10:**47.
30. Kabyemela ER, Fried M, Kurtis JD, Mutabingwa TK, Duffy PE: **Decreased susceptibility to Plasmodium falciparum infection in pregnant women with iron deficiency.** *J Infect Dis* 2008, **198:**163-166.
31. Senga EL, Koshy G, Brabin BJ: **Zinc erythrocyte protoporphyrin as marker of malaria risk in pregnancy - a retrospective cross-sectional and longitudinal study.** *Malar J* 2012, **11:**249.
32. Lamola AA, Yamane T: **Zinc protoporphyrin in the erythrocytes of patients with lead intoxication and iron deficiency anemia.** *Science* 1974, **186:**936-938.
33. Kung'u JK, Wright VJ, Haji HJ, Ramsan M, Goodman D, Tielsch JM, Bickle QD, Raynes JG, Stoltzfus RJ: **Adjusting for the acute phase response is essential to interpret iron status indicators among young Zanzibari children prone to chronic malaria and helminth infections.** *J Nutr* 2009, **139:**2124-2131.
34. Clark MA, Goheen MM, Cerami C: **Influence of host iron status on Plasmodium falciparum infection.** *Front Pharmacol* 2014, **5:**84.
35. Manning L, Laman M, Rosanas-Urgell A, Michon P, Aipit S, Bona C, Siba P, Mueller I, Davis TM: **Severe anemia in Papua New Guinean children from a malaria-endemic area: a case-control etiologic study.** *PLoS neglected tropical diseases* 2012, **6:**e1972.
36. Raventos-Suarez C, Pollack S, Nagel RL: **Plasmodium falciparum: inhibition of in vitro growth by desferrioxamine.** *Am J Trop Med Hyg* 1982, **31:**919-922.
37. Ferrer P, Tripathi AK, Clark MA, Hand CC, Rienhoff HY, Jr., Sullivan DJ, Jr.: **Antimalarial iron chelator, FBS0701, shows asexual and gametocyte Plasmodium falciparum activity and single oral dose cure in a murine malaria model.** *PLoS One* 2012, **7:**e37171.
38. Fritsch G, Treumer J, Spira DT, Jung A: **Plasmodium vinckei: suppression of mouse infections with desferrioxamine B.** *Experimental parasitology* 1985, **60:**171-174.
39. Pollack S, Rossan RN, Davidson DE, Escajadillo A: **Desferrioxamine suppresses Plasmodium falciparum in Aotus monkeys.** *Proc Soc Exp Biol Med* 1987, **184:**162-164.

40. Mabeza GF, Loyevsky M, Gordeuk VR, Weiss G: **Iron chelation therapy for malaria: a review.** *Pharmacol Ther* 1999, **81:**53-75.
41. Traore O, Carnevale P, Kaptue-Noche L, M'Bede J, Desfontaine M, Elion J, Labie D, Nagel RL: **Preliminary report on the use of desferrioxamine in the treatment of Plasmodium falciparum malaria.** *American journal of hematology* 1991, **37:**206-208.
42. Thuma PE, Mabeza GF, Biemba G, Bhat GJ, McLaren CE, Moyo VM, Zulu S, Khumalo H, Mabeza P, M'Hango A, et al: **Effect of iron chelation therapy on mortality in Zambian children with cerebral malaria.** *Transactions of the Royal Society of Tropical Medicine and Hygiene* 1998, **92:**214-218.
43. Smith HJ, Meremikwu M: **Iron chelating agents for treating malaria.** *Cochrane Database Syst Rev* 2003**:**CD001474.
44. Rodriguez MH, Jungery M: **A protein on Plasmodium falciparum-infected erythrocytes functions as a transferrin receptor.** *Nature* 1986, **324:**388-391.
45. Hershko C, Peto TE: **Deferoxamine inhibition of malaria is independent of host iron status.** *J Exp Med* 1988, **168:**375-387.
46. McDermid JM, Prentice AM: **Iron and infection: effects of host iron status and the iron-regulatory genes haptoglobin and NRAMP1 (SLC11A1) on host-pathogen interactions in tuberculosis and HIV.** *Clin Sci (Lond)* 2006, **110:**503-524.
47. Matsuzaki-Moriya C, Tu L, Ishida H, Imai T, Suzue K, Hirai M, Tetsutani K, Hamano S, Shimokawa C, Hisaeda H: **A critical role for phagocytosis in resistance to malaria in iron-deficient mice.** *European journal of immunology* 2011, **41:**1365-1375.
48. Nweneka CV, Doherty CP, Cox S, Prentice A: **Iron delocalisation in the pathogenesis of malarial anaemia.** *Transactions of the Royal Society of Tropical Medicine and Hygiene* 2010, **104:**175-184.
49. Abboud S, Haile DJ: **A novel mammalian iron-regulated protein involved in intracellular iron metabolism.** *J Biol Chem* 2000, **275:**19906-19912.
50. Donovan A, Brownlie A, Zhou Y, Shepard J, Pratt SJ, Moynihan J, Paw BH, Drejer A, Barut B, Zapata A, et al: **Positional cloning of zebrafish ferroportin1 identifies a conserved vertebrate iron exporter.** *Nature* 2000, **403:**776-781.
51. McKie AT, Marciani P, Rolfs A, Brennan K, Wehr K, Barrow D, Miret S, Bomford A, Peters TJ, Farzaneh F, et al: **A novel duodenal iron-regulated transporter, IREG1, implicated in the basolateral transfer of iron to the circulation.** *Molecular cell* 2000, **5:**299-309.
52. Pigeon C, Ilyin G, Courselaud B, Leroyer P, Turlin B, Brissot P, Loreal O: **A new mouse liver-specific gene, encoding a protein homologous to human antimicrobial peptide hepcidin, is overexpressed during iron overload.** *The Journal of biological chemistry* 2001, **276:**7811-7819.
53. Krause A, Neitz S, Magert HJ, Schulz A, Forssmann WG, Schulz-Knappe P, Adermann K: **LEAP-1, a novel highly disulfide-bonded human peptide, exhibits antimicrobial activity.** *FEBS Lett* 2000, **480:**147-150.

54. Park CH, Valore EV, Waring AJ, Ganz T: **Hepcidin, a urinary antimicrobial peptide synthesized in the liver.** *J Biol Chem* 2001, **276:**7806-7810.
55. Nemeth E, Tuttle MS, Powelson J, Vaughn MB, Donovan A, Ward DM, Ganz T, Kaplan J: **Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization.** *Science* 2004, **306:**2090-2093.
56. Nicolas G, Bennoun M, Devaux I, Beaumont C, Grandchamp B, Kahn A, Vaulont S: **Lack of hepcidin gene expression and severe tissue iron overload in upstream stimulatory factor 2 (USF2) knockout mice.** *Proc Natl Acad Sci U S A* 2001, **98:**8780-8785.
57. Roetto A, Papanikolaou G, Politou M, Alberti F, Girelli D, Christakis J, Loukopoulos D, Camaschella C: **Mutant antimicrobial peptide hepcidin is associated with severe juvenile hemochromatosis.** *Nat Genet* 2003, **33:**21-22.
58. Bridle KR, Frazer DM, Wilkins SJ, Dixon JL, Purdie DM, Crawford DH, Subramaniam VN, Powell LW, Anderson GJ, Ramm GA: **Disrupted hepcidin regulation in HFE-associated haemochromatosis and the liver as a regulator of body iron homoeostasis.** *Lancet* 2003, **361:**669-673.
59. Drakesmith H, Schimanski LM, Ormerod E, Merryweather-Clarke AT, Viprakasit V, Edwards JP, Sweetland E, Bastin JM, Cowley D, Chinthammitr Y, et al: **Resistance to hepcidin is conferred by hemochromatosis-associated mutations of ferroportin.** *Blood* 2005, **106:**1092-1097.
60. Nicolas G, Bennoun M, Porteu A, Mativet S, Beaumont C, Grandchamp B, Sirito M, Sawadogo M, Kahn A, Vaulont S: **Severe iron deficiency anemia in transgenic mice expressing liver hepcidin.** *Proceedings of the National Academy of Sciences of the United States of America* 2002, **99:**4596-4601.
61. Finberg KE, Heeney MM, Campagna DR, Aydinok Y, Pearson HA, Hartman KR, Mayo MM, Samuel SM, Strouse JJ, Markianos K, et al: **Mutations in TMPRSS6 cause iron-refractory iron deficiency anemia (IRIDA).** *Nat Genet* 2008, **40:**569-571.
62. Armitage AE, Eddowes LA, Gileadi U, Cole S, Spottiswoode N, Selvakumar TA, Ho LP, Townsend AR, Drakesmith H: **Hepcidin regulation by innate immune and infectious stimuli.** *Blood* 2011.
63. Howard CT, McKakpo US, Quakyi IA, Bosompem KM, Addison EA, Sun K, Sullivan D, Semba RD: **Relationship of hepcidin with parasitemia and anemia among patients with uncomplicated Plasmodium falciparum malaria in Ghana.** *Am J Trop Med Hyg* 2007, **77:**623-626.
64. de Mast Q, Syafruddin D, Keijmel S, Riekerink TO, Deky O, Asih PB, Swinkels DW, van der Ven AJ: **Increased serum hepcidin and alterations in blood iron parameters associated with asymptomatic P. falciparum and P. vivax malaria.** *Haematologica* 2010, **95:**1068-1074.
65. de Mast Q, Nadjm B, Reyburn H, Kemna EH, Amos B, Laarakkers CM, Silalye S, Verhoef H, Sauerwein RW, Swinkels DW, van der Ven AJ: **Assessment of urinary concentrations of hepcidin provides novel insight into disturbances in iron homeostasis during malarial infection.** *J Infect Dis* 2009, **199:**253-262.

66. de Mast Q, van Dongen-Lases EC, Swinkels DW, Nieman AE, Roestenberg M, Druilhe P, Arens TA, Luty AJ, Hermsen CC, Sauerwein RW, van der Ven AJ: **Mild increases in serum hepcidin and interleukin-6 concentrations impair iron incorporation in haemoglobin during an experimental human malaria infection.** *Br J Haematol* 2009, **145:**657-664.
67. Portugal S, Carret C, Recker M, Armitage AE, Goncalves LA, Epiphanio S, Sullivan D, Roy C, Newbold CI, Drakesmith H, Mota MM: **Host-mediated regulation of superinfection in malaria.** *Nature medicine* 2011, **17:**732-737.
68. Wang HZ, He YX, Yang CJ, Zhou W, Zou CG: **Hepcidin is regulated during blood-stage malaria and plays a protective role in malaria infection.** *Journal of immunology* 2011, **187:**6410-6416.
69. Cercamondi CI, Egli IM, Ahouandjinou E, Dossa R, Zeder C, Salami L, Tjalsma H, Wiegerinck E, Tanno T, Hurrell RF, et al: **Afebrile Plasmodium falciparum parasitemia decreases absorption of fortification iron but does not affect systemic iron utilization: a double stable-isotope study in young Beninese women.** *The American journal of clinical nutrition* 2010, **92:**1385-1392.
70. Prentice AM, Doherty CP, Abrams SA, Cox SE, Atkinson SH, Verhoef H, Armitage AE, Drakesmith H: **Hepcidin is the major predictor of erythrocyte iron incorporation in anemic African children.** *Blood* 2012.
71. Doherty CP, Cox SE, Fulford AJ, Austin S, Hilmers DC, Abrams SA, Prentice AM: **Iron incorporation and post-malaria anaemia.** *PLoS One* 2008, **3:**e2133.
72. Theurl M, Nairz M, Schroll A, Sonnweber T, M AB, Haschka D, Seifert M, Willenbacher W, Wilflingseder D, Posch W, et al: **Hepcidin as a predictive factor and therapeutic target in erythropoiesis-stimulating agent treatment for anemia of chronic disease in rats.** *Haematologica* 2014.
73. Atkinson SH, Armitage AE, Khandwala S, Mwangi TW, Uyoga S, Bejon PA, Williams TN, Prentice AM, Drakesmith H: **Combinatorial effects of malaria season, iron deficiency and inflammation determine plasma hepcidin concentration in African children.** *Blood* 2014.
74. van Santen S, de Mast Q, Swinkels DW, van der Ven AJ: **The iron link between malaria and invasive non-typhoid Salmonella infections.** *Trends in parasitology* 2013, **29:**220-227.
75. Mabey DC, Brown A, Greenwood BM: **Plasmodium falciparum malaria and Salmonella infections in Gambian children.** *J Infect Dis* 1987, **155:**1319-1321.
76. Cunnington AJ, de Souza JB, Walther M, Riley EM: **Malaria impairs resistance to Salmonella through heme- and heme oxygenase-dependent dysfunctional granulocyte mobilization.** *Nat Med* 2012, **18:**120-127.
77. Nicolas G, Chauvet C, Viatte L, Danan JL, Bigard X, Devaux I, Beaumont C, Kahn A, Vaulont S: **The gene encoding the iron regulatory peptide hepcidin is regulated by anemia, hypoxia, and inflammation.** *J Clin Invest* 2002, **110:**1037-1044.

78. Corradini E, Meynard D, Wu Q, Chen S, Ventura P, Pietrangelo A, Babitt JL: **Serum and liver iron differently regulate the bone morphogenetic protein 6 (BMP6)-SMAD signaling pathway in mice.** *Hepatology* 2011, **54:**273-284.
79. Babitt JL, Huang FW, Wrighting DM, Xia Y, Sidis Y, Samad TA, Campagna JA, Chung RT, Schneyer AL, Woolf CJ, et al: **Bone morphogenetic protein signaling by hemojuvelin regulates hepcidin expression.** *Nat Genet* 2006, **38:**531-539.
80. Wang RH, Li C, Xu X, Zheng Y, Xiao C, Zerfas P, Cooperman S, Eckhaus M, Rouault T, Mishra L, Deng CX: **A role of SMAD4 in iron metabolism through the positive regulation of hepcidin expression.** *Cell metabolism* 2005, **2:**399-409.
81. Meynard D, Kautz L, Darnaud V, Canonne-Hergaux F, Coppin H, Roth MP: **Lack of the bone morphogenetic protein BMP6 induces massive iron overload.** *Nature genetics* 2009, **41:**478-481.
82. Andriopoulos B, Jr., Corradini E, Xia Y, Faasse SA, Chen S, Grgurevic L, Knutson MD, Pietrangelo A, Vukicevic S, Lin HY, Babitt JL: **BMP6 is a key endogenous regulator of hepcidin expression and iron metabolism.** *Nature genetics* 2009, **41:**482-487.
83. Truksa J, Peng H, Lee P, Beutler E: **Bone morphogenetic proteins 2, 4, and 9 stimulate murine hepcidin 1 expression independently of Hfe, transferrin receptor 2 (Tfr2), and IL-6.** *Proc Natl Acad Sci U S A* 2006, **103:**10289-10293.
84. Babitt JL, Huang FW, Xia Y, Sidis Y, Andrews NC, Lin HY: **Modulation of bone morphogenetic protein signaling in vivo regulates systemic iron balance.** *J Clin Invest* 2007, **117:**1933-1939.
85. Silvestri L, Pagani A, Nai A, De Domenico I, Kaplan J, Camaschella C: **The serine protease matriptase-2 (TMPRSS6) inhibits hepcidin activation by cleaving membrane hemojuvelin.** *Cell metabolism* 2008, **8:**502-511.
86. Du X, She E, Gelbart T, Truksa J, Lee P, Xia Y, Khovananth K, Mudd S, Mann N, Moresco EM, et al: **The serine protease TMPRSS6 is required to sense iron deficiency.** *Science* 2008, **320:**1088-1092.
87. Silvestri L, Guillem F, Pagani A, Nai A, Oudin C, Silva M, Toutain F, Kannengiesser C, Beaumont C, Camaschella C, Grandchamp B: **Molecular mechanisms of the defective hepcidin inhibition in TMPRSS6 mutations associated with iron-refractory iron deficiency anemia.** *Blood* 2009, **113:**5605-5608.
88. Melis MA, Cau M, Congiu R, Sole G, Barella S, Cao A, Westerman M, Cazzola M, Galanello R: **A mutation in the TMPRSS6 gene, encoding a transmembrane serine protease that suppresses hepcidin production, in familial iron deficiency anemia refractory to oral iron.** *Haematologica* 2008, **93:**1473-1479.
89. Nemeth E, Valore EV, Territo M, Schiller G, Lichtenstein A, Ganz T: **Hepcidin, a putative mediator of anemia of inflammation, is a type II acute-phase protein.** *Blood* 2003, **101:**2461-2463.

90. Nemeth E, Rivera S, Gabayan V, Keller C, Taudorf S, Pedersen BK, Ganz T: **IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin.** *J Clin Invest* 2004, **113:**1271-1276.
91. Verga Falzacappa MV, Vujic Spasic M, Kessler R, Stolte J, Hentze MW, Muckenthaler MU: **STAT3 mediates hepatic hepcidin expression and its inflammatory stimulation.** *Blood* 2007, **109:**353-358.
92. Wrighting DM, Andrews NC: **Interleukin-6 induces hepcidin expression through STAT3.** *Blood* 2006, **108:**3204-3209.
93. Besson-Fournier C, Latour C, Kautz L, Bertrand J, Ganz T, Roth MP, Coppin H: **Induction of activin B by inflammatory stimuli up-regulates expression of the iron-regulatory peptide hepcidin through Smad1/5/8 signaling.** *Blood* 2012, **120:**431-439.
94. Mayeur C, Lohmeyer LK, Leyton P, Kao SM, Pappas AE, Kolodziej SA, Spagnolli E, Yu B, Galdos RL, Yu PB, et al: **The type I BMP receptor, Alk3, is required for the induction of hepatic hepcidin gene expression by interleukin-6.** *Blood* 2014.
95. Steinbicker AU, Sachidanandan C, Vonner AJ, Yusuf RZ, Deng DY, Lai CS, Rauwerdink KM, Winn JC, Saez B, Cook CM, et al: **Inhibition of bone morphogenetic protein signaling attenuates anemia associated with inflammation.** *Blood* 2011, **117:**4915-4923.
96. Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, Lopez R, McWilliam H, Remmert M, Soding J, et al: **Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega.** *Mol Syst Biol* 2011, **7:**539.
97. Ramey G, Deschemin JC, Vaulont S: **Cross-talk between the mitogen activated protein kinase and bone morphogenetic protein/hemojuvelin pathways is required for the induction of hepcidin by holotransferrin in primary mouse hepatocytes.** *Haematologica* 2009, **94:**765-772.
98. Poli M, Luscieti S, Gandini V, Maccarinelli F, Finazzi D, Silvestri L, Roetto A, Arosio P: **Transferrin receptor 2 and HFE regulate furin expression via mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/Erk) signaling. Implications for transferrin-dependent hepcidin regulation.** *Haematologica* 2010, **95:**1832-1840.
99. Wallace DF, Summerville L, Crampton EM, Frazer DM, Anderson GJ, Subramaniam VN: **Combined deletion of Hfe and transferrin receptor 2 in mice leads to marked dysregulation of hepcidin and iron overload.** *Hepatology* 2009, **50:**1992-2000.
100. Oliveira SJ, Pinto JP, Picarote G, Costa VM, Carvalho F, Rangel M, de Sousa M, de Almeida SF: **ER stress-inducible factor CHOP affects the expression of hepcidin by modulating C/EBPalpha activity.** *PLoS One* 2009, **4:**e6618.
101. Vecchi C, Montosi G, Zhang K, Lamberti I, Duncan SA, Kaufman RJ, Pietrangelo A: **ER stress controls iron metabolism through induction of hepcidin.** *Science* 2009, **325:**877-880.
102. Mleczko-Sanecka K, Roche F, da Silva AR, Call D, D'Alessio F, Ragab A, Lapinski PE, Ummanni R, Korf U, Oakes C, et al: **Unbiased RNAi screen for hepcidin regulators links hepcidin suppression to proliferative**

Ras/RAF and nutrient-dependent mTOR signaling. *Blood* 2014, **123:**1574-1585.

103. Burte F, Brown BJ, Orimadegun AE, Ajetunmobi WA, Afolabi NK, Akinkunmi F, Kowobari O, Omokhodion S, Osinusi K, Akinbami FO, et al: **Circulatory hepcidin is associated with the anti-inflammatory response but not with iron or anemic status in childhood malaria.** *Blood* 2013, **121:**3016-3022.
104. Casals-Pascual C, Huang H, Lakhal-Littleton S, Thezenas ML, Kai O, Newton CR, Roberts DJ: **Hepcidin demonstrates a biphasic association with anemia in acute Plasmodium falciparum malaria.** *Haematologica* 2012, **97:**1695-1698.
105. Jonker FA, Calis JC, Phiri K, Kraaijenhagen RJ, Brabin BJ, Faragher B, Wiegerinck ET, Tjalsma H, Swinkels DW, Boele van Hensbroek M: **Low hepcidin levels in severely anemic malawian children with high incidence of infectious diseases and bone marrow iron deficiency.** *PLoS One* 2013, **8:**e78964.
106. Peyssonnaux C, Zinkernagel AS, Schuepbach RA, Rankin E, Vaulont S, Haase VH, Nizet V, Johnson RS: **Regulation of iron homeostasis by the hypoxia-inducible transcription factors (HIFs).** *J Clin Invest* 2007, **117:**1926-1932.
107. Sonnweber T, Nachbaur D, Schroll A, Nairz M, Seifert M, Demetz E, Haschka D, Mitterstiller AM, Kleinsasser A, Burtscher M, et al: **Hypoxia induced downregulation of hepcidin is mediated by platelet derived growth factor BB.** *Gut* 2014.
108. Liu Q, Davidoff O, Niss K, Haase VH: **Hypoxia-inducible factor regulates hepcidin via erythropoietin-induced erythropoiesis.** *J Clin Invest* 2012, **122:**4635-4644.
109. Nicolas G, Viatte L, Bennoun M, Beaumont C, Kahn A, Vaulont S: **Hepcidin, a new iron regulatory peptide.** *Blood cells, molecules & diseases* 2002, **29:**327-335.
110. Ashby DR, Gale DP, Busbridge M, Murphy KG, Duncan ND, Cairns TD, Taube DH, Bloom SR, Tam FW, Chapman R, et al: **Erythropoietin administration in humans causes a marked and prolonged reduction in circulating hepcidin.** *Haematologica* 2010, **95:**505-508.
111. Pak M, Lopez MA, Gabayan V, Ganz T, Rivera S: **Suppression of hepcidin during anemia requires erythropoietic activity.** *Blood* 2006, **108:**3730-3735.
112. Tanno T, Porayette P, Sripichai O, Noh SJ, Byrnes C, Bhupatiraju A, Lee YT, Goodnough JB, Harandi O, Ganz T, et al: **Identification of TWSG1 as a second novel erythroid regulator of hepcidin expression in murine and human cells.** *Blood* 2009, **114:**181-186.
113. Tanno T, Bhanu NV, Oneal PA, Goh SH, Staker P, Lee YT, Moroney JW, Reed CH, Luban NL, Wang RH, et al: **High levels of GDF15 in thalassemia suppress expression of the iron regulatory protein hepcidin.** *Nat Med* 2007, **13:**1096-1101.
114. Casanovas G, Vujic Spasic M, Casu C, Rivella S, Strelau J, Unsicker K, Muckenthaler MU: **The murine growth differentiation factor 15 is not**

essential for systemic iron homeostasis in phlebotomized mice. *Haematologica* 2013, **98:**444-447.
115. Theurl I, Finkenstedt A, Schroll A, Nairz M, Sonnweber T, Bellmann-Weiler R, Theurl M, Seifert M, Wroblewski VJ, Murphy AT, et al: **Growth differentiation factor 15 in anaemia of chronic disease, iron deficiency anaemia and mixed type anaemia.** *Br J Haematol* 2010, **148:**449-455.
116. Kanda J, Mizumoto C, Kawabata H, Tsuchida H, Tomosugi N, Matsuo K, Uchiyama T: **Serum hepcidin level and erythropoietic activity after hematopoietic stem cell transplantation.** *Haematologica* 2008, **93:**1550-1554.
117. Kautz L, Jung G, Valore EV, Rivella S, Nemeth E, Ganz T: **Identification of erythroferrone as an erythroid regulator of iron metabolism.** *Nat Genet* 2014.
118. Awandare GA, Kempaiah P, Ochiel DO, Piazza P, Keller CC, Perkins DJ: **Mechanisms of erythropoiesis inhibition by malarial pigment and malaria-induced proinflammatory mediators in an in vitro model.** *American journal of hematology* 2011, **86:**155-162.
119. Thawani N, Tam M, Bellemare MJ, Bohle DS, Olivier M, de Souza JB, Stevenson MM: **Plasmodium products contribute to severe malarial anemia by inhibiting erythropoietin-induced proliferation of erythroid precursors.** *J Infect Dis* 2014, **209:**140-149.
120. Kabyemela E, Goncalves BP, Prevots DR, Morrison R, Harrington W, Gwamaka M, Kurtis JD, Fried M, Duffy PE: **Cytokine profiles at birth predict malaria severity during infancy.** *PLoS One* 2013, **8:**e77214.
121. Rehu M, Punnonen K, Ostland V, Heinonen S, Westerman M, Pulkki K, Sankilampi U: **Maternal serum hepcidin is low at term and independent of cord blood iron status.** *Eur J Haematol* 2010, **85:**345-352.
122. Young MF, Griffin I, Pressman E, McIntyre AW, Cooper E, McNanley T, Harris ZL, Westerman M, O'Brien KO: **Maternal hepcidin is associated with placental transfer of iron derived from dietary heme and nonheme sources.** *J Nutr* 2012, **142:**33-39.
123. Briana DD, Boutsikou T, Baka S, Boutsikou M, Stamati L, Hassiakos D, Gourgiotis D, Malamitsi-Puchner A: **Perinatal role of hepcidin and iron homeostasis in full-term intrauterine growth-restricted infants.** *Eur J Haematol* 2013, **90:**37-44.
124. Lorenz L, Herbst J, Engel C, Peter A, Abele H, Poets CF, Westerman M, Franz AR: **Gestational Age-Specific Reference Ranges of Hepcidin in Cord Blood.** *Neonatology* 2014, **106:**133-139.
125. Van Santen S, de Mast Q, Luty AJ, Wiegerinck ET, Van der Ven AJ, Swinkels DW: **Iron homeostasis in mother and child during placental malaria infection.** *Am J Trop Med Hyg* 2011, **84:**148-151.
126. Driss A, Hibbert JM, Wilson NO, Iqbal SA, Adamkiewicz TV, Stiles JK: **Genetic polymorphisms linked to susceptibility to malaria.** *Malar J* 2011, **10:**271.
127. Hogg K, Price EM, Hanna CW, Robinson WP: **Prenatal and perinatal environmental influences on the human fetal and placental epigenome.** *Clin Pharmacol Ther* 2012, **92:**716-726.

128. Curtin JA, Simpson A, Belgrave D, Semic-Jusufagic A, Custovic A, Martinez FD: **Methylation of IL-2 promoter at birth alters the risk of asthma exacerbations during childhood.** *Clin Exp Allergy* 2013, **43:**304-311.
129. Hermsdorff HH, Mansego ML, Campion J, Milagro FI, Zulet MA, Martinez JA: **TNF-alpha promoter methylation in peripheral white blood cells: relationship with circulating TNFalpha, truncal fat and n-6 PUFA intake in young women.** *Cytokine* 2013, **64:**265-271.
130. Liu HW, Lin HL, Yen JH, Tsai WC, Chiou SS, Chang JG, Ou TT, Wu CC, Chao NC: **Demethylation within the proximal promoter region of human estrogen receptor alpha gene correlates with its enhanced expression: Implications for female bias in lupus.** *Mol Immunol* 2014, **61:**28-37.
131. Sandoval J, Heyn H, Moran S, Serra-Musach J, Pujana MA, Bibikova M, Esteller M: **Validation of a DNA methylation microarray for 450,000 CpG sites in the human genome.** *Epigenetics* 2011, **6:**692-702.
132. Armitage AE, Pinches R, Eddowes LA, Newbold CI, Drakesmith H: **Plasmodium falciparum infected erythrocytes induce hepcidin (HAMP) mRNA synthesis by peripheral blood mononuclear cells.** *Br J Haematol* 2009, **147:**769-771.
133. Goodman AL, Forbes EK, Williams AR, Douglas AD, de Cassan SC, Bauza K, Biswas S, Dicks MD, Llewellyn D, Moore AC, et al: **The utility of Plasmodium berghei as a rodent model for anti-merozoite malaria vaccine assessment.** *Sci Rep* 2013, **3:**1706.
134. Kautz L, Besson-Fournier C, Meynard D, Latour C, Roth MP, Coppin H: **Iron overload induces BMP6 expression in the liver but not in the duodenum.** *Haematologica* 2011, **96:**199-203.
135. Motulsky HJ, Brown RE: **Detecting outliers when fitting data with nonlinear regression - a new method based on robust nonlinear regression and the false discovery rate.** *BMC Bioinformatics* 2006, **7:**123.
136. Hollnagel A, Oehlmann V, Heymer J, Ruther U, Nordheim A: **Id genes are direct targets of bone morphogenetic protein induction in embryonic stem cells.** *The Journal of biological chemistry* 1999, **274:**19838-19845.
137. Grcevic D, Kusec R, Kovacic N, Lukic A, Lukic IK, Ivcevic S, Nemet D, Seiwerth RS, Ostojic SK, Croucher PI, Marusic A: **Bone morphogenetic proteins and receptors are over-expressed in bone-marrow cells of multiple myeloma patients and support myeloma cells by inducing ID genes.** *Leuk Res* 2010, **34:**742-751.
138. Maes K, Nemeth E, Roodman GD, Huston A, Esteve F, Freytes C, Callander N, Katodritou E, Tussing-Humphreys L, Rivera S, et al: **In anemia of multiple myeloma, hepcidin is induced by increased bone morphogenetic protein 2.** *Blood* 2010, **116:**3635-3644.
139. Jones KL, Mansell A, Patella S, Scott BJ, Hedger MP, de Kretser DM, Phillips DJ: **Activin A is a critical component of the inflammatory response, and its binding protein, follistatin, reduces mortality in endotoxemia.** *Proc Natl Acad Sci U S A* 2007, **104:**16239-16244.
140. Wu H, Chen Y, Winnall WR, Phillips DJ, Hedger MP: **Acute regulation of activin A and its binding protein, follistatin, in serum and tissues**

following lipopolysaccharide treatment of adult male mice. *Am J Physiol Regul Integr Comp Physiol* 2012, **303:**R665-675.
141. Hagihara K, Nishikawa T, Sugamata Y, Song J, Isobe T, Taga T, Yoshizaki K: **Essential role of STAT3 in cytokine-driven NF-kappaB-mediated serum amyloid A gene expression.** *Genes Cells* 2005, **10:**1051-1063.
142. Zhang Z, Fuller GM: **Interleukin 1beta inhibits interleukin 6-mediated rat gamma fibrinogen gene expression.** *Blood* 2000, **96:**3466-3472.
143. Albrecht U, Yang X, Asselta R, Keitel V, Tenchini ML, Ludwig S, Heinrich PC, Haussinger D, Schaper F, Bode JG: **Activation of NF-kappaB by IL-1beta blocks IL-6-induced sustained STAT3 activation and STAT3-dependent gene expression of the human gamma-fibrinogen gene.** *Cell Signal* 2007, **19:**1866-1878.
144. Xia Y, Schneyer AL: **The biology of activin: recent advances in structure, regulation and function.** *J Endocrinol* 2009, **202:**1-12.
145. Hedger MP, Winnall WR, Phillips DJ, de Kretser DM: **The regulation and functions of activin and follistatin in inflammation and immunity.** *Vitam Horm* 2011, **85:**255-297.
146. Carrancio S, Markovics J, Wong P, Leisten J, Castiglioni P, Groza MC, Raymon HK, Heise C, Daniel T, Chopra R, Sung V: **An activin receptor IIA ligand trap promotes erythropoiesis resulting in a rapid induction of red blood cells and haemoglobin.** *Br J Haematol* 2014.
147. Stewart AG, Milborrow HM, Ring JM, Crowther CE, Forage RG: **Human inhibin genes. Genomic characterisation and sequencing.** *FEBS Lett* 1986, **206:**329-334.
148. Lau AL, Kumar TR, Nishimori K, Bonadio J, Matzuk MM: **Activin betaC and betaE genes are not essential for mouse liver growth, differentiation, and regeneration.** *Mol Cell Biol* 2000, **20:**6127-6137.
149. Jones KL, de Kretser DM, Clarke IJ, Scheerlinck JP, Phillips DJ: **Characterisation of the rapid release of activin A following acute lipopolysaccharide challenge in the ewe.** *J Endocrinol* 2004, **182:**69-80.
150. Michel U, Ebert S, Phillips D, Nau R: **Serum concentrations of activin and follistatin are elevated and run in parallel in patients with septicemia.** *Eur J Endocrinol* 2003, **148:**559-564.
151. Petrakou E, Fotopoulos S, Anagnostakou M, Anatolitou F, Samitas K, Semitekolou M, Xanthou G, Xanthou M: **Activin-A exerts a crucial anti-inflammatory role in neonatal infections.** *Pediatr Res* 2013, **74:**675-681.
152. Elsammak MY, Amin GM, Khalil GM, Ragab WS, Abaza MM: **Possible contribution of serum activin A and IGF-1 in the development of hepatocellular carcinoma in Egyptian patients suffering from combined hepatitis C virus infection and hepatic schistosomiasis.** *Clin Biochem* 2006, **39:**623-629.
153. de Kretser DM, Bensley JG, Pettila V, Linko R, Hedger MP, Hayward S, Allan CA, McLachlan RI, Ludlow H, Phillips DJ: **Serum activin A and B levels predict outcome in patients with acute respiratory failure: a prospective cohort study.** *Crit Care* 2013, **17:**R263.

154. Wu H, Chen Y, Winnall WR, Phillips DJ, Hedger MP: **Regulation of activin A release from murine bone marrow-derived neutrophil precursors by tumour necrosis factor-alpha and insulin.** *Cytokine* 2013, **61:**199-204.
155. Robson NC, Phillips DJ, McAlpine T, Shin A, Svobodova S, Toy T, Pillay V, Kirkpatrick N, Zanker D, Wilson K, et al: **Activin-A: a novel dendritic cell-derived cytokine that potently attenuates CD40 ligand-specific cytokine and chemokine production.** *Blood* 2008, **111:**2733-2743.
156. Eramaa M, Hurme M, Stenman UH, Ritvos O: **Activin A/erythroid differentiation factor is induced during human monocyte activation.** *J Exp Med* 1992, **176:**1449-1452.
157. Mason AJ, Berkemeier LM, Schmelzer CH, Schwall RH: **Activin B: precursor sequences, genomic structure and in vitro activities.** *Mol Endocrinol* 1989, **3:**1352-1358.
158. Phillips DJ, Brauman JN, Mason AJ, de Kretser DM, Hedger MP: **A sensitive and specific in vitro bioassay for activin using a mouse plasmacytoma cell line, MPC-11.** *J Endocrinol* 1999, **162:**111-116.
159. Brown CW, Houston-Hawkins DE, Woodruff TK, Matzuk MM: **Insertion of Inhbb into the Inhba locus rescues the Inhba-null phenotype and reveals new activin functions.** *Nat Genet* 2000, **25:**453-457.
160. Brown CW, Li L, Houston-Hawkins DE, Matzuk MM: **Activins are critical modulators of growth and survival.** *Mol Endocrinol* 2003, **17:**2404-2417.
161. Bonomi L, Brown M, Ungerleider N, Muse M, Matzuk MM, Schneyer A: **Activin B regulates islet composition and islet mass but not whole body glucose homeostasis or insulin sensitivity.** *Am J Physiol Endocrinol Metab* 2012, **303:**E587-596.
162. Ludlow H, Phillips DJ, Myers M, McLachlan RI, de Kretser DM, Allan CA, Anderson RA, Groome NP, Hyvonen M, Duncan WC, Muttukrishna S: **A new 'total' activin B enzyme-linked immunosorbent assay (ELISA): development and validation for human samples.** *Clin Endocrinol (Oxf)* 2009, **71:**867-873.
163. Derynck R, Zhang YE: **Smad-dependent and Smad-independent pathways in TGF-beta family signalling.** *Nature* 2003, **425:**577-584.
164. Xia Y, Babitt JL, Sidis Y, Chung RT, Lin HY: **Hemojuvelin regulates hepcidin expression via a selective subset of BMP ligands and receptors independently of neogenin.** *Blood* 2008, **111:**5195-5204.
165. Nakamura T, Takio K, Eto Y, Shibai H, Titani K, Sugino H: **Activin-binding protein from rat ovary is follistatin.** *Science* 1990, **247:**836-838.
166. Phillips DJ, de Kretser DM: **Follistatin: a multifunctional regulatory protein.** *Front Neuroendocrinol* 1998, **19:**287-322.
167. Schneyer A, Schoen A, Quigg A, Sidis Y: **Differential binding and neutralization of activins A and B by follistatin and follistatin like-3 (FSTL-3/FSRP/FLRG).** *Endocrinology* 2003, **144:**1671-1674.
168. Phillips DJ, Hedger MP, McFarlane JR, Klein R, Clarke IJ, Tilbrook AJ, Nash AD, de Kretser DM: **Follistatin concentrations in male sheep increase following sham castration/castration or injection of interleukin-1 beta.** *J Endocrinol* 1996, **151:**119-124.

169. Klein R, Clarke IJ, Hedger MP, Robertson DM: **Plasma follistatin concentrations increase following lipopolysaccharide administration in sheep.** *Clin Exp Pharmacol Physiol* 1996, **23:**754-755.
170. Dieelberg C, Ribes S, Michel U, Redlich S, Bruck W, Nau R, Schutze S: **Follistatin does not influence the course of Escherichia coli K1 sepsis in a mouse model.** *Shock* 2012, **38:**615-619.
171. Matzuk MM, Lu N, Vogel H, Sellheyer K, Roop DR, Bradley A: **Multiple defects and perinatal death in mice deficient in follistatin.** *Nature* 1995, **374:**360-363.
172. Lee SJ, Lee YS, Zimmers TA, Soleimani A, Matzuk MM, Tsuchida K, Cohn RD, Barton ER: **Regulation of muscle mass by follistatin and activins.** *Mol Endocrinol* 2010, **24:**1998-2008.
173. McPherron AC, Lawler AM, Lee SJ: **Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member.** *Nature* 1997, **387:**83-90.
174. Lee SJ, McPherron AC: **Regulation of myostatin activity and muscle growth.** *Proc Natl Acad Sci U S A* 2001, **98:**9306-9311.
175. Masaratana P, Patel N, Latunde-Dada GO, Vaulont S, Simpson RJ, McKie AT: **Regulation of iron metabolism in Hamp (-/-) mice in response to iron-deficient diet.** *Eur J Nutr* 2013, **52:**135-143.
176. Cuny GD, Yu PB, Laha JK, Xing X, Liu JF, Lai CS, Deng DY, Sachidanandan C, Bloch KD, Peterson RT: **Structure-activity relationship study of bone morphogenetic protein (BMP) signaling inhibitors.** *Bioorg Med Chem Lett* 2008, **18:**4388-4392.
177. Kimura F, Sidis Y, Bonomi L, Xia Y, Schneyer A: **The follistatin-288 isoform alone is sufficient for survival but not for normal fertility in mice.** *Endocrinology* 2010, **151:**1310-1319.
178. Holtzhausen A, Golzio C, How T, Lee YH, Schiemann WP, Katsanis N, Blobe GC: **Novel bone morphogenetic protein signaling through Smad2 and Smad3 to regulate cancer progression and development.** *FASEB J* 2014, **28:**1248-1267.
179. Wang Y, Ho CC, Bang E, Rejon CA, Libasci V, Pertchenko P, Hebert TE, Bernard DJ: **Bone morphogenetic protein 2 stimulates noncanonical SMAD2/3 signaling via the BMP type 1A receptor in gonadotrope-like cells: implications for FSH synthesis.** *Endocrinology* 2014, **155:**1970-1981.
180. Schaap CC, Hendriks JC, Kortman GA, Klaver SM, Kroot JJ, Laarakkers CM, Wiegerinck ET, Tjalsma H, Janssen MC, Swinkels DW: **Diurnal rhythm rather than dietary iron mediates daily hepcidin variations.** *Clinical chemistry* 2013, **59:**527-535.
181. Angleton P, Chandler WL, Schmer G: **Diurnal variation of tissue-type plasminogen activator and its rapid inhibitor (PAI-1).** *Circulation* 1989, **79:**101-106.
182. Humphries A, Klein D, Baler R, Carter DA: **cDNA array analysis of pineal gene expression reveals circadian rhythmicity of the dominant negative helix-loop-helix protein-encoding gene, Id-1.** *J Neuroendocrinol* 2002, **14:**101-108.

183. Sun CC, Vaja V, Chen S, Theurl I, Stepanek A, Brown DE, Cappellini MD, Weiss G, Hong CC, Lin HY, Babitt JL: **A hepcidin lowering agent mobilizes iron for incorporation into red blood cells in an adenine-induced kidney disease model of anemia in rats.** *Nephrol Dial Transplant* 2013, **28:**1733-1743.
184. Pearsall RS, Canalis E, Cornwall-Brady M, Underwood KW, Haigis B, Ucran J, Kumar R, Pobre E, Grinberg A, Werner ED, et al: **A soluble activin type IIA receptor induces bone formation and improves skeletal integrity.** *Proc Natl Acad Sci U S A* 2008, **105:**7082-7087.
185. Ruckle J, Jacobs M, Kramer W, Pearsall AE, Kumar R, Underwood KW, Seehra J, Yang Y, Condon CH, Sherman ML: **Single-dose, randomized, double-blind, placebo-controlled study of ACE-011 (ActRIIA-IgG1) in postmenopausal women.** *J Bone Miner Res* 2009, **24:**744-752.
186. Dussiot M, Maciel TT, Fricot A, Chartier C, Negre O, Veiga J, Grapton D, Paubelle E, Payen E, Beuzard Y, et al: **An activin receptor IIA ligand trap corrects ineffective erythropoiesis in beta-thalassemia.** *Nat Med* 2014, **20:**398-407.
187. Macbride HJ, Templeton WL: **The Treatment of General Paralysis of the Insane by Malaria.** *J Neurol Psychopathol* 1924, **5:**13-27.
188. Vogel G: **Malaria as lifesaving therapy.** *Science* 2013, **342:**686.
189. Sauerwein RW, Roestenberg M, Moorthy VS: **Experimental human challenge infections can accelerate clinical malaria vaccine development.** *Nat Rev Immunol* 2011, **11:**57-64.
190. Pasricha SR, Atkinson SH, Armitage AE, Khandwala S, Veenemans J, Cox SE, Eddowes LA, Hayes T, Doherty CP, Demir AY, et al: **Expression of the iron hormone hepcidin distinguishes different types of anemia in African children.** *Sci Transl Med* 2014, **6:**235re233.
191. Swinkels DW, Girelli D, Laarakkers C, Kroot J, Campostrini N, Kemna EH, Tjalsma H: **Advances in quantitative hepcidin measurements by time-of-flight mass spectrometry.** *PLoS One* 2008, **3:**e2706.
192. Kroot JJ, van Herwaarden AE, Tjalsma H, Jansen RT, Hendriks JC, Swinkels DW: **Second round robin for plasma hepcidin methods: first steps toward harmonization.** *American journal of hematology* 2012, **87:**977-983.
193. Kroot JJ, Kemna EH, Bansal SS, Busbridge M, Campostrini N, Girelli D, Hider RC, Koliaraki V, Mamalaki A, Olbina G, et al: **Results of the first international round robin for the quantification of urinary and plasma hepcidin assays: need for standardization.** *Haematologica* 2009, **94:**1748-1752.
194. Ewer KJ, O'Hara GA, Duncan CJ, Collins KA, Sheehy SH, Reyes-Sandoval A, Goodman AL, Edwards NJ, Elias SC, Halstead FD, et al: **Protective CD8+ T-cell immunity to human malaria induced by chimpanzee adenovirus-MVA immunisation.** *Nat Commun* 2013, **4:**2836.
195. Sheehy SH, Duncan CJ, Elias SC, Choudhary P, Biswas S, Halstead FD, Collins KA, Edwards NJ, Douglas AD, Anagnostou NA, et al: **ChAd63-MVA-vectored blood-stage malaria vaccines targeting MSP1 and AMA1: assessment of efficacy against mosquito bite challenge in humans.** *Mol Ther* 2012, **20:**2355-2368.

196. Hodgson SH EK, Bliss CM, Edwards NJ, Rampling T, Anagnostou NA, Havelock T, Poulton ID, de Cassan S, Mange P, Collins KA, Roberts R, De Barra E, Bowyer G, Altman D, Berrie E, Moyle S, Colloca S, Cortese R, Sinden RE, Gilbert SC, Bejon P, Lawrie AM, Nicosia A, Faust SN, Hill AVS: **Evaluation of the Efficacy of ChAd63-MVA Vectored Vaccines Expressing CS & ME-TRAP Against Controlled Human Malaria Infection in Malaria Naïve Individuals.** *Submitted J Infect Dis* 2014.
197. Harrison-Findik DD: **Gender-related variations in iron metabolism and liver diseases.** *World J Hepatol* 2010, **2:**302-310.
198. Galesloot TE, Vermeulen SH, Geurts-Moespot AJ, Klaver SM, Kroot JJ, van Tienoven D, Wetzels JF, Kiemeney LA, Sweep FC, den Heijer M, Swinkels DW: **Serum hepcidin: reference ranges and biochemical correlates in the general population.** *Blood* 2011, **117:**e218-225.
199. Wu H, Wu M, Chen Y, Allan CA, Phillips DJ, Hedger MP: **Correlation between blood activin levels and clinical parameters of type 2 diabetes.** *Exp Diabetes Res* 2012, **2012:**410579.
200. Kwiatkowski D, Nowak M: **Periodic and chaotic host-parasite interactions in human malaria.** *Proc Natl Acad Sci U S A* 1991, **88:**5111-5113.
201. Kwiatkowski D, Cannon JG, Manogue KR, Cerami A, Dinarello CA, Greenwood BM: **Tumour necrosis factor production in Falciparum malaria and its association with schizont rupture.** *Clinical and experimental immunology* 1989, **77:**361-366.
202. Corrigan RA, Rowe JA: **Strain variation in early innate cytokine induction by Plasmodium falciparum.** *Parasite immunology* 2010, **32:**512-527.
203. de Souza JB, Runglall M, Corran PH, Okell LC, Kumar S, Gowda DC, Couper KN, Riley EM: **Neutralization of malaria glycosylphosphatidylinositol in vitro by serum IgG from malaria-exposed individuals.** *Infection and immunity* 2010, **78:**3920-3929.
204. Krishnegowda G, Hajjar AM, Zhu J, Douglass EJ, Uematsu S, Akira S, Woods AS, Gowda DC: **Induction of proinflammatory responses in macrophages by the glycosylphosphatidylinositols of Plasmodium falciparum: cell signaling receptors, glycosylphosphatidylinositol (GPI) structural requirement, and regulation of GPI activity.** *The Journal of biological chemistry* 2005, **280:**8606-8616.
205. Gowda DC: **TLR-mediated cell signaling by malaria GPIs.** *Trends in parasitology* 2007, **23:**596-604.
206. Kumar S, Gowda NM, Wu X, Gowda RN, Gowda DC: **CD36 modulates proinflammatory cytokine responses to Plasmodium falciparum glycosylphosphatidylinositols and merozoites by dendritic cells.** *Parasite immunology* 2012, **34:**372-382.
207. Schofield L, Hackett F: **Signal transduction in host cells by a glycosylphosphatidylinositol toxin of malaria parasites.** *The Journal of experimental medicine* 1993, **177:**145-153.
208. Sharma S, Deoliveira RB, Kalantari P, Parroche P, Goutagny N, Jiang Z, Chan J, Bartholomeu DC, Lauw F, Hall JP, et al: **Innate Immune Recognition of an**

AT-Rich Stem-Loop DNA Motif in the Plasmodium falciparum Genome. *Immunity* 2011, **35:**194-207.

209. Parroche P, Lauw FN, Goutagny N, Latz E, Monks BG, Visintin A, Halmen KA, Lamphier M, Olivier M, Bartholomeu DC, et al: **Malaria hemozoin is immunologically inert but radically enhances innate responses by presenting malaria DNA to Toll-like receptor 9.** *Proceedings of the National Academy of Sciences of the United States of America* 2007, **104:**1919-1924.
210. Gowda NM, Wu X, Gowda DC: **The nucleosome (histone-DNA complex) is the TLR9-specific immunostimulatory component of Plasmodium falciparum that activates DCs.** *PLoS One* 2011, **6:**e20398.
211. Wu X, Gowda NM, Kumar S, Gowda DC: **Protein-DNA complex is the exclusive malaria parasite component that activates dendritic cells and triggers innate immune responses.** *Journal of immunology* 2010, **184:**4338-4348.
212. Delabranche X, Berger A, Boisrame-Helms J, Meziani F: **Microparticles and infectious diseases.** *Med Mal Infect* 2012, **42:**335-343.
213. Diamant M, Tushuizen ME, Sturk A, Nieuwland R: **Cellular microparticles: new players in the field of vascular disease?** *Eur J Clin Invest* 2004, **34:**392-401.
214. Regev-Rudzki N, Wilson DW, Carvalho TG, Sisquella X, Coleman BM, Rug M, Bursac D, Angrisano F, Gee M, Hill AF, et al: **Cell-cell communication between malaria-infected red blood cells via exosome-like vesicles.** *Cell* 2013, **153:**1120-1133.
215. Johnstone RM, Adam M, Hammond JR, Orr L, Turbide C: **Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes).** *J Biol Chem* 1987, **262:**9412-9420.
216. Nantakomol D, Dondorp AM, Krudsood S, Udomsangpetch R, Pattanapanyasat K, Combes V, Grau GE, White NJ, Viriyavejakul P, Day NP, Chotivanich K: **Circulating red cell-derived microparticles in human malaria.** *J Infect Dis* 2011, **203:**700-706.
217. Campos FM, Franklin BS, Teixeira-Carvalho A, Filho AL, de Paula SC, Fontes CJ, Brito CF, Carvalho LH: **Augmented plasma microparticles during acute Plasmodium vivax infection.** *Malar J* 2010, **9:**327.
218. Couper KN, Barnes T, Hafalla JC, Combes V, Ryffel B, Secher T, Grau GE, Riley EM, de Souza JB: **Parasite-derived plasma microparticles contribute significantly to malaria infection-induced inflammation through potent macrophage stimulation.** *PLoS pathogens* 2010, **6:**e1000744.
219. Combes V, Coltel N, Alibert M, van Eck M, Raymond C, Juhan-Vague I, Grau GE, Chimini G: **ABCA1 gene deletion protects against cerebral malaria: potential pathogenic role of microparticles in neuropathology.** *The American journal of pathology* 2005, **166:**295-302.
220. Mantel PY, Hoang AN, Goldowitz I, Potashnikova D, Hamza B, Vorobjev I, Ghiran I, Toner M, Irimia D, Ivanov AR, et al: **Malaria-infected erythrocyte-derived microvesicles mediate cellular communication within the**

parasite population and with the host immune system. *Cell Host Microbe* 2013, **13:**521-534.
221. Johnson D, Gunther K, Ansorge I, Benting J, Kent A, Bannister L, Ridley R, Lingelbach K: **Characterization of membrane proteins exported from Plasmodium falciparum into the host erythrocyte.** *Parasitology* 1994, **109 (Pt 1):**1-9.
222. Gardner JP, Pinches RA, Roberts DJ, Newbold CI: **Variant antigens and endothelial receptor adhesion in Plasmodium falciparum.** *Proceedings of the National Academy of Sciences of the United States of America* 1996, **93:**3503-3508.
223. Pasvol G, Wilson RJ, Smalley ME, Brown J: **Separation of viable schizont-infected red cells of Plasmodium falciparum from human blood.** *Annals of tropical medicine and parasitology* 1978, **72:**87-88.
224. Guo M, Mathieu PA, Linebaugh B, Sloane BF, Reiners JJ, Jr.: **Phorbol ester activation of a proteolytic cascade capable of activating latent transforming growth factor-betaL a process initiated by the exocytosis of cathepsin B.** *J Biol Chem* 2002, **277:**14829-14837.
225. Yoshimori T, Yamamoto A, Moriyama Y, Futai M, Tashiro Y: **Bafilomycin A1, a specific inhibitor of vacuolar-type H(+)-ATPase, inhibits acidification and protein degradation in lysosomes of cultured cells.** *J Biol Chem* 1991, **266:**17707-17712.
226. Alexopoulou L, Holt AC, Medzhitov R, Flavell RA: **Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3.** *Nature* 2001, **413:**732-738.
227. Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, Matsumoto M, Hoshino K, Wagner H, Takeda K, Akira S: **A Toll-like receptor recognizes bacterial DNA.** *Nature* 2000, **408:**740-745.
228. Heil F, Hemmi H, Hochrein H, Ampenberger F, Kirschning C, Akira S, Lipford G, Wagner H, Bauer S: **Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8.** *Science* 2004, **303:**1526-1529.
229. Jurk M, Heil F, Vollmer J, Schetter C, Krieg AM, Wagner H, Lipford G, Bauer S: **Human TLR7 or TLR8 independently confer responsiveness to the antiviral compound R-848.** *Nat Immunol* 2002, **3:**499.
230. Zhu X, Pan Y, Li Y, Jiang Y, Shang H, Gowda DC, Cui L, Cao Y: **Targeting Toll-like receptors by chloroquine protects mice from experimental cerebral malaria.** *Int Immunopharmacol* 2012, **13:**392-397.
231. Franklin BS, Ishizaka ST, Lamphier M, Gusovsky F, Hansen H, Rose J, Zheng W, Ataide MA, de Oliveira RB, Golenbock DT, Gazzinelli RT: **Therapeutical targeting of nucleic acid-sensing Toll-like receptors prevents experimental cerebral malaria.** *Proc Natl Acad Sci U S A* 2011, **108:**3689-3694.
232. Akira S, Takeda K: **Toll-like receptor signalling.** *Nat Rev Immunol* 2004, **4:**499-511.
233. Mackinnon MJ, Mwangi TW, Snow RW, Marsh K, Williams TN: **Heritability of malaria in Africa.** *PLoS Med* 2005, **2:**e340.

234. Gambling L, Czopek A, Andersen HS, Holtrop G, Srai SK, Krejpcio Z, McArdle HJ: **Fetal iron status regulates maternal iron metabolism during pregnancy in the rat.** *Am J Physiol Regul Integr Comp Physiol* 2009, **296:**R1063-1070.
235. Cornock R, Gambling L, Langley-Evans SC, McArdle HJ, McMullen S: **The effect of feeding a low iron diet prior to and during gestation on fetal and maternal iron homeostasis in two strains of rat.** *Reprod Biol Endocrinol* 2013, **11:**32.
236. Cizmeci MN, Kara S, Kanburoglu MK, Simavli S, Duvan CI, Tatli MM: **Detection of cord blood hepcidin levels as a biomarker for early-onset neonatal sepsis.** *Med Hypotheses* 2014, **82:**310-312.
237. Gun Eryilmaz O, Tavil B, Turan S, Yumusak O, Doganay M, Uzunlar O, Akar S, Eyi EG: **Hepcidin and erythropoietin measurements in the cord blood of neonates with meconium-stained amniotic fluid.** *J Obstet Gynaecol Res* 2013, **39:**175-179.
238. Wu TW, Tabangin M, Kusano R, Ma Y, Ridsdale R, Akinbi H: **The utility of serum hepcidin as a biomarker for late-onset neonatal sepsis.** *The Journal of pediatrics* 2013, **162:**67-71.
239. Domellof M, Lonnerdal B, Abrams SA, Hernell O: **Iron absorption in breast-fed infants: effects of age, iron status, iron supplements, and complementary foods.** *Am J Clin Nutr* 2002, **76:**198-204.
240. Apinjoh TO, Anchang-Kimbi JK, Njua-Yafi C, Mugri RN, Ngwai AN, Rockett KA, Mbunwe E, Besingi RN, Clark TG, Kwiatkowski DP, Achidi EA: **Association of cytokine and Toll-like receptor gene polymorphisms with severe malaria in three regions of Cameroon.** *PLoS One* 2013, **8:**e81071.
241. Manjurano A, Clark TG, Nadjm B, Mtove G, Wangai H, Sepulveda N, Campino SG, Maxwell C, Olomi R, Rockett KR, et al: **Candidate human genetic polymorphisms and severe malaria in a Tanzanian population.** *PLoS One* 2012, **7:**e47463.
242. Sunahori K, Juang YT, Kyttaris VC, Tsokos GC: **Promoter hypomethylation results in increased expression of protein phosphatase 2A in T cells from patients with systemic lupus erythematosus.** *Journal of immunology* 2011, **186:**4508-4517.
243. Karmaus W, Ziyab AH, Everson T, Holloway JW: **Epigenetic mechanisms and models in the origins of asthma.** *Curr Opin Allergy Clin Immunol* 2013, **13:**63-69.
244. Wei Y, Yang CR, Wei YP, Zhao ZA, Hou Y, Schatten H, Sun QY: **Paternally induced transgenerational inheritance of susceptibility to diabetes in mammals.** *Proc Natl Acad Sci U S A* 2014, **111:**1873-1878.
245. Bird A: **DNA methylation patterns and epigenetic memory.** *Genes Dev* 2002, **16:**6-21.
246. Reddington JP, Pennings S, Meehan RR: **Non-canonical functions of the DNA methylome in gene regulation.** *The Biochemical journal* 2013, **451:**13-23.
247. Smith ZD, Meissner A: **DNA methylation: roles in mammalian development.** *Nat Rev Genet* 2013, **14:**204-220.

248. Rauch TA, Wu X, Zhong X, Riggs AD, Pfeifer GP: **A human B cell methylome at 100-base pair resolution.** *Proc Natl Acad Sci U S A* 2009, **106:**671-678.
249. Ball MP, Li JB, Gao Y, Lee JH, LeProust EM, Park IH, Xie B, Daley GQ, Church GM: **Targeted and genome-scale strategies reveal gene-body methylation signatures in human cells.** *Nat Biotechnol* 2009, **27:**361-368.
250. Lorincz MC, Dickerson DR, Schmitt M, Groudine M: **Intragenic DNA methylation alters chromatin structure and elongation efficiency in mammalian cells.** *Nat Struct Mol Biol* 2004, **11:**1068-1075.
251. Lister R, Pelizzola M, Dowen RH, Hawkins RD, Hon G, Tonti-Filippini J, Nery JR, Lee L, Ye Z, Ngo QM, et al: **Human DNA methylomes at base resolution show widespread epigenomic differences.** *Nature* 2009, **462:**315-322.
252. Mutabingwa TK, Bolla MC, Li JL, Domingo GJ, Li X, Fried M, Duffy PE: **Maternal malaria and gravidity interact to modify infant susceptibility to malaria.** *PLoS Med* 2005, **2:**e407.
253. Goncalves BP, Huang CY, Morrison R, Holte S, Kabyemela E, Prevots DR, Fried M, Duffy PE: **Parasite burden and severity of malaria in Tanzanian children.** *N Engl J Med* 2014, **370:**1799-1808.
254. Marabita F, Almgren M, Lindholm ME, Ruhrmann S, Fagerstrom-Billai F, Jagodic M, Sundberg CJ, Ekstrom TJ, Teschendorff AE, Tegner J, Gomez-Cabrero D: **An evaluation of analysis pipelines for DNA methylation profiling using the Illumina HumanMethylation450 BeadChip platform.** *Epigenetics* 2013, **8:**333-346.
255. Yousefi P, Huen K, Schall RA, Decker A, Elboudwarej E, Quach H, Barcellos L, Holland N: **Considerations for normalization of DNA methylation data by Illumina 450K BeadChip assay in population studies.** *Epigenetics* 2013, **8:**1141-1152.
256. Du P, Kibbe WA, Lin SM: **lumi: a pipeline for processing Illumina microarray.** *Bioinformatics* 2008, **24:**1547-1548.
257. Pilsner JR, Hall MN, Liu X, Ilievski V, Slavkovich V, Levy D, Factor-Litvak P, Yunus M, Rahman M, Graziano JH, Gamble MV: **Influence of prenatal arsenic exposure and newborn sex on global methylation of cord blood DNA.** *PLoS One* 2012, **7:**e37147.
258. Kundakovic M, Lim S, Gudsnuk K, Champagne FA: **Sex-specific and strain-dependent effects of early life adversity on behavioral and epigenetic outcomes.** *Front Psychiatry* 2013, **4:**78.
259. Kundakovic M, Gudsnuk K, Franks B, Madrid J, Miller RL, Perera FP, Champagne FA: **Sex-specific epigenetic disruption and behavioral changes following low-dose in utero bisphenol A exposure.** *Proc Natl Acad Sci U S A* 2013, **110:**9956-9961.
260. Petani S, Topic E, Turcic G, Daschner M: **Clinical evaluation of the Cell-Dyn 1700CS blood counter.** *Clinical chemistry* 1997, **43:**1085-1088.
261. **The Single Nucleotide Polymorphism Database (dbSNP) of Nucleotide Sequence Variation.** [http://www.ncbi.nlm.nih.gov/books/NBK21088/]
262. Jacoby M, Gohrbandt S, Clausse V, Brons NH, Muller CP: **Interindividual variability and co-regulation of DNA methylation differ among blood cell populations.** *Epigenetics* 2012, **7:**1421-1434.

263. Hellman A, Chess A: **Extensive sequence-influenced DNA methylation polymorphism in the human genome.** *Epigenetics Chromatin* 2010, **3:**11.
264. Price ME, Cotton AM, Lam LL, Farre P, Emberly E, Brown CJ, Robinson WP, Kobor MS: **Additional annotation enhances potential for biologically-relevant analysis of the Illumina Infinium HumanMethylation450 BeadChip array.** *Epigenetics Chromatin* 2013, **6:**4.
265. Abrams ET, Kwiek JJ, Mwapasa V, Kamwendo DD, Tadesse E, Lema VM, Molyneux ME, Rogerson SJ, Meshnick SR: **Malaria during pregnancy and foetal haematological status in Blantyre, Malawi.** *Malar J* 2005, **4:**39.
266. Muehlenbachs A, Fried M, Lachowitzer J, Mutabingwa TK, Duffy PE: **Genome-wide expression analysis of placental malaria reveals features of lymphoid neogenesis during chronic infection.** *Journal of immunology* 2007, **179:**557-565.
267. Florio P, Calonaci G, Luisi S, Severi FM, Ignacchiti E, Palumbo M, Bocchi C, Petraglia F: **Inhibin A, inhibin B and activin A concentrations in umbilical cord artery and vein.** *Gynecol Endocrinol* 2003, **17:**181-185.
268. Florio P, Reis FM, Severi FM, Luisi S, Imperatore A, Palumbo MA, Bagnoli F, Gioffre W, Petraglia F: **Umbilical cord serum activin A levels are increased in pre-eclampsia with impaired blood flow in the uteroplacental and fetal circulation.** *Placenta* 2006, **27:**432-437.
269. Bellissima V, Visser GH, Ververs TF, van Bel F, Termote JU, van der Heide M, Florio P, Li Volti G, Gazzolo D: **Antenatal maternal antidepressants drugs affect Activin A concentrations in maternal blood, in amniotic fluid and in fetal cord blood.** *J Matern Fetal Neonatal Med* 2011, **24 Suppl 2:**31-34.
270. Fiala M, Baumert M, Walencka Z, Paprotny M: **Umbilical activin A concentration as an early marker of perinatal hypoxia.** *J Matern Fetal Neonatal Med* 2012, **25:**2098-2101.
271. Mupfudze TG, Stoltzfus RJ, Rukobo S, Moulton LH, Humphrey JH, Prendergast AJ: **Hepcidin decreases over the first year of life in healthy African infants.** *Br J Haematol* 2014, **164:**150-153.
272. Jaeggi T, Moretti D, Kvalsvig J, Holding PA, Tjalsma H, Kortman GA, Joosten I, Mwangi A, Zimmermann MB: **Iron status and systemic inflammation, but not gut inflammation, strongly predict gender-specific concentrations of serum hepcidin in infants in rural Kenya.** *PLoS One* 2013, **8:**e57513.
273. Domellof M, Lonnerdal B, Dewey KG, Cohen RJ, Rivera LL, Hernell O: **Sex differences in iron status during infancy.** *Pediatrics* 2002, **110:**545-552.
274. Pasricha SR, Black J, Muthayya S, Shet A, Bhat V, Nagaraj S, Prashanth NS, Sudarshan H, Biggs BA, Shet AS: **Determinants of anemia among young children in rural India.** *Pediatrics* 2010, **126:**e140-149.
275. Roque ME, Sandoval MJ, Aggio MC: **Serum erythropoietin and its relation with soluble transferrin receptor in patients with different types of anaemia in a locally defined reference population.** *Clin Lab Haematol* 2001, **23:**291-295.
276. Huang H, Lamikanra AA, Alkaitis MS, Thezenas ML, Ramaprasad A, Moussa E, Roberts DJ, Casals-Pascual C: **Interleukin-10 regulates hepcidin in Plasmodium falciparum malaria.** *PLoS One* 2014, **9:**e88408.

277. Girelli D, Trombini P, Busti F, Campostrini N, Sandri M, Pelucchi S, Westerman M, Ganz T, Nemeth E, Piperno A, Camaschella C: **A time course of hepcidin response to iron challenge in patients with HFE and TFR2 hemochromatosis.** *Haematologica* 2011, **96:**500-506.
278. Zimmermann MB, Troesch B, Biebinger R, Egli I, Zeder C, Hurrell RF: **Plasma hepcidin is a modest predictor of dietary iron bioavailability in humans, whereas oral iron loading, measured by stable-isotope appearance curves, increases plasma hepcidin.** *Am J Clin Nutr* 2009, **90:**1280-1287.
279. Tobi EW, Lumey LH, Talens RP, Kremer D, Putter H, Stein AD, Slagboom PE, Heijmans BT: **DNA methylation differences after exposure to prenatal famine are common and timing- and sex-specific.** *Human molecular genetics* 2009, **18:**4046-4053.
280. Houseman EA, Accomando WP, Koestler DC, Christensen BC, Marsit CJ, Nelson HH, Wiencke JK, Kelsey KT: **DNA methylation arrays as surrogate measures of cell mixture distribution.** *BMC Bioinformatics* 2012, **13:**86.
281. Atkinson SH, Rockett KA, Morgan G, Bejon PA, Sirugo G, O'Connell MA, Hanchard N, Kwiatkowski DP, Prentice AM: **Tumor necrosis factor SNP haplotypes are associated with iron deficiency anemia in West African children.** *Blood* 2008, **112:**4276-4283.
282. McGuire W, Hill AV, Allsopp CE, Greenwood BM, Kwiatkowski D: **Variation in the TNF-alpha promoter region associated with susceptibility to cerebral malaria.** *Nature* 1994, **371:**508-510.
283. Stirnadel HA, Stockle M, Felger I, Smith T, Tanner M, Beck HP: **Malaria infection and morbidity in infants in relation to genetic polymorphisms in Tanzania.** *Trop Med Int Health* 1999, **4:**187-193.
284. Gertz J, Varley KE, Reddy TE, Bowling KM, Pauli F, Parker SL, Kucera KS, Willard HF, Myers RM: **Analysis of DNA methylation in a three-generation family reveals widespread genetic influence on epigenetic regulation.** *PLoS Genet* 2011, **7:**e1002228.
285. Zhi D, Aslibekyan S, Irvin MR, Claas SA, Borecki IB, Ordovas JM, Absher DM, Arnett DK: **SNPs located at CpG sites modulate genome-epigenome interaction.** *Epigenetics* 2013, **8:**802-806.
286. Liu Y, Li X, Aryee MJ, Ekstrom TJ, Padyukov L, Klareskog L, Vandiver A, Moore AZ, Tanaka T, Ferrucci L, et al: **GeMes, clusters of DNA methylation under genetic control, can inform genetic and epigenetic analysis of disease.** *Am J Hum Genet* 2014, **94:**485-495.
287. Baliraine FN, Afrane YA, Amenya DA, Bonizzoni M, Menge DM, Zhou G, Zhong D, Vardo-Zalik AM, Githeko AK, Yan G: **High prevalence of asymptomatic plasmodium falciparum infections in a highland area of western Kenya: a cohort study.** *J Infect Dis* 2009, **200:**66-74.
288. Portugal S, Moebius J, Skinner J, Doumbo S, Doumtabe D, Kone Y, Dia S, Kanakabandi K, Sturdevant DE, Virtaneva K, et al: **Exposure-dependent control of malaria-induced inflammation in children.** *PLoS Pathog* 2014, **10:**e1004079.
289. Clark MA, Goheen MM, Fulford A, Prentice AM, Elnagheeb MA, Patel J, Fisher N, Taylor SM, Kasthuri RS, Cerami C: **Host iron status and iron**

supplementation mediate susceptibility to erythrocytic stage Plasmodium falciparum. *Nat Commun* 2014, **5:**4446.
290. Poli M, Asperti M, Ruzzenenti P, Regoni M, Arosio P: **Hepcidin antagonists for potential treatments of disorders with hepcidin excess.** *Front Pharmacol* 2014, **5:**86.
291. Lamikanra AA, Brown D, Potocnik A, Casals-Pascual C, Langhorne J, Roberts DJ: **Malarial anemia: of mice and men.** *Blood* 2007, **110:**18-28.